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**STRUCTURE-FUNCTION RELATIONSHIPS OF RICIN A-CHAIN.**

**A thesis submitted by**

**John Andrew Chaddock B.Sc. (Hons) Dunelm**

**for the degree of**

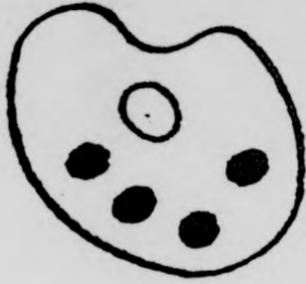
**Doctor of Philosophy**



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**February, 1992**

NUMEROUS ORIGINALS  
IN COLOUR



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DECLARATION

All the results presented in this thesis were obtained by the author unless specifically indicated in the text. This work has not been used in any previous application for a degree. All sources of information have been specifically acknowledged by means of reference.

*J. A. Chaddock*  
JOHN A. CHADDOCK

### ABBREVIATIONS

|                  |  |
|------------------|--|
| $\text{\AA}$     | Angstroms  |
| ADP              | adenosine diphosphate                              |
| approx.          | approximately                                      |
| ATP              | adenosine triphosphate                             |
| APS              | ammonium persulphate                               |
| Bis              | N'-methylene-bisacrylamide                         |
| bp               | base-pairs   |
| BSA              | bovine serum albumin                               |
| Cl               | curie  |
| CM-              | carboxy-methyl-                                    |
| [ ]              | concentration                                      |
| cpm              | counts per minute                                  |
| Da               | dalton   |
| DEAE-            | diethylaminoethyl                                  |
| DNA              | deoxyribonucleic acid                              |
| dNTPs            | deoxyribonucleoside triphosphates                  |
| DTT              | dithiothreitol                                     |
| <u>E. coli</u>   | <u>Escherichia coli</u>                            |
| EDTA             | diaminoethanetetra-acetic acid, disodium salt      |
| EGTA             | 1,2-Di(2-aminoethoxy)ethane-NNN'-tetra-acetic acid |
| Fig.             | Figure   |
| g                | gram   |
| HCl              | hydrochloric acid                                  |
| HPLC             | high performance liquid chromatography             |
| IC <sub>50</sub> | cytotoxicity index                                 |

|               |  |
|---------------|--|
| ICI           | Imperial Chemical Industries Plc.            |
| IPTG          | isopropyl- $\beta$ -D-thio galactopyranoside |
| kDa           | Kilodalton                                   |
| M             | molar  |
| mA            | milliamps                                    |
| MIR           | multiple isomorphous replacement             |
| MOPS          | 3-[N-Morpholino]propanesulphonic acid        |
| ng            | nanogram                                     |
| nm            | nanometres                                   |
| NSWY          | non-salt washed yeast ribosomes              |
| $^{\circ}$ C  | degrees centigrade                           |
| OD            | optical density                              |
| oligos        | oligonucleotides                             |
| PAGE          | polyacrylamide gel electrophoresis           |
| PBS           | phosphate buffered saline                    |
| PMSF          | phenylmethylsulphonylfluoride                |
| %             | percent                                      |
| pp $\alpha$ F | preproalpha factor                           |
| RF            | replicative form                             |
| RIP           | ribosome inactivating protein                |
| RNA           | ribonucleic acid                             |
| RNase         | ribonuclease                                 |
| rpm           | revolutions per minute                       |
| rRNA          | ribosomal RNA                                |
| rRTA          | recombinant ricin A-chain                    |
| RTA           | ricin A-chain                                |
| S-            | Sulphonyl-                                   |

|       |   |
|-------|---|
| SDS   | sodium dodecyl sulphate                 |
| SWR   | salt washed reticulocyte ribosomes      |
| SWY   | salt washed yeast ribosomes             |
| TCA   | trichloroacetic acid                    |
| TEMED | NNN'N'-tetramethylethylenediamine       |
| Tris  | tris (hydroxymethyl) aminomethane       |
| µg    | microgram                               |
| µl    | microlitres                             |
| µM    | micromolar                              |
| UV    | ultraviolet                             |
| V     | volts                                   |
| v/v   | volume/volume                           |
| W     | Watts                                   |
| w/v   | weight/volume                           |
| xg    | fold greater than gravity               |
| X-gal | 5-bromo-4-chloro-3-indolyl βgalactoside |

Restriction endonuclease abbreviations:

B = BamHI

Bg = BglII

C = ClaI

H = HindIII

P = PstI

R = EcoRI

S = SalI

Sm = SmaI

X = XhoI

Xb = XbaI

To Mum and Dad

## SUMMARY

This thesis represents a study of the relationships between the structure and function of the A-chain of ricin. Ricin is one member of a family of ribosome-inactivating proteins (RIPs) that inactivate protein synthesis by modification of the ribosomal RNA. The mechanism of this catalytic effect is unknown. This project was designed to investigate which amino acid residues were important in the mechanism of catalysis.

Using site-directed mutagenesis of specific ricin A-chain residues, eight mutants were created for biochemical analysis. Residues were targeted for mutagenesis by analysis of the position of conserved amino acids in the ricin 3-D structure. Glu177 was mutated to Lys, Ala and Asp, and Arg180 was mutated to Gln, Ala and Met. The arginine residue at position 29 was altered to an alanine, and a deletion of residues Ser176 to Arg180 was performed.

Mutant recombinant ricin A-chain (rRTA) constructs were prepared for *in vitro* and *in vivo* expression in the vectors pGEN1 and pDS573 respectively. Initially, mutants were translated in a cell-free wheatgerm translation system to assess the size of the mutant polypeptides. All the constructs produced polypeptides of the correct size. The N-glycosidase activity of the mutants was then assessed with two methods of analysis using rabbit reticulocyte ribosomes. It was shown that some of the mutants were devoid of detectable activity and some had reduced activity.

Mutant constructs were expressed in *Escherichia coli* in order to isolate protein for quantitative activity measurements. Crude *E. coli* extracts containing rRTA and rTA mutants were tested for activity. It was found that the relative activities of mutant proteins produced in *E. coli* was similar to that seen previously with the *in vitro* measurements. Soluble, active protein was recovered for a few of the mutant proteins, although no expression was observed from some constructs. Various purification procedures were assessed and ion-exchange chromatography was determined to be most suitable.

Mutant D177 was tested for its N-glycosidase activity towards salt-washed yeast ribosomes and related to the activity of wild-type rRTA. It was shown that the activity of D177 was approximately 60 fold lower than that of wild-type rRTA with the majority of the difference being observed with the *k<sub>cat</sub>* of the reaction. It was also shown that the data produced in this study correlated with a recently published putative mechanism of action of ricin A-chain.

*Chapter One*

**INTRODUCTION**

## 1.1 General Introduction

In the late 19<sup>th</sup> century it was observed that seed extracts from certain plant species were cytotoxic. In the late 20<sup>th</sup> century this phenomenon is being investigated worldwide. Why is there such interest in the products from these seeds? Why is the knowledge gained of use to mankind? It is the purpose of this introduction to try to answer these questions and give the reader an appreciation of a topic which has much significance in the areas of biochemistry, cell biology and the treatment of disease.

Early observations by Stillmark (1888) on seed extracts from the castor oil plant Ricinus communis, demonstrated the presence of a protein (he called this ricin) which was a haemagglutinin. With the advent of more sophisticated protein purification techniques the properties of agglutination and toxicity were found to belong to two distinct protein entities (Olsson & Pihl, 1973). The protein possessing strong agglutination activity, but poor cytotoxicity, was termed Ricinus communis agglutinin I (RCA). The protein now referred to as ricin is a relatively weak haemagglutinin (Olsson et al., 1974) but is a highly potent cytotoxin. This thesis is a study of one of the subunits of ricin.

Ricin is one of the most toxic substances known. It has the ability to inhibit protein synthesis in eukaryotic cells by irreversibly inactivating the 60S ribosomal

subunit. It has been reported that just one molecule of ricin is necessary to enter a cell cytoplasm to cause cell death (Eiklid et al., 1980). In addition to ricin there are numerous other protein toxins found naturally in plants, bacteria and fungi that can inactivate protein synthesis to this degree. Hence ricin is just one member of a family of proteins that have been termed ribosome-inactivating proteins (RIPs). The original criterion proposed for inclusion of proteins within this group was that the protein must catalytically inactivate the 60S subunit of eukaryotic ribosomes rendering them incapable of binding elongation factor 2 (eEF-2) (Stirpe, 1982). A list of selected protein toxins that fit the above criterion is shown in Table 1. Even though the fungal toxin  $\alpha$ -sarcin is essentially unrelated to the other RIPs, since it has an alternative catalytic mechanism and an unrelated protein structure, it still fits the criteria for inclusion in the RIP group.

The proteins described as RIPs utilize a method of protein synthesis inactivation that has been adopted by a wide variety of unrelated species. It is the apparently ubiquitous feature of these proteins (and the mechanism of action) that has led to suggestions that this type of protein may be present in all plant species (Gasperi-Campani, et al., 1985). However the structure, activity

Table 1. Selected examples of ribosome-inactivating proteins (RIPs). Those RIPs highlighted with a \* are glycosylated.

| RIP  | Mr     | Source                       |
|--|--------|------------------------------|
| <b>Single-chain RIPs (Type 1):</b>         |        |                              |
| PAP (Pokeweed anti-viral protein)          | 30000  | <u>Phytolacca americana</u>  |
| Tritin                                     | 30000  | <u>Triticum aestivum</u>     |
| Gelonin                                    | 30000* | <u>Gelonium multiflorum</u>  |
| Dianthin30                                 | 30000* | <u>Dianthus carvophyllus</u> |
| Saporin                                    | 29500  | <u>Saponaria officinalis</u> |
| MAP ( <u>Mirabilis</u> anti-viral protein) | 28000  | <u>Mirabilis jalapa</u>      |
| <b>Heterodimeric (Type 2):</b>             |        |                              |
| Ricin                                      | 65000* | <u>Ricinus communis</u>      |
| Abrin                                      | 65000* | <u>Abrus precatorius</u>     |
| Nodeccin                                   | 63000* | <u>Adenia digitata</u>       |
| Viscumin                                   | 60000* | <u>Viscum album</u>          |

and substrate specificities of these proteins are not identical. There is therefore an interest in how this class of protein evolved to be present in such a wide range of species. It has been suggested that these proteins may have evolved from a common ancestral gene (Ready et al., 1984) .

This introduction has been organised such that the reader is firstly given information about the source, synthesis and structure of native ricin. Following this is a description of the mechanism of protein synthesis inhibition, from the binding of ricin to the target cell to the actual depurination reaction. The A-chain is examined in more detail and results that have been published before, and during this project, are referred to. Later, the activity, uses, and therapeutic importance of the A-chain is discussed. Throughout the introduction comparisons are continually made with other A-chain-like proteins to give the reader an appreciation of the field of research as a whole. Additional introductory statements are found at the beginning of each section of the Results chapter.

#### 1.1.1 Project goals

This thesis represents a study of the A-chain of ricin. In particular, an examination of the relationship between protein structure and the ribosome-inactivating

activity of ricin A-chain. By careful analysis of a series of specifically constructed mutant A-chains, the project aims to provide information as to the importance of these residues in the mechanism of ribosome inactivation.

## 1.2 Classification of ribosome-inactivating proteins

Ribosome-inactivating proteins are classified into two subgroups (Type 1 and Type 2) on the basis of their quaternary protein structure. Type 2 proteins, such as ricin, are heterodimers of an A- and a B-chain linked by a single disulphide bond and have a molecular weight of approximately 60000-65000 Da. Type 1 RIPs (average Mr=30000 Da) resemble the A-chain of the Type 2 RIPs but do not possess the additional B-chain.

Although Type 1 RIPs are highly toxic to ribosomes in vitro they are not cytotoxic to intact cells since they lack the requisite cell binding domains. Type 2 RIPs are highly cytotoxic ( $IC_{50}$   $3 \times 10^{-12}M$  for CEM cells (a human leukaemia cell line) according to Shire et al., 1990) because they possess a cell binding subunit (the B-chain) in addition to a ribosome-inactivating subunit. For the Type 2 RIP to be active against ribosomes, the catalytic A-chain domain must be released from the cell binding domain after binding to the cell and internalisation of the heterodimer (Olson & Pihl, 1982).

The heterodimeric structure of the Type 2 RIPs has

been suggested to be the result of a gene fusion between DNA coding for a ribosome-inactivating polypeptide (analogous to a Type 1 RIP) and a gene encoding a lectin.

### 1.3 Biosynthesis and trafficking of ricin in *Ricinus communis*

The synthesis of ricin, and the related agglutinin RCA, occurs simultaneously in the endosperm cells of maturing castor bean seeds (Gifford et al., 1982). Synthesis occurs during and after testa formation when the organelles are being rapidly formed (Roberts & Lord, 1981a). Work by Butterworth & Lord (1983) has shown that the functionally distinct A and B subunits of ricin are derived from large polypeptide precursors. Examination of the cDNA (Lamb et al., 1985) of the precursor, preproricin, has shown that the polypeptide is composed of a 35 amino acid N-terminal presequence peptide preceding the A-chain (267 amino acids), a 12 amino acid linking region and 262 amino acids of the B-chain.

The preproprotein undergoes extensive modification as it travels from its site of synthesis to its ultimate destination, the protein bodies. An N-terminal signal peptide, believed to lie within the first 22 residues of the presequence, is cleaved during cotranslational translocation of the precursor across the membranes of the endoplasmic reticulum. At the same time the precursor is

glycosylated by the addition of core oligosaccharide side-chains to appropriate asparagine residues. There are 4 such potential glycosylation sites, 2 on the A-chain (residues 10 and 236) and 2 on the B-chain (residues 374 and 414) (numbering here, and throughout the thesis, according to Lamb et al., 1985 - see appendix). The formation of disulphide bonds is also achieved at this stage (Roberts & Lord, 1981b). Five bonds are catalysed : four within the B-chain and one between the A-chain and the B-chain.

The glycosylated precursor, now known as proricin, then travels by vesicular transport to the Golgi and finally by vesicles which fuse with the newly formed protein bodies. The sequence information required to target ricin to the protein bodies is not yet known, though recent work excludes the remaining residues of the N-terminal prosequence after signal peptide cleavage (N. Westby, PhD. thesis, 1991). Since the carbohydrate side-chains are also known not to affect this targeting, the possibility remains that the targeting signal is within the mature subunits or within the linker peptide. The proricin precursor is endoproteolytically cleaved within the protein bodies to remove both the 12 amino acid linker sequence and a prosequence at the N-terminus of the A-chain to create mature ricin molecules.

The synthesis scheme as outlined above ensures that the highly toxic A-chain does not come into contact with

the protein synthesis machinery of the endosperm cells. Only within the protein bodies is the mature form of ricin finally produced (Harley & Lord, 1985). Expression of preproricin transcripts in Xenopus laevis oocytes (Richardson et al., 1989) demonstrated segregation of the product into the oocyte endomembrane system, core glycosylation and removal of the N-terminal signal peptide. Soluble recombinant proricin with an active B-chain function was purified from oocyte homogenates. However this proricin polypeptide could not inactivate rabbit reticulocyte ribosomes, demonstrating that the active form of ricin is only produced in the final post-translational modification step in the protein bodies.

A genomic clone of ricin has been obtained (Halling et al., 1985) which has been shown not to contain introns. This feature has been observed in a number of other (non-homologous) lectin genes that have been sequenced (e.g. soybean lectin gene (Vodkin et al., 1983) and Phaseolus vulgaris lectin gene (Hoffman, 1984)). The ricin gene has been proposed to be a member of a small multigene family.

### 1.3.1 Biosynthesis and trafficking of other RIPs

The genomic cloning of preproabrin from the leaves of Abrus precatorius has recently been described (Wood et al., 1991). This is the only other Type 2 RIP to be cloned and reported. The gene lacks introns and produces a

polypeptide precursor with a 34 amino acid pre-sequence and a 14 amino acid linker which in this case contains a potential glycosylation site. From the primary structure, it is clear that abrin is similar to ricin. Mature protein is also sequestered in the protein body organelles of the Abrus precatorius seed tissue.

Many Type 1 RIPs have been studied recently in order to obtain a DNA sequence that can be used for characterization of their properties. Genomic sequences have been reported for  $\alpha$ -trichosanthin (Chow et al., 1990) and saporin (Fordham-Skelton et al., 1991). Also the genomic sequences of the A-chain of the Type 2 RIP abrin (Evensen et al., 1991) and the fungal toxin restrictocin (Lamy & Davies, 1991) have been determined. In addition cDNA sequences have been reported for dianthin 30 (Legname et al., 1991), Mirabilis anti-viral protein (MAP) (Kataoka et al., 1991), momordin (Ho et al., 1991), saporin-6 (Benatti et al., 1989) and pokeweed anti-viral protein (PAP) (Lin et al., 1991). A DNA sequence for the Escherichia coli shiga-like toxin gene has also been reported (Calderwood et al., 1987). Chemically synthesised genes have been produced for ricin A-chain (Shire et al., 1990), MAP (Habuka et al., 1990) and  $\alpha$ -sarcin (Hanze et al., 1990).

Examination of some of these sequences has revealed the likelihood of N-terminal signal peptides which would be involved in targeting the Type 1 RIPs into the

endoplasmic reticulum (E.R.) from where they may be taken to the vacuole or other internal structures, or to the cell surface. PAP has been shown to be localised in the cell wall (Ready et al., 1986). Those proteins, such as PAP, presumably follow the secretory pathway and would therefore only require a signal peptide to allow translocation into the E.R. In contrast, RIPs destined for an intracellular location require additional sorting information.

The sequence and structure of these signals are largely unknown. With many of the RIPs there is the hypothetical potential of targeting signals within the N-terminal or C-terminal propeptides which are ultimately removed, or in the carbohydrate modifications which are removed in some RIPs upon final proteolytic processing. For example,  $\alpha$ -trichosanthin, dianthin 30 and saporin-6 possess a C-terminal extension which may contain vacuolar targeting signals. 19 amino acid residues are removed from the C-terminus of  $\alpha$ -trichosanthin to form the mature protein (Chow et al., 1990) and 22 C-terminal amino acids are removed in saporin-6 (Benatti et al., 1991). A similar C-terminal region in dianthin also contains a N-glycosylation site and shows homology to a propeptide present in several plant vacuolar proteins e.g. barley lectin (Wilkins et al., 1990) and wheat germ agglutinin (Raikhel & Wilkins, 1987), the former having been shown to be involved in vacuolar targeting (Bednarek et al., 1990).

#### 1.4 Internalisation and intracellular trafficking of ricin

The process of internalisation of protein toxins has been reviewed by Olsnes & Sandvig (1988) and provides a good overview of the binding and routing of toxins through cells during cellular intoxication.

Ricin, as with other heterodimeric glycoprotein toxins, can enter cells by a choice of routes. Since ricin and ricin A-chain possesses typical mannose-rich plant carbohydrate side-chains, uptake can proceed via the mannose receptors located on macrophages and cells of the reticuloendothelium leading to cell death (Simmons et al., 1986). However, more commonly, ricin utilises the lectin properties of the B-chain to gain entry to the cell. The B-chain has an affinity for galactose and N-acetylgalactosamine which allows it to bind to the plasma membrane glycoproteins. There are  $3 \times 10^7$  such sites for ricin per HeLa cell (Sandvig et al., 1976). Ricin binds evenly and opportunistically over the surface of the cell, including areas of coated and uncoated pits. Interestingly, Shiga toxin from Shigella dysenteriae 1 and the Shiga-like toxins from E. coli bind to particular glycolipids and are also largely endocytosed from coated pits (Sandvig et al., 1989).

The opportunistic binding of ricin leads to endocytosis of a proportion of the bound heterodimer and transport of it through the endosomal region of the cell

to the Golgi (van Deurs et al., 1986). It is well established that ricin is taken up by endocytosis by studies using ricin labelled with horseradish peroxidase or colloidal gold (van Deurs et al., 1985). Recently a fluorescent labelled ricin has been used to observe ricin entry into the cell (Ballelli et al., 1990). However the region where ricin crosses the membrane into the cytosol is presently unknown, although evidence is accumulating to suggest the trans Golgi network (TGN) or a pre-Golgi compartment may be sites of ricin A-chain membrane translocation.

If recycling of ricin back to the cell surface is inhibited, ricin accumulates in the TGN. Evidence presented by Youle & Colombatti (1987) using hybridoma cells expressing anti-ricin A-chain antibodies, showed that these cells were up to 300 fold more resistant to ricin than control hybridoma cells. They hypothesized that the monoclonal antibodies being secreted interact with the internalised ricin in a Golgi compartment. The TGN is believed to be common to both secretory and endocytic pathways. The role of the Golgi has also been hypothesized by Yoshida et al. (1990) while examining the cytotoxicity of ricin in a mutant Chinese hamster ovary (CHO) cell line which is reported to be defective in Golgi functions/structures.

Later studies have used brefeldin A (BFA) which disrupts the Golgi apparatus causing the Golgi contents

and membranes to redistribute to the endoplasmic reticulum. Using this system it has been proposed by Yoshida et al. (1991) that ricin is transported from the endosomes to the Golgi, from where it can be released into the cytoplasm. This is in contrast to diphtheria toxin (DT) which is translocated from acidified endosomes without involving the Golgi region. Acidification of the cell potentiates DT release whereas the translocation of ricin, abrin and viscumin is increased by the addition of agents to increase the pH (Sandvig & Olsnes, 1982). It is believed that the Golgi region of the cell has a higher pH than that within the endosomes.

#### 1.5 Role of ricin B-chain in membrane translocation

It has been indicated above that the B-chain of heterodimeric RIPs has an important function in binding the holotoxin to the cell surface. The B-chain was also reported to be essential for A-chain toxicity as it possessed an, as yet unidentified, translocation domain. Translocation of the toxin into the cytosol has been reported to be the rate-limiting step during the decline in protein synthesis (Hudson & Neville, 1987). The Type 1 RIPs do not possess a cell binding domain. Under certain circumstances, non-glycosylated Type 1-like RIPs may still enter cells and have a toxic effect, as observed by using

ricin A-chain - antibody conjugates to kill target cells (Vitetta & Thorpe, 1991). However, in the case of some conjugates, the presence of the B-chain (with the cell-binding domains blocked) appears to potentiate A-chain immunoconjugate activity, indicating that the B-chain must be capable of aiding the A-chain in some way. It is also interesting to note that the binding and internalisation functions of the B-chain can be mimicked by alternative conjugated toxins. It has been reported that a conjugate of epidermal growth factor (EGF) and ricin A-chain is as toxic to A431 cells as ricin (Herschman, 1984) and that the heavy chain of tetanus toxin can mediate the entry of the Type 1 RIP gelonin into intact HT29 cells (Johnstone et al., 1990). In addition the B-chain moiety from a Type 2 RIP (ricin) has been conjugated to a single chain barley RIP to produce an active cytotoxic conjugate (Ovadia et al., 1988).

It has been suggested that the B-chain may act to target or concentrate the toxin in a compartment of the cell that is competent for the translocation of the A-chain (Lord et al., 1991). Recent, as yet unpublished results from this laboratory clearly indicate that the galactose-binding sites on ricin B-chain are important for this potentiation property. However, it does not appear that the sugar binding sites on the B-chain are directly involved in the membrane translocation event itself. Thus membrane translocation of the toxic subunit may involve

structural information residing entirely in the A-chain. It is important to bear in mind this fact when performing A-chain mutagenesis, since mutagenesis may have a detrimental effect on the ability of the A-chain to translocate. This may affect the effectiveness of immunoconjugates. Though not an aim of this project, the study of the potential translocation properties of the A-chain would be an interesting and useful project since, as yet, there is no information available on this topic.

#### 1.6 Structural analysis of RIPs

Various chemical and biochemical techniques have been applied to elucidate the structure of RIPs. The problem of characterising RIP structure is complicated by the fact that several isoforms of RIPs can be isolated (Olsnes & Pihl, 1982). In the case of ricin, at least three forms of ricin and two of Ricinus agglutinin have been demonstrated (Cawley et al., 1978 ; J. Tregear, PhD thesis, 1990). Southern analysis of the Ricinus genomic DNA by Halling et al. (1985) and J. Tregear, PhD. thesis, (1990), has shown that ricin is a member of a small multigene family. The purification of three toxins and two agglutinins from Abrus precatorius seeds using lectamylsapharose affinity chromatography has recently been reported (Hegde et al., 1991) and the isolation of two distinct genomic DNA sequences encoding two distinct abrin

A-chains has recently been reported by Evensen et al. (1991). Thus isoforms of RIFs can be isolated from seeds of differing origin and even from within seeds of the same source, making the process of obtaining an accurate structural model more difficult.

### 1.7 The structure of ricin

As a member of the Type 2 group of ribosome-inactivating proteins, ricin is a heterodimer consisting of a catalytic glycoprotein (the A-chain, apparent molecular weight 32000Da) linked by a single disulphide bond to a glycosylated lectin (the B-chain, apparent molecular weight 34000Da) (Olsnes & Pihl, 1973). The B-chain is a lectin which binds much more strongly (by a factor of at least  $10^4$ ) to complex galactosides from cell surface carbohydrates than to simple sugars (Baensiger, 1979). The disulphide bond serves to maintain stability of the heterodimer at low protein concentrations, however it does not appear to be essential for cytotoxicity (Lewis & Youle, 1986).

The three-dimensional crystal structure for ricin D was first reported by Montfort et al. in 1987. This initial model (Fig. 1.1), based on a 2.8Å MIR electron density map, proved to be an invaluable tool for examination of the ricin structure. A recent refinement of this structural model to 2.5Å has been reported (Rutenber



Fig. 1.1 A representation of the three-dimensional structure of ricin.

The heterodimeric structure of ricin (determined to  $2.8\text{\AA}$ ) is depicted by a ribbon representation of the A-chain (in orange) and the B-chain (in purple). The single disulphide bond linking the two chains is indicated. The B-chain intrachain disulphide bonds, the glycosylation sites and the sugar-binding sites are not depicted in the interests of clarity. The highly organised secondary structure of ricin A-chain can easily be visualised, as can the two similar galactose-binding domains of ricin B-chain. Crystal structure representations were generated using Polygen Quanta software.

et al., 1991) which has indicated slight changes in the spatial orientation of several amino acid residues. It has become clear that a more refined model will be required in order to assess the role of key residues or to rationalise the results of chemical or genetic changes to the wild type protein.

#### 1.7.1 The structure of ricin A-chain

From the original  $2.8\text{\AA}$  structure (Fig. 1.1) it was shown that ricin A-chain is a globular protein of dimensions  $55\text{\AA} \times 45\text{\AA} \times 35\text{\AA}$  exhibiting a substantial amount of secondary structure (Fig. 1.2). 30% of this polypeptide is helical (consisting of seven alpha helices totalling approximately 80 amino acid residues) and 15% is  $\beta$ -structure (consisting of a five-stranded  $\beta$ -sheet). The A-chain folds into three domains. The five-stranded  $\beta$ -sheet dominates the first domain (residues 1-117) whereas the second domain (residues 118-210) is dominated by 5 alpha helices. On the second helix of this second domain lies the only free sulphhydryl group (Cys171) in this polypeptide. Domain three (residues 211-267) interacts with the first two domains and is also involved in strong interaction with the B-chain.

It is generally considered that the A and B chains are held together by non-polar forces and by measuring the free energy of association it can be shown that the



Fig. 1.2 The structure of ricin A-chain.

A representation of the three-dimensional structure of ricin A-chain (to  $2.8\text{\AA}$ ) is shown. The structure is displayed in a 'protein cartoon' format with areas of secondary structure highlighted. The extensive  $\alpha$ -helices are indicated with purple solid lines and the  $\beta$ -sheet is depicted in yellow. The remaining areas of the molecule are shown in white.



Fig. 1.2 The structure of ricin A-chain.

A representation of the three-dimensional structure of ricin A-chain (to  $2.8\text{\AA}$ ) is shown. The structure is displayed in a 'protein cartoon' format with areas of secondary structure highlighted. The extensive  $\alpha$ -helices are indicated with purple solid lines and the  $\beta$ -sheet is depicted in yellow. The remaining areas of the molecule are shown in white.

interface need only involve a few hydrophobic contacts. From the position of amino acid residues in the structural model, various A-chain residues have been proposed to interact with the B-chain (Rutenber & Robertus, 1991). The two potential glycosylation sites in the A-chain are sometimes occupied (Foxwell et al., 1985) although from studies in our laboratory ricin A-chain appears to always have 2 associated carbohydrate side-chains. It would appear that N-glycosylation of the A-chain is not required for toxicity since recombinant ricin A-chain purified from E. coli is as toxic to ribosomes as native A-chain (O'Hare et al., 1987).

Examination of this A-chain structure has revealed a cleft created at the interface of all three domains (Fig. 1.3). The actual structure of this cleft cannot be accurately mapped from this representation since this model was created from the crystallisation of whole ricin and not of the isolated ricin A-chain. It has been suggested that the A-chain of ricin may undergo a conformational change when it is released from the B-chain which may affect the conformation of this putative active site (Robertus et al., 1987). Crystals of A-chain have been produced (Robertus et al., 1987) and are presently undergoing analysis.

Fig. 1.3 The putative active-site cleft of ricin A-chain.

The putative active-site cleft is highlighted by selected residues (in A and B) and by a solvent surface model (in C).

In Figure A the polypeptide backbone is represented in 'protein cartoon' style, highlighting areas of  $\alpha$ -helix and  $\beta$ -sheet. Selected conserved residues are represented by the coloured side-chain structures. The residues selected are Tyr80 (brown), Tyr123 (orange), Glu177 (red), Ala178 (purple), Arg180 (blue), Asn209 (yellow) and Trp211 (green). The active-site is postulated to lie in the vicinity of these residues in an orientation perpendicular to the plane of the photograph.

Figures B and C represent an alternative view of the putative active-site cleft from the direction indicated by an arrow in Figure A. The residues indicated in B are as described for A. Figure C represents a 'solvent surface' view of Figure B. A radius of  $18\text{\AA}$  was specified, centred around Arg180, and an assessment of the accessibility of the structure to water molecules was made. The solvent surface is denoted by yellow dots overlaid on the protein structure depicted in orange.



**A**



**B**

**C**

### 1.7.2 The structure of ricin B-chain

Ricin B-chain has previously been shown to be a gene duplication product (Villafranca & Robertus, 1981) and this is seen in the 3-D structure as two sugar-binding domains with identical folding topologies. Lactose was included during crystallisation and this can be visualised in the electron density map. There are two glycosylation sites on ricin B-chain, both of which are usually occupied. The two sugar binding domains are separated by 70Å making it impossible for a single biantennary cell surface carbohydrate molecule to bind both sites.

### 1.8 Structural models for other RIPs

Using the original ricin A-chain structure, models have been proposed for abrin and trichosanthin. It has been shown that the sequence differences between ricin A-chain and these related toxins could be accommodated into the structural model with little perturbation in the chain folding (Collins et al., 1990). Thus, although there are differences in primary structure between these proteins, the overall tertiary structures would appear to be surprisingly similar. An alternative model for the Type 1 RIP trichosanthin has been proposed (Pan et al., 1987) suggesting that the structural similarity between trichosanthin and ricin is lower than that suggested by

Collins. However additional evidence from circular dichroism measurements (Kubota et al., 1987) found nearly identical results for each protein, enhancing the belief that proteins in this class are structurally similar, even if their parent plants are taxonomically unrelated.

#### 1.8.1 Structural similarities between plant RIPs and other proteins

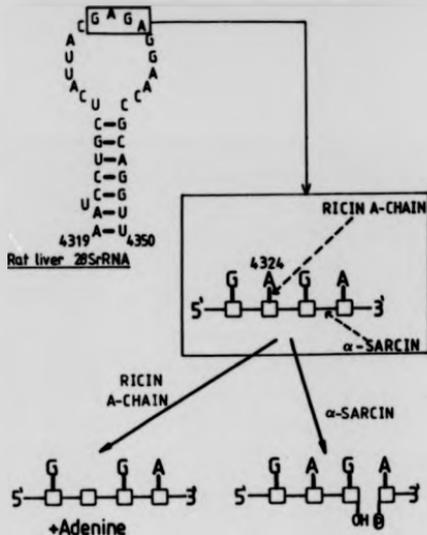
It has been shown by Ready et al. (1988) that some plant ribosome-inactivating proteins are homologous at the domain level to RNase H from Escherichia coli and to two regions of the pol gene product of retroviral reverse transcriptases. It is plausible that this represents an ancient folding unit capable of binding and catalysing hydrolytic reactions on nucleic acids. Since there is no evidence of RIP activity in plants below angiosperms, and yet RIP proteins have been found in bacteria, it is suggested that the RIP gene (at least for domains 2 and 3) may have entered the genome of an ancestral angiosperm by transfer from a bacterium or virus.

#### 1.9 The mode and site of action of ricin and other RIPs

The inhibition of protein synthesis by ribosome-inactivating proteins is a highly specific, irreversible catalytic event. An early observation (Obrig et al.,

1985) had suggested that RIPs were ribonucleases but this was later disproved. In 1987 Endo and colleagues clearly demonstrated that ricin A-chain acted directly on a specific glycosidic bond within the large rRNA. It was shown that the enzyme removed adenine 4324 from the 28S rRNA from rat liver ribosomes leaving the phosphodiester backbone of the RNA intact (Fig. 1.4). Endo demonstrated that the depurinated rRNA was then susceptible to cleavage at this site by treatment with the amine base aniline at acidic pH. In this way ricin A-chain action on the ribosomes could be visualised experimentally rather than observing the indirect effect of inhibition of protein synthesis. It was shown that 1 mole of adenine was released from 1 mole of ribosome after incubation of rat liver ribosomes with a catalytic amount of ricin A-chain (Endo & Tsurugi, 1987).

The N-glycosidic bond between the adenine residue and the rRNA backbone could be cleaved by a phosphorolysis or hydrolysis mechanism. The non-incorporation of <sup>32</sup>P phosphate during incubation of ricin A-chain with ribosomes demonstrated that the mechanism of bond breakage did not proceed by phosphorolysis, since a phosphate would be incorporated into the RNA (as ribose 1-phosphate) and radioactivity would be found in the ribosome fraction. Thus it would appear that ricin and other RIPs (with the exception of  $\alpha$ -sarcin), cleave this particular N-glycosidic bond in a hydrolytic fashion. N-glycosidase



**Fig. 1.4** Differential sites of action shown by ricin A-chain and  $\alpha$ -sarcin.

It has been shown that ricin A-chain, in common with most RIPs studied, cleaves a single N-glycosidic bond between a specific adenine residue and the phosphodiester backbone of ribosomal RNA.  $\alpha$ -sarcin cleaves the phosphodiester bond to the 3' of a specific guanosine nucleotide within the same conserved region.

enzymes have been found for a variety of diverse substrates e.g. uridine (Magni et al., 1975), NAD<sup>+</sup> (Kaplan et al., 1951) and uracil-DNA (Lindahl et al., 1977). The ability of RIPs to distinguish which bond to hydrolyse out of the estimated 7000 N-glycosidic bonds in eukaryotic rRNA is unknown, although several interesting experiments have been performed. As discussed in section 1.10, the target site of ricin A-chain action is highly conserved amongst ribosomal RNA substrates.

#### 1.9.1 The catalytic site of ricin A-chain

As previously mentioned, examination of the crystal structure of ricin A-chain revealed a cleft region that has been proposed to be the catalytic site. However the catalytic site of ricin A-chain has never been mapped to a particular location in the structure, nor is the mechanism of action understood. In order to address this problem work has been performed, including the results within this thesis, to elucidate the residues involved in catalysis and the structure of the active site. By a combination of chemical alteration of amino acids and specific mutagenesis, various residues have been implicated as being of importance and this will be discussed fully elsewhere. However it is appropriate at this stage to mention that there is conservation of amino acids around this cleft area (e.g. Trp211 and Tyr80) and conservation

of residues concentrated at the base of the cleft (Glu177, Ala178 and Arg180).

Experiments have been performed to attempt to crystallise ricin A-chain with a variety of ligands (e.g. adenine, G-A etc.) however there are no reports of any success at present. Since the nature of the interactions between ricin A-chain and its substrate are unknown, it is possible that the hypothesis of a single region of contact around the putative active site is a simplistic view. Interactions with the ribosome may occur in areas distant from the cleft which may affect catalysis.

#### 1.9.2 The mode and site of action of $\alpha$ -sarcin

The fungal ribosome-inactivating protein  $\alpha$ -sarcin (from Aspergillus giganteus) does not inhibit protein synthesis by the mechanism of N-glycosidic bond breakage as described above, however the rRNA target area is the same (Chan et al., 1983).  $\alpha$ -sarcin acts as a ribonuclease and hydrolyses the phosphodiester backbone of the rRNA at a specific site to the 3' of G 4325 in rat 28S rRNA releasing a fragment from the 3' end (Fig. 1.4). This cleavage inactivates the ribosomes, indicating again the importance of this region of the rRNA.

1.10 The ribosomal substrate for N-glycosidase activity

The site of action of ricin, other RIPs and  $\alpha$ -sarcin is located within a purine-rich 14 ribonucleotide segment of rRNA that is almost universally conserved (Wool, 1986 ; Raus *et al.*, 1988) as shown in Fig. 1.5 below.

Fig. 1.5 Conservation of the RIP rRNA target site

| rRNA               | Sequence                                | Reference     |
|--------------------|---|---------------|
|                    | 4325                                    |               |
|                    |   |               |
| Rat 28S            | UCCUGCUCAGU <b>ACGAGAGGA</b> ACCGCAGGUU | Chan, 1983    |
| <u>X. laevis</u>   | UCCUGCUCAGU <b>ACGAGAGGA</b> ACCGCAGGUU | Clark, 1984   |
| Yeast 26S          | UUGAACUUAGU <b>ACGAGAGGA</b> ACAGUUAU   | Veldman, 1981 |
| <u>E. coli</u> 23S | CUGCUCUAGU <b>ACGAGAGGA</b> ACCGGAGUGGA | Brosius, 1980 |

Work performed by Endo & Tsurugi (1988) originally showed that ricin A-chain recognised a specific structure in the ribosomal RNA. This structure is thought to fold into a stem-loop conformation with the conserved purine-rich region being in the exposed loop. The sequence GAGA lies at the centre of the loop. The adenine indicated previously in bold is the adenine removed as a result of ricin A-chain action.

At present there is controversy over the importance of the ribosomal proteins in the interaction between the RIP and the rRNA. It would appear that the presence of this loop is not the sole requirement for effective N-glycosidase activity. Removal of ribosomal proteins from rat liver 28S rRNA (i.e. 'naked' rRNA) decreases the rate of depurination by a factor of at least  $10^5$ , although the apparent Michaelis constant ( $K_m$ ) was similar to that determined using native ribosomes as substrate. This would indicate that the ribosomal proteins modulate the depurination reaction in some way but that they do not affect the binding of the enzyme to its substrate. It was also shown that intact 28S rRNA was not required since a 553 nucleotide 3' fragment containing the ricin sensitive site also serves as a substrate.

The stem-loop structure proposed as being the substrate requirement for ricin A-chain appears in other rRNA sources e.g. E. coli 23SrRNA. It had been previously demonstrated (Gale et al., 1981) that ricin A-chain does not inactivate prokaryotic ribosomes, nor does it cleave an N-glycosidic bond in the rRNA of these ribosomes (Endo & Tsurugi, 1987). However the target site is present in E. coli and is almost identical to the stem-loop sequence as found in eukaryotes. Incubation of naked rRNA from E. coli with ricin A-chain at concentrations similar to that used for naked eukaryotic rRNA produced an unexpected result as both the E. coli 23S and the 16S rRNA were depurinated at

a specific site. This would suggest that the r-proteins in E. coli ribosomes normally prevent interaction between ricin A-chain and the target RNA sequences, maybe by masking the target site.

With these data Endo suggested a consensus site for ricin A-chain attack. This was a stem-loop structure in the rRNA with the sequence GAGA in the loop and 6-7 bp in the stem. Ricin, and other RIPs tested, depurinate the ribosomes at the adenine (highlighted in bold) rendering the ribosomes unable to sustain protein synthesis. The depurinated rRNA is now susceptible to experimental cleavage with reagents such as acetic aniline. Later experiments by Endo et al. (1991) examined the effect of the nucleotide content and sequence on depurination to give a clearer understanding of the target site of RIP action (see 1.11.2).

#### 1.10.1 Interaction of RIPs with different ribosomal substrates

As mentioned above, ricin A-chain has different activities on naked rRNA and on rRNA from E. coli. Despite great similarities between RIPs from many species and the high conservation of sequence around the target site in 28S/26S/23S rRNAs it is at present unknown why some RIPs are active towards some ribosomes though less active or inactive towards others. When compared to activities

determined with rabbit reticulocyte ribosomes, ricin A-chain is approximately 1000 fold less active on wheatgerm ribosomes (Osborn, 1991). This difference was shown to be as a result of a  $10^3$  decrease in the kcat for the reaction. Thus it has been shown for a variety of RIPs against a variety of ribosomal substrates that there is a wide range of ribosome-inactivating activities.

Recent studies have revealed that some single-chain RIPs are active towards intact ribosomes from Escherichia coli (Hartley et al., 1991; Habuka et al., 1990). Thus despite extensive sequence homologies between the RIPs as a whole, it is clearly evident that important structural differences, however subtle, do exist and can make a crucial difference in their ability to interact and depurinate the target adenine. Additional differences in the structure and conformation of ribosomes from different sources make the enzyme-substrate interaction an interesting, though complex problem.

In conclusion, it seems that RIPs can interact directly with the rRNA substrate and not necessarily with the ribosomal proteins, although the r-proteins are important since naked rRNA (with the exception of E. coli 23S rRNA) is never as good a substrate for any RIP as the whole ribosome (e.g. for ricin A-chain the kcat decreases from 1777 rat liver ribosomes per minute, to 0.02 ribosomes/min for naked rRNA). Endo has suggested a consensus target sequence for RIPs that takes the form of

a stem-loop structure with 6-7 base pairs in the stem, and the sequence GAGA at the centre of the exposed loop.

### 1.11 Minimum substrate requirement

#### 1.11.1 For $\alpha$ -sarcin

Oligonucleotides reproducing the conserved stem-loop structure have been used to investigate the target site recognised by ricin and  $\alpha$ -sarcin. According to Endo et al. (1990), a synthetic radioactive oligoribonucleotide (35mer) serves as an in vitro substrate for  $\alpha$ -sarcin action. From studying a number of closely related 'mutant' oligonucleotides it is reported that  $\alpha$ -sarcin has an absolute requirement for a helical stem (which can be reduced from 7 to 3 base pairs). The nature of the base pairs in the stem modifies but does not abolish recognition. The bulged nucleotide below the loop structure apparently does not contribute to identification of the target site. Changing the position of the conserved tetranucleotide GAGA within the loop leads to loss of recognition by the toxin and the cleavage of the backbone is affected by altering the nucleotides surrounding G-4325. Specific cleavage of this synthetic structure occurs at an  $\alpha$ -sarcin concentration of  $2.94 \times 10^{-8}M$  but at higher concentrations ( $2.94 \times 10^{-6}M$ ), cleavage occurs at all the purines and it is no longer possible to

distinguish between specific and nonspecific hydrolysis. The immediate 5' adenosine (i.e. the ricin A-chain target site) has no influence on RNA identification. This has been shown previously since ribosomes preincubated with ricin A-chain are still susceptible to cleavage by  $\alpha$ -sarcin. In fact, ricin-induced depurination increases the susceptibility of rat liver ribosomes to cleavage by  $\alpha$ -sarcin (Terao et al., 1988).

#### 1.11.2 For ricin

Recently this oligoribonucleotide approach has been applied to the study of the structural motifs recognised by ricin A-chain (Endo et al., 1991). The wild-type 35mer and a number of mutants were produced from an in vitro transcription system as before. The  $K_m$  of ricin A-chain for the oligo was 13.55 $\mu$ M and the  $k_{cat}$  was 0.023min<sup>-1</sup>. Thus the  $K_m$  value is approximately 5 fold greater for the oligo and the  $k_{cat}$  is 10<sup>5</sup> smaller suggesting that the A-chain binds to the oligo with less affinity than to ribosomes. This may possibly be explained by the 3-D structural differences of the oligo. It has been recognised that the value of these observations was debatable because of doubts over the ability of the oligo to form the correct structure. However, NMR spectroscopy of the oligoribonucleotide indicated that the loop has a definite structure (although not identified) that is

stable at 40°C (Szewczak et al., 1991). The reason for the difference in  $K_m$  between this oligo and naked RNA has not been explained. It has been shown that the ricin A-chain is ineffective on an oligo designed to have no stem therefore there is an absolute requirement for a stem structure (though this can be reduced from 7 to 3 base pairs). There is also an absolute requirement for an adenosine at the position that corresponds to position 4324. Changing the position of the GAGA sequence in the loop or altering the nucleotides in the universal sequence surrounding A4324 drastically affects substrate recognition.

#### 1.12 Ribosome-inactivating proteins and protein synthesis

The way ricin A-chain specifically deurinates eukaryotic (and naked prokaryotic) rRNA in a catalytic mechanism has been described above. There are many stem-loop structures in ribosomal RNA (Noller, 1984), but the effectiveness of this mechanism of inhibition is due to the importance of the conserved loop structure to the protein synthesising system of the organism, and the importance of the quaternary structure of the ribosome. It is interesting to note that if ribosomes are incubated with  $\alpha$ -sarcin to cleave the target phosphodiester bond and then subsequently treated with ricin A-chain, specific deurination of the rRNA still occurs. However hydrolysis

of an oligoribonucleotide with  $\alpha$ -sarcin prevents subsequent recognition by ricin A-chain, suggesting that in intact ribosomes the resultant rRNA ends following  $\alpha$ -sarcin cleavage are in some way constrained, potentially by interaction with ribosomal proteins.

#### 1.12.1 The conformation of modified ribosomes

Inhibition of protein synthesis by ricin A-chain is associated with, and possibly caused by, conformational changes in the ribosome. Terao et al. (1988) have observed differential labelling of ribosomal proteins of toxin-treated ribosomes when they are incubated with radioactive N-ethylmaleimide, compared to the r-proteins labelled in untreated ribosomes. It was found that labelling of protein L14 with N-ethylmaleimide (Ghosh & Moore, 1979) was specifically reduced after treatment of the rat liver ribosomes with ricin A-chain when compared to untreated control ribosomes. Similarly, labelling of proteins L3 and L4 was reduced following incubation with  $\alpha$ -sarcin. Thus toxin treatment of ribosomes may cause a conformational change in the rRNA causing a difference in the conformation, and therefore accessibility, of ribosomal proteins. Proteins L3 and L4 are believed to play important roles in protein synthesis- L4 is known to be located within the partial A site (Soshin et al., 1979) and L3 within the partial P site (Fabijanski et al., 1981) and

they can be cross-linked with elongation factor 2.

Additional evidence of a toxin-induced conformational change comes from reports that protein synthesis inhibition can be reversed by high concentrations of  $Mg^{2+}$  (Skorve et al., 1977; Terao et al., 1988). It was also observed (Paleologue et al., 1986) that incubation of 60S ribosomal subunits with ricin A-chain reduced their stability during heat treatment i.e. the thermal denaturation curve of the subunits was shifted towards lower temperatures. An increased amount of 5S rRNA- r-protein L5 complex was released after heating toxin treated subunits leading to the hypothesis that a conformational change had been induced by the toxin in the region of protein L5.

#### 1.12.2 The functional role of the conserved rRNA sequence

Aside from the potential effects of conformational change on the function of the ribosome, it has also been demonstrated that the conserved stretch of 14 nucleotide residues has a role in binding various factors crucial to protein synthesis. There is general agreement that ricin A-chain inhibits the binding of elongation factor 2 to ribosomes (Nilsson & Nygard, 1986; Montanaro et al., 1975) and affects both EF-1 and EF-2 GTPase activities (Sperti et al., 1975; Nygard & Nilsson, 1989). For a review of the translational dynamics of the protein synthesis system and

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a summary of the characteristics of the components of the system, the reader is directed to Nygard & Nilsson (1990). The observations outlined above are consistent with the finding of Moazed et al. (1988) that in Escherichia coli EF-G and EF-Tu interact with a loop in 23SrRNA which corresponds to the conserved loop structure found in 26S/28S rRNA.

It has been observed by Fernandez-Puentes et al. (1976a) that one could protect rabbit reticulocyte ribosomes from ricin A-chain inactivation by treatment with elongation factor 2 in the presence or absence of GTP. The protection could be overcome by increasing the amounts of toxin, indicating that the binding between the ribosomes and the EF-2 was an equilibrium. Replacing GTP with a non-hydrolyzable analogue prevented ricin A-chain inactivation. Thus it would appear that the elongation factors and the RIFs share a common rRNA binding region.

While there is agreement that RIFs inhibit the elongation cycle of protein synthesis, some evidence has been presented for inhibition of translation initiation by both ricin and abrin on a wheatgerm extract (Osborn & Hartley, 1990; Skorge et al., 1977). Recent experiments performed on rabbit reticulocyte lysate have investigated all the stages of protein synthesis that are inhibited by ricin A-chain (Osborn & Hartley, 1990). Using the ribosome 'shift' assay (Darnbrough et al., 1973) the authors observed a six-fold decrease in the rate of the initiation

of translation when compared to that of diphtheria toxin treated ribosomes (diphtheria toxin acts solely at the elongation phase of protein synthesis). Since only the 60S subunit of the translational apparatus is affected by ricin A-chain (Fernandez-Puentes & Vazquez, 1977), and the only role of the 60S subunit in initiation is the formation of the 80S initiation complex, the authors concluded that the joining of the 60S subunit to the 40S initiation complex was the most likely step to be inhibited by ricin A-chain. However, the primary effect of ricin A-chain on ribosomes was shown to be inhibition of the translocation step of protein synthesis.

### 1.13 Roles of ribosome-inactivating proteins

#### 1.13.1 Physiological roles

It is difficult to state unequivocally the actual roles RIPs play in the survival of the organism. As the properties of these proteins become clearer, more suggestions are proposed for their roles. For several of the plant RIPs, including ricin and abrin, it would appear that they serve as storage proteins since they are synthesised in the endosperm or storage tissue of maturing seeds where they accumulate in large amounts (Lord, 1985). The RIP genes show identical tissue and developmental specificity of expression to the genes for storage

proteins. In *Ricinus communis* seeds, storage proteins and ricin are broken down simultaneously during germination (Tulley & Beavers, 1976). However the role of Type 2 RIPs cannot be limited to that of an amino acid store because of the cytotoxic function that has evolved and been conserved in these proteins. It is suggested the RIPs perform a defensive role against predators and pathogens. In a recent study three barley seed proteins with antifungal activity were isolated. Two of these (a chitinase and a (1-3)- $\beta$ -glucanase) inhibit fungal growth by degrading major cell wall polysaccharides in growing hyphae. The third is a single chain RIP which appears to act synergistically in vitro with the two hydrolases to inhibit fungal growth. Presumably the permeabilised hyphae is susceptible to the RIP which inactivates the fungal ribosomes leading to rapid cell death (Leah et al., 1991). The RIP, lacking a cell binding domain, presumably depends on such permeabilisation events to gain access to the cytoplasm. It may be the case that other cell wall located Type 1 RIPs act in a coordinated fashion with other proteins to gain access to the ribosomes of the invading pathogens.

#### 1.13.2 Antiviral effects

The first Type 1 RIP to be characterised was not initially studied because of its protein synthesis

inhibition properties but because of its apparent antiviral effects. It had been shown that leaf extracts from several species of plant, including Phytolacca americana (pokeweed), were capable of preventing the mechanical transmission of viral infection to other plants (Wyatt & Shepherd, 1969). Pokeweed antiviral protein (PAP) also prevented animal virus replication in mammalian cells, where it was found to inhibit protein synthesis by virally infected cells at a PAP concentration which did not affect normal cells (Ussery et al., 1977). How this apparent selection takes place is uncertain, however it has been suggested by Fernandez-Puentes & Carrasco (1980) that viral infection alters the permeability of the plasma membrane of the host cell and allows the toxins to invade the cytoplasm.

Once both antiviral and ribosome-inactivating effects had been established, it was of interest to explain how the antiviral effects were produced. Early studies proposed that certain (RIP containing) plant extracts could protect other plant species from viral attack by inactivation of the heterologous ribosomes, but were believed to be ineffective against the ribosomes of the plant producing the RIP (Barbiari & Stirpe, 1982). However an improved model has been proposed recently as a consequence of the observation that pokeweed leaf ribosomes are highly sensitive to the PAP produced in the pokeweed plant (Taylor & Irvin, 1990). PAP is sequestered

in the cell walls of pokeweed cells (Ready at al., 1986) and may thus be part of a suicide system to protect the plant. It is envisaged that damage to the cell wall during viral infection may bring the RIP into contact with the cytosol where it can depurinate the pokeweed ribosomes and prevent viral replication. Direct interaction of the RIP with the virus cannot be ruled out. In fact recent studies, that will be discussed below, suggest that RIPs may interact with a human virus selectively, leaving the protein synthesis machinery intact.

#### 1.14 Utilization of RIPs

##### 1.14.1 Therapeutics

As described above, some RIPs have been shown to exhibit antiviral effects. It has recently been reported by McGrath at al. (1989) that trichosanthin, from the root tubers of Trichosanthes kirilowii, can effectively inhibit the replication of type 1 human immunodeficiency virus (HIV-1) in acutely infected lymphoblastoid cells or chronically infected macrophages. The dose of toxin required to inhibit HIV-1 propagation is significantly lower than required for inhibition of protein synthesis however the toxin is still active towards the ribosomes. A second antiviral protein from Trichosanthes kirilowii has been recently isolated (Lee-Huang at al., 1991) called

TAP-29 which is reported to have a therapeutic index at least two orders of magnitude higher than trichosanthin. It has been shown that all the RIPs tested (including ricin A-chain) have antiviral activities towards HIV-1 (Lifson et al., 1988; Lee-Huang et al., 1990). It is interesting to speculate why RIPs should have activity against HIV-1. Since HIV-1 has an RNA genome it maybe that there are RNA binding sites on the RIP that have evolved to interact with plant viruses (these mainly have RNA genomes) which may interact strongly with the HIV-1 genome, preventing the activity of reverse transcriptase. The application of mutagenesis studies, as described in this thesis, may help to explain the anti-viral properties of RIPs.

The therapeutic value of RIPs is not a recent phenomenon however, but has been recognised for thousands of years. Trichosanthin, for instance, has been used for centuries in China for inducing abortions by placental destruction. Additionally certain tumour cell types have been shown to have increased sensitivity to RIPs than normal cells and virally infected macrophages can take up RIPs by generalised pinocytosis. The use of native RIPs for therapeutics has limited application due to the extreme toxicity of the RIP to normal cells. A regime to harness the potential of RIPs is described in the next section.

#### 1.14.1.1 Construction of RIP immunocjugates

One of the most important therapeutic uses for RIPs was recognised as early as the beginning of this century by Paul Ehrlich who visualised the concept of 'magic bullets' (Ehrlich, 1891). The potency of Type 2 RIPs such as ricin had been observed by Ehrlich for some time and the potential for the treatment of disease was exciting. However it was not until the production of monoclonal antibodies was established (Kohler & Milstein, 1975) that effective treatment became a possibility. Hybrid molecules could be produced consisting of an RIP fused to an antibody and such a molecule, in theory, would be directed to a target cell by the antibody moiety where the toxin would act specifically and kill the cell. These hybrids were termed immunotoxins. Many reviews have been presented relating to this topic (Lord et al., 1988; Vitetta et al., 1987; Pastan et al., 1986) which describe the approaches that have been taken for the construction of active immunotoxins and problems that have been encountered.

Construction of an ideal therapeutic immunotoxin has been hampered by various problems and gaps in our understanding; the choice of target antigen to give specificity to the immunotoxin; the large size of the hybrid limiting its use; the route of entry of the hybrid into the cell being important; the lack of understanding of the mechanism of toxin translocation into the cytosol

and toxin routing in the cell; the rapid blood clearance of the hybrid; the effect on toxicity of the RIP after conjugation etc. However, even with these problems the important benefits that can be derived from the use of immunotoxins has generated a worldwide research initiative to examine their use.

Many different toxins have been used for the construction of immunotoxins including the prototype ricin A-chain and others eg. abrin A-chain, gelonin, momordin, (Wawrsynczak et al. 1990),  $\alpha$ -sarcin (Wawrsynczak et al. 1991) and the bacterial toxins diphtheria toxin and pseudomonas exotoxin. For a review of the ricin-containing immunotoxins being used in clinical trials and the diseases that they are effective against, the reader is directed to Vitetta & Thorpe (1991) and Byars & Baldwin (1991).

Study of the A-chain of ricin, and other RIPs, will lead to a better understanding of the residues and domains that are important for its activity. With this knowledge it should be possible to construct smaller, less immunogenic, yet highly potent toxins that could be used for the preparation of more efficient immunotoxins.

1.14.2 Use of RIFs in understanding protein synthesis and  
in cell biology

In addition to the medicinal benefits of RIFs, their use has provided much information about non-clinical fields. One of the most important areas to have benefited from the use of RIFs is the examination of the mechanism of protein synthesis. Study of the mode of action of RIFs and an examination of their substrate target sites has provided information regarding the role of the ribosome and rRNA in protein synthesis and the interaction of rRNA with ribosomal proteins and other factors important to protein synthesis. Further studies may examine the concept of protein-RNA recognition domains and shed light on ribosome structure-function.

RIFs have also proved to be interesting from the aspect of intracellular routing and the processes of membrane permeabilisation and translocation. An alternative application of RIFs has been in the construction of toxigenes (Evans, 1991). Toxigenes are artificial genes created through genetic engineering which produce a native or modified toxin under the control of a tissue- or developmental-stage specific regulatory element. Programmed cell death may be used to study developmental cell lineages and to create animal models of degenerative diseases.

### 1.15 Aims of the project

The aim of this project was to examine the structure-function relationships of ricin A-chain. Using information derived from RIP protein sequence homology studies and examination of the 3D structure of ricin, mutagenesis reactions were performed at specific amino acid residues. Characterisation of the resultant A-chain mutants was facilitated by in vitro and in vivo expression of mutant A-chain cDNAs and examination of the properties of the mutants. By comparison with 'wild-type' recombinant ricin A-chain, it was therefore possible to ascertain the importance of the mutated residues in the mechanism of ribosome inactivation. More detailed kinetic experiments using purified A-chain would assist in the elucidation of the roles of the targeted amino acids within catalysis.

Analysis of the effect of mutagenesis at the specific loci chosen for this study, in addition to results from other workers, would be crucial to the determination of the mechanism of action of RIPs. As the roles of the amino acids in the RIPs becomes established, more information will become available for improving the therapeutic uses of these RIPs eg. for the construction of improved immunotoxins or utilisation of the anti-viral properties in a controlled approach.

In Chapter 3 I present the results of my study of ricin A-chain. The work has been subdivided into sections

containing a brief introduction for each subsection. Here I will present the information derived from mutagenesis, in vitro expression, in vivo expression, protein purification, and kinetic analysis. Chapter 4 will discuss the results obtained and relate them to other published work.

*Chapter Two*

**MATERIALS AND  
METHODS**

## MATERIALS

Unless specified below, all chemicals were of Analar grade obtained from BDH (Poole, Dorset, UK) or Fisons (FSA laboratory supplies, Loughborough, UK).

Bactoagar, bactopectone, bactotryptone and caseamino acids were obtained from Difco laboratories (Detroit, USA). Yeast extract was from Beta lab (Surrey, UK).

Boric acid, EDTA and glycerol were obtained from Prolabo (Paris). Agarosa, ampicillin, anti-sheep IgG - alkaline phosphatase conjugate, bovine gamma globulins, BSA, coomassie brilliant blue R-250, DTT, ethidium bromide, HEPES, IPTG, lysozyme, magnesium acetate, MOPS, PMSF, RNase A, rubidium chloride, spermidine, spermine, TEMED, thiamine and X-gal were all obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

Ether and sodium dihydrogen orthophosphate were supplied by May & Baker (Dagenham, England), silver nitrate was from Johnson Mathey (Herts, England).

Prestained molecular weight markers (low range), protein assay concentrate, Biogel P6 and Affigel 10 were purchased from Biorad (California, USA). Pharmacia (Great Britain) Ltd. supplied all chromatography columns, column matrices, polybuffer 96, micrococcal nuclease and capping agent (7mG(5')ppp(5')G).

Amersham International plc. supplied 'Amplify', Hybond C, <sup>14</sup>C-labelled protein markers, in vitro

mutagenesis kit, RNasin, various restriction and modification enzymes, and all radioisotopes; ( $^{32}\text{P}$ -ATP (7000 Ci/mmol),  $^{35}\text{S}$ -dATP (1200 Ci/mmol),  $^{35}\text{S}$ -methionine (1000 Ci/mmol).

The Sequenase sequencing reagents were obtained from United States Biochemical (Ohio, USA). BCIP, NBT and some rabbit reticulocyte lysate were supplied by Promega. Tween 20 and ethanalamine were from Aldrich Chemical Co., and Whatmann International Ltd. supplied DE81 paper and various types of filter paper.

## METHODS

### 2.1 DNA manipulations

#### 2.1.1 Growth and maintenance of bacteria

##### 2.1.1.1 Bacterial strains

DNA manipulations were performed using various strains of Escherichia coli as host, as described overleaf.

Certain strains of Escherichia coli were used for specific experiments and this is indicated by the letter;

- a -for routine DNA manipulations.
- b -for M13 DNA manipulations.
- c -for protein expression.

| <u>Strain</u> | <u>Use</u> | <u>Genotype</u>  |
|---------------|------------|--|
| 71.18         | a, c       | <u>supE</u> thiA(lac-proAB)<br>F'[proAB <sup>+</sup> lacI <sup>q</sup> lacZAM15]   |
| TG1           | a, b, c    | <u>supE</u> hadA5thiA(lac-proAB)<br>F'[traD36proAB <sup>+</sup> lacI <sup>q</sup> lacZAM15]  |
| JM101         | c          | <u>supE</u> thiA(lac-proAB)<br>F'[traD36proAB <sup>+</sup> lacI <sup>q</sup> lacZAM15]   |
| JM109         | c          | <u>recA1</u> supE44endA1hdR17zvrA96relA1<br>thiA(lac-proAB)<br>F'[traD36proAB <sup>+</sup> lacI <sup>q</sup> lacZAM15]                             |
| DH5 $\alpha$  | a          | <u>supE44A1</u> lacU169( $\phi$ 80lacZAM15)hdR17<br><u>recA1</u> endA1zvrA96thi-1relA1   |
| XL1-Blue      | c          | <u>supE44</u> hdR17 <u>recA1</u> endA1zvrA46thi<br>relA1lac <sup>-</sup><br>F'[proAB <sup>+</sup> lacI <sup>q</sup> lacZAM15Tn(tet <sup>r</sup> )] |

#### 2.1.1.2 Bacterial culture

Culture of Escherichia coli strains for plasmid DNA manipulation was performed in L-broth (Luria-Bertani medium : 1% (w/v) NaCl, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract). For the culture of M13 virally infected Escherichia coli. 2xTY medium was used (0.5% (w/v) NaCl, 1.6% (w/v) bactotryptone, 1% (w/v) yeast extract). For some expression experiments, and the preparation of agar

plates for Escherichia coli strains 71.18, JM101, JM109, TG1, minimal media was prepared consisting of 1x M9 salts (12.8% (w/v)  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 3% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.5% NaCl, 1% (w/v)  $\text{NH}_4\text{Cl}$ ), 2 $\mu\text{g}/\text{ml}$  thiamine, 4% (w/v) glucose, 0.1M  $\text{CaCl}_2$ , 10mM  $\text{MgCl}_2$ , 10mM  $\text{MgSO}_4$ . For protein expression casamino acids were added to 1% (w/v).

Selection pressure for the retention of a plasmid within the Escherichia coli strain was provided by the inclusion of ampicillin to a final concentration of 50 $\mu\text{g}/\text{ml}$  in liquid culture, or 100 $\mu\text{g}/\text{ml}$  in agar plates.

#### 2.1.1.3 Maintenance of Escherichia coli strains

Bacterial strains were maintained on agar plates at 4°C for up to 1 month before restreaking onto fresh plates. Agar plates were prepared by the addition of bactoagar (1.5% (w/v)) to the appropriate culture medium.

Escherichia coli culture stocks were stored at -20°C in 50% (v/v) glycerol.

#### 2.1.2 Preparation of competent cells

##### 2.1.2.1 Calcium chloride method

-for the preparation of fresh cells.

10ml of L-broth was inoculated with the specific strain of E. coli required. The culture was incubated at 37°C overnight with shaking (200rpm). 1ml of this culture

was used to inoculate 49ml of fresh L-broth, which was incubated at 37°C / 200rpm until the optical density at 550nm was 0.45-0.5. The cells were harvested (2000 xg / 10 minutes / 4°C) and the supernatant discarded. The cell pellet was resuspended in 25ml ice cold, sterile 50mM CaCl<sub>2</sub> and left on ice for 30 minutes. The cells were pelleted at 2000 xg / 10 minutes / 4°C. The cell pellet was resuspended in 2ml ice cold, sterile 50mM CaCl<sub>2</sub> and left on ice before transformation (2.1.3). Cells prepared by this method were used within 24 hours.

#### 2.1.2.2 Rubidium chloride method

-for the preparation of cells for storage.

10ml of L-broth was inoculated with the specific strain of E. coli required. The culture was incubated at 37°C overnight with shaking (200rpm). 1ml of this culture was used to inoculate 100ml of prewarmed L-broth, which was incubated at 37°C / 200rpm until the optical density at 550nm was 0.45-0.5. The cells were cooled on ice for 10 minutes before harvesting by centrifugation at 1500 xg / 4°C / 5 minutes. The supernatant was discarded and the cell pellet resuspended in 30ml ice-cold RF1 and left on ice for 15 minutes. The cells were harvested at 1000 xg / 4°C / 5 minutes and the cell pellet resuspended in 8 ml ice-cold RF2. The cell suspension was left on ice for approximately 10 minutes before aliquoting into tubes for

storage at  $-80^{\circ}\text{C}$ . Cells were thawed on ice before transformation (2.1.3).

RF1 : 1.2% (w/v) RbCl + 0.99% (w/v)  $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$  + 30mM potassium acetate + 0.15% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  + 15% (w/v) glycerol.

pH 5.8 (0.2M acetic acid) , filter sterilised.

RF2 : 0.12% (w/v) RbCl + 1.1% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  + 10mM MOPS + 15% (w/v) glycerol.

pH 6.8 (NaOH) , filter sterilised.

### 2.1.3 Transformation of competent cells

#### 2.1.3.1 Transformation of plasmid DNA

100 $\mu\text{l}$  of ice cold competent cells (2.1.2) were used for each transformation. To the cells were added 10 $\mu\text{l}$  aliquots of a range of concentrations of plasmid DNA and incubated on ice for 30 minutes with occasional agitation. The cells were placed in a  $42^{\circ}\text{C}$  water bath for 2 minutes then cooled on ice for 5 minutes. The cells were plated onto dry agar plates and incubated overnight at  $37^{\circ}\text{C}$ .

#### 2.1.3.2 Transformation of M13 DNA

To 100 $\mu\text{l}$  ice-cold, competent TG1 cells were added a range of M13 DNA concentrations (single or double-stranded) and incubated on ice for 30 minutes. The cells

were placed in a 42°C water bath for 2 minutes then cooled on ice for 5 minutes. 3ml B-top (0.8% (w/v) bactotryptone, 0.5% (w/v) NaCl, 0.6% (w/v) bactoagar), 100µl log-phase TG1, 10µl 100mM IPTG, 25µl X-GAL (50mg/ml) were added to sterile glass tubes and maintained at 42°C. The transformed cells were added to the molten agar, mixed, and poured onto dry B-agar plates (0.8% (w/v) bactotryptone, 0.5% (w/v) NaCl, 2% (w/v) bactoagar) and incubated overnight at 37°C.

#### 2.1.4 Preparation of DNA

##### 2.1.4.1 Alkaline lysis miniprep

The method for preparation of DNA in small quantities was adapted from that described by Birnboim & Doly (1979).

A single colony was used to inoculate 10ml L-broth (+ 50µg/ml ampicillin) which was incubated overnight at 37°C with shaking (200rpm). 4.5ml of culture was harvested by centrifugation at 8000 xg for 2 minutes at room temperature. The cell pellet was resuspended in 80µl SET (1% (w/v) sucrose, 10mM EDTA, 25mM Tris-HCl (pH 8.0)) and placed on ice. To this suspension was added 10µl freshly made lysozyme (20mg/ml in SET) with mixing, and placed on ice for 5 minutes. 200µl of a freshly prepared lysis mix (0.2M NaOH + 1% (w/v) SDS) was added to the cell suspension with vortexing and placed on ice for 10

were placed in a 42°C water bath for 2 minutes then cooled on ice for 5 minutes. 3ml B-top (0.8% (w/v) bactotryptone, 0.5% (w/v) NaCl, 0.6% (w/v) bactoagar), 100µl log-phase TGI, 10µl 100mM IPTG, 25µl X-GAL (50mg/ml) were added to sterile glass tubes and maintained at 42°C. The transformed cells were added to the molten agar, mixed, and poured onto dry B-agar plates (0.8% (w/v) bactotryptone, 0.5% (w/v) NaCl, 2% (w/v) bactoagar) and incubated overnight at 37°C.

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minutes. 150 $\mu$ l 3M sodium acetate (pH 4.8) was added with vortexing and placed on ice for 15 minutes. The supernatant was cleared by centrifugation at 8000 xg for 10 minutes at room temperature and the pellet discarded. The supernatant was extracted twice with phenol/chloroform (2.1.5) and the aqueous layer precipitated with 3M sodium acetate (pH 6.0) (2.1.6). The washed, dried pellet was resuspended in 30 $\mu$ l T.E. and treated with 1 $\mu$ l RNase A (10mg/ml) for at least 1 hour at 37°C.

DNA prepared in this way was suitable for modification by restriction enzymes (2.1.7) and further transformation (2.1.3).

#### 2.1.4.2 Miniprep DNA for sequencing

Plasmid sequencing of miniprep DNA was facilitated by the use of an alternative DNA preparation method to the one previously described (2.1.4.1). This is a modification of the boiling lysis method described by Holmes and Quigley (1981).

10ml of L-broth (+ampicillin (50 $\mu$ g/ml)) was inoculated with 1 colony from an agar plate and incubated overnight at 37°C with shaking (200rpm). The culture was centrifuged at 3500rpm in a Beckman GPR bench centrifuge for 10 minutes at room temperature and the supernatant discarded. The cell pellet was resuspended in 100 $\mu$ l 25% (w/v) sucrose / 50mM Tris-HCl (pH 8) and the suspension

minutes. 150 $\mu$ l 3M sodium acetate (pH 4.8) was added with vortexing and placed on ice for 15 minutes. The supernatant was cleared by centrifugation at 8000 xg for 10 minutes at room temperature and the pellet discarded. The supernatant was extracted twice with phenol/chloroform (2.1.5) and the aqueous layer precipitated with 3M sodium acetate (pH 6.0) (2.1.6). The washed, dried pellet was resuspended in 30 $\mu$ l T.E. and treated with 1 $\mu$ l RNase A (10mg/ml) for at least 1 hour at 37°C.

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transferred to an Eppendorf microcentrifuge tube. A lysis solution was prepared consisting of 600 $\mu$ l M STET (5% (w/v) sucrose + 50mM Tris-HCl (pH 8) + 5% (v/v) Triton X-100) + 7 $\mu$ l freshly made 50mg/ml lysozyme (in 50mM Tris-HCl (pH 8)) for each cell sample. This lysis solution was added to the 100 $\mu$ l cell suspension, mixed by inversion then boiled for 60 seconds in a boiling water bath. The tubes were placed on ice for 5 minutes before spinning at 8000 xg at room temperature for 45-60 minutes until the cell pellet was tight. The pellet was discarded. To the supernatant was added 1 $\mu$ l RNase A (10mg/ml) and incubated at 37°C for 30 minutes. The supernatant was extracted twice with 300 $\mu$ l phenol/chloroform and the aqueous layer precipitated at -20°C for 30 minutes with 1/10th volume 3M sodium acetate (pH 6.0) + equal volume of isopropanol. The precipitate was spun at 8000 xg for 10 minutes at room temperature. The pellet was resuspended in 100 $\mu$ l low T.E. (10mM Tris-HCl (pH 7.5), 0.1mM EDTA) and reprecipitated twice with 3M sodium acetate (pH 6) and 100% ethanol (2.1.6). The final pellet was resuspended in 25 $\mu$ l T.E.. An aliquot (2-3 $\mu$ l) was digested with an appropriate restriction enzyme before visualisation on agarose gels (2.1.8).

The DNA was rapidly purified in a spin dialysis step using Sepharose CL-6B or Biogel P6. A column of this material was constructed in a microeppendorf tube, previously punctured at its tip (with a needle (23G x 1 $\frac{1}{2}$ )). The hole was blocked with acid-washed Ballantine No.

1 glass beads and the column material packed on top by centrifugation at 3500rpm for 3 minutes in a bench centrifuge.

Approximately 10 $\mu$ g of DNA was denatured by the addition of 1 $\mu$ l 5M NaOH and incubated at room temperature for 5 minutes. The DNA was placed on the surface of the column and centrifuged at 3000 xg for 3 minutes at room temperature. The DNA that was recovered was directly used for sequencing (2.1.12.2).

#### 2.1.4.3 Large scale DNA preparation-alkaline lysis

The method followed was an adaptation of the alkaline lysis preparation of miniprep DNA (Birnboim & Doly (1979)).

40ml of L-broth (+ ampicillin (50 $\mu$ g/ml)), inoculated with an E. coli strain containing a plasmid of interest, was incubated overnight at 37°C with shaking (200rpm). The cells were pelleted by centrifugation at 2000 xg for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 2ml ice cold SET (15% (w/v) sucrose, 10mM EDTA, 25mM Tris-HCl (pH 8.0)). To this cell suspension was added 0.2ml freshly prepared lysozyme (20mg/ml) and placed on ice for 5 minutes. 4ml of freshly made lysis mix (0.2M NaOH, 1% (w/v) SDS) was added with vortexing and left on ice for 10 minutes. 3ml of 3M sodium

acetate (pH 4.8) was added with vortexing and placed on ice for a further 15 minutes. The cell debris was pelleted by centrifugation at 17,500 xg for 20 minutes at 4°C and the supernatant carefully removed. The supernatant was extracted twice with a phenol/chloroform mixture and back extracted once with T.E. (2.1.5). The DNA was precipitated by the addition of a 1/10th volume of 3M sodium acetate (pH 6.0) + 2 volumes of 100% ethanol to the aqueous layer and placed on dry ice for 20 minutes. The DNA was pelleted by centrifugation at 8000 xg for 15 minutes at 4°C. The DNA pellet was washed with 800µl 70% ethanol, transferred to an eppendorf tube, dried in vacuo. and resuspended in 100µl T.E.. To this was added 3µl RNase A (10mg/ml) and incubated at 37°C for at least 1 hour.

DNA prepared in this way was suitable for restriction enzyme modification, further cloning procedures and transformation. For use in transcription reactions (2.3.1) the DNA was further cleaned by 2 additional phenol/chloroform extractions. Approximately 2.5µg of DNA per ml culture was produced.

#### 2.1.4.4 Large scale DNA preparation-lysis by boiling

400ml of L-broth (+ ampicillin to 50µg/ml) was inoculated with an E. coli strain containing a DNA plasmid of interest. This culture was incubated overnight in a 2 litre conical flask at 37°C with shaking (200rpm). The

cells were harvested by centrifugation at 2000 xg for 5 minutes at 4°C and the supernatant discarded. The cell pellet was resuspended in 7.2ml SET (25mM Tris-HCl (pH 8.0), 10mM EDTA, 15% (v/v) sucrose). To this was added 7.2 ml lysozyme (4mg/ml in SET) and incubated at room temperature for 5 minutes. 12ml of 10% (v/v) Triton X-100 was added and the mix heated over a bunsen flame with constant swirling until the cell suspension started to boil. The flask was plunged into an ice/water bath, swirled to cool and then cooled on ice for 10 minutes. The cell debris was pelleted by centrifugation at 31,500 xg for 30 minutes at 4°C and the pellet discarded. To the supernatant was added ¼ volume 7.5M ammonium acetate and incubated on ice for 20 minutes. The suspension was centrifuged at 17,500 xg for 10 minutes at 4°C to pellet the protein and the pellet discarded.

To the supernatant was added 0.7 volumes of isopropanol and the precipitating mix placed at -20°C for at least 10 minutes. The DNA was pelleted by centrifugation at 17,500 xg for 10 minutes at 4°C and the supernatant discarded. The DNA pellet was resuspended in 4ml low T.E. (10mM Tris-HCl (pH 8.0), 0.1mM EDTA). Into this DNA was dissolved 4.3g caesium chloride + 0.5ml ethidium bromide (5mg/ml). The solution was transferred to an ultracentrifuge tube (Beckman Quick-seal). Separation of the plasmid DNA from the chromosomal DNA, protein, and RNA was achieved by centrifugation of the DNA preparation

through the caesium chloride gradient formed during centrifugation at 52000rpm for 12 hours at 20°C (Vti 65.1 rotor in Beckman L8-70 ultracentrifuge).

The plasmid DNA was visualised under UV light and the DNA carefully removed using a syringe and needle. The plasmid solution was transferred to an eppendorf tube and the ethidium bromide removed by repeated extraction with a solution of isopropanol equilibrated with caesium chloride saturated T.E.. The tube was filled with this solution, shaken, and the upper pink layer discarded. When the lower phase was no longer pink the top phase was discarded and 4 volumes of T.E. was added. The plasmid DNA was precipitated by the addition of 1/10th volume 3M sodium acetate (pH 6.0) + 2 volumes of 100% ethanol and placed at -20°C for at least 2 hours. The DNA was pelleted by centrifugation at 17,500 xg for 10 minutes at 4°C and the supernatant discarded. The DNA was washed in 70% ethanol, dried in vacuo and resuspended in 400µl T.E.

DNA prepared by this method was highly purified and was suitable for restriction enzyme modification, transformation and transcription reactions.

#### 2.1.4.5 Preparation of single-stranded M13 DNA

M13 DNA in the replicative form (RF) consists of + and - strand DNA. Single-stranded M13 DNA represents the + strand. To prepare large amounts of this strand for

sequencing (2.1.12.1) or mutagenesis (2.1.11) the following protocol was used.

An overnight culture of Escherichia coli TG1 was prepared in 10ml L-broth. To sterile, narrow-necked glass bottles was added 3.5ml L-broth, 100 $\mu$ l of the overnight TG1 culture and a plaque plug (removed from the agar plate using a 200 $\mu$ l Gilson tip) from an agar plate from an M13 transformation (2.1.3.2). This culture was incubated at 37°C for 5 $\frac{1}{2}$  hours with shaking (300rpm). 1.5ml of culture was centrifuged at 8000 xg at room temperature for 5 minutes. The supernatant was decanted into a clean eppendorf and the pellet discarded. To the supernatant was added 150 $\mu$ l 20% (w/v) PEG 6000 / 2.5M NaCl, mixed, and incubated at room temperature for at least 20 minutes to aggregate the viral particles. The solution was centrifuged for 5 minutes at 8000 xg at room temperature and the PEG solution removed. The tubes were respun and the remaining PEG solution removed by aspiration. The pellet was resuspended in 120 $\mu$ l T.E., to which was added 50 $\mu$ l phenol / T.E. (2.1.5). This solution was vortexed vigorously and centrifuged at 8000 xg for 5 minutes at room temperature. 100 $\mu$ l of the aqueous layer was transferred into a clean eppendorf. If the aqueous layer was cloudy an ether extraction was performed. 1.4ml of ether was added to the aqueous layer and mixed vigorously until the aqueous layer was clear. The majority of the ether was removed by pipette and the excess removed by

evaporation under a stream of nitrogen gas.

The DNA was precipitated from the aqueous layer (2.1.6) and the dried pellet resuspended in 22 $\mu$ l T.E.. 2 $\mu$ l of this solution was examined by electrophoresis in an agarose gel (2.1.8.1).

#### 2.1.4.6 Preparation of double-stranded M13 DNA

25 $\mu$ l of log phase Escherichia coli TG1 was used to inoculate 10ml L-broth, into which was added a plaque plug removed from an agar plate with a 200 $\mu$ l pipette tip. This culture, containing M13 virus, was incubated overnight at 37°C with shaking (200rpm). 40ml of L-broth was inoculated with 1ml of a TG1 overnight culture and incubated at 37°C / 200rpm until the optical density at 550nm was 0.5-0.6. 0.1ml from the M13 containing overnight was added and the culture incubated at 37°C for 4 hours at 300rpm. The viral double-stranded DNA was isolated from this culture using the alkaline lysis protocol as described in section 2.1.4.3.

#### 2.1.5 Preparation and use of phenol

##### 2.1.5.1 Preparation of phenol and phenol/chloroform

Highly pure phenol crystals were melted at 65°C before equilibration with Tris-HCl (pH 8.0) as described

in Sambrook et al. (1989). Preparation of phenol/T.E. and phenol/chloroform was as described in Sambrook. Phenol solutions were stored in darkened bottles at 4°C.

#### 2.1.5.2 Use of phenol solutions

In order to partition nucleic acids and protein, an equal volume of phenol/chloroform was added to the sample and mixed vigorously for 20 seconds. To separate the aqueous and organic (phenol) layers the sample was centrifuged for 5 minutes at 13000rpm (appendorf tubes) or 2 minutes at 4000rpm (Beckman J2-21M/E). The upper aqueous layer was transferred to a clean tube and an equal volume of phenol/chloroform added, mixed, and centrifuged. The aqueous layer was removed and the DNA/RNA precipitated (2.1.6).

For some preparation methods a 'back extraction' with T.E. (10mM Tris-HCl (pH 7.5), 1mM EDTA) was performed. After the first phenol/chloroform extraction a  $\frac{1}{4}$  volume of T.E. was added to the phenol layer, mixed, centrifuged, and the resultant aqueous phase added to the first aqueous layer removed. The combined aqueous phase was then extracted with phenol/chloroform as described.

### 2.1.6 Precipitation of nucleic acids

DNA was generally precipitated from aqueous solutions by the addition of 1/10th volume 3M sodium acetate (pH 6.0) and 2 volumes of 100% ethanol (-20°C). The solutions were mixed and placed at -20°C for at least 2 hours, or onto dry ice for 20 minutes. The DNA was pelleted by centrifugation at 8000 xg for 15 minutes and the ethanol supernatant discarded. 70% (v/v) ethanol (-20°C) was added to the DNA pellet and centrifuged for 5 minutes at 8000 xg to wash the pellet. The ethanol solution was discarded and the DNA pellet dried in vacuo for 5 minutes. The DNA was resuspended in T.E. (10mM Tris-HCl (pH 7.5), 1mM EDTA) and stored at -20°C.

RNA was precipitated with 2M sodium acetate (pH 6.0) and was generally precipitated for 30 minutes before centrifugation. Further alterations to the above method are outlined in the other method sections.

### 2.1.7 Enzymatic modification of DNA

#### 2.1.7.1 Restriction endonuclease digestion

Modification of DNA by restriction endonucleases was performed at 37°C for 2 hours. A 10x stock of three buffers was prepared consisting of 0.5M Tris-HCl (pH 7.5), 0.1M MgCl<sub>2</sub>, 10mM DTT + 1M NaCl, or + 0.5M NaCl, or with no

NaCl added (stored at  $-20^{\circ}\text{C}$ ). In digestion experiments, buffers were chosen to be nearest the NaCl concentration which gave the optimum activity of the enzyme.

#### 2.1.7.2 Ligation

DNA fragments with compatible cohesive termini were ligated together using T4 DNA ligase. Equimolar amounts of DNA (usually 50-100ng) to be ligated were added to a solution of 10mM DTT, 1mM ATP, 10mM  $\text{MgCl}_2$ , 50mM Tris-HCl (pH 7.4) and 2 units of T4 DNA ligase. The mix was incubated for 2 hours at room temperature or overnight at  $16^{\circ}\text{C}$ .

#### 2.1.7.3 Phosphorylation of oligonucleotides

Oligonucleotides were phosphorylated before use in mutagenesis reactions (2.1.11) and also in order to check the integrity of the DNA (by phosphorylation of the 5' terminus of the DNA with radioactive phosphate and visualising by autoradiography). A kinase buffer was prepared to 10x working concentration ; 1M Tris-HCl (pH 8.0), 100mM  $\text{MgCl}_2$ , 70mM DTT, 10mM ATP (omitted when labelling DNA with radioactive phosphate) and stored at  $-20^{\circ}\text{C}$ . 2.5 $\mu\text{l}$  oligonucleotide (5  $\text{OD}_{260}/\text{ml}$ ) was added to 3 $\mu\text{l}$  10x kinase buffer, 25 $\mu\text{l}$  water and 0.5 $\mu\text{l}$  T4 DNA polynucleotide kinase. For the preparation of radioactive

DNA, 0.5 $\mu$ l (10 $\mu$ Ci) gamma <sup>32</sup>P ATP was added. The kinase was incubated at 37°C for 15 minutes before inactivation by incubation at 70°C for 10 minutes.

## 2.1.8 Electrophoresis of DNA and oligonucleotides

### 2.1.8.1 Agarose gel electrophoresis of DNA

DNA samples were mixed with a volume of 10x loading buffer (30% (v/v) glycerol in 10x running buffer + 0.25% (w/v) bromophenol blue). An agarose gel was cast in a 11cm x 14cm gel former using a concentration of agarose (0.8-2.0% (w/v)) appropriate to the range of DNA fragment sizes that were to be separated. The agarose was dissolved in 1xTAE (40mM Tris-HCl (pH 8.0), 20mM sodium acetate, 1mM EDTA) for gels to be used for DNA isolation (2.1.11) or in 1xTBE (90mM Tris, 90mM boric acid, 2mM EDTA) for routine DNA analysis. Ethidium bromide was added to a final concentration of 0.5 $\mu$ g/ml. Samples were subjected to horizontal electrophoresis in BRL series H5 apparatus. The agarose gels were submerged in a tank buffer of 1xTAE or 1xTBE with ethidium bromide at 0.5 $\mu$ g/ml and electrophoresis performed at a constant voltage of 50-120V until the fragments had separated. DNA was visualised using a UV transilluminator and the gel photographed using Polaroid 667 film (f11 for 1 second).

### 2.1.8.2 DNA polyacrylamide gels

For separation and visualisation of small DNA fragments (<1000bp) a polyacrylamide gel was used. The gel was cast in BRL apparatus (16cm x 20cm) with 1mm spacers. For a 10% gel the following mix was prepared:

|   |          |
|---|----------|
| 10x TBE                                 | 3.0 ml   |
| (0.9M Tris, 0.9M boric acid, 20mM EDTA) |          |
| 30% (w/v) acrylamide /                  | 10.06 ml |
| 0.8% (w/v) bis-acrylamide               |          |
| 50% (v/v) glycerol                      | 3.0 ml   |
| Distilled water                         | 13.94 ml |

Polymerisation was initiated by the addition of 14.4µl TEMED + 192µl freshly made 10% (w/v) APS. The cast gel was placed in a vertical electrophoresis tank with 1x TBE as buffer in the top and bottom reservoirs. DNA samples were mixed with 0.2 volumes of a 5x loading buffer (7mg bromophenol blue + 7mg xylene cyanol + 2ml 0.2M EDTA + 7.5ml 50% (v/v) glycerol) and loaded into the wells of the gel with a Hamilton syringe. Electrophoresis was performed at a constant current of 45mA until the dye fronts had migrated sufficiently through the gel (with a 10% gel the bromophenol blue migrates at a position equivalent to approximately 33 bp DNA, xylene cyanol equivalent to 110bp).

The gel was removed from the casting apparatus and stained in 500ml 1x TBE (+ 50µl ethidium bromide (5µg/ml)) for 20 minutes. After staining the gel was destained for 5 minutes by shaking in 500ml distilled water. The DNA bands were visualised with UV light.

### 2.1.8.3 Sequencing gel electrophoresis

Products from sequencing reactions (2.1.12) were subjected to vertical electrophoresis through a 38cm polyacrylamide / urea gradient gel. The gradient mixes were prepared as indicated below:

|   | 0.5x  | 5.0x  |
|---|-------|-------|
| 40% (w/v) acrylamide                              | 75ml  | 30ml  |
| 2% (w/v) bis-acrylamide                           |       |       |
| 10xTBE (0.9M Tris,<br>0.9M boric acid, 20mM EDTA) | 25ml  | 100ml |
| Urea  | 230g  | 92g   |
| Bromophenol blue                                  | 0     | 10mg  |
| Made up with water to                             | 500ml | 200ml |

From the above mixes, two solutions were prepared :

Solution A. 30ml 0.5x mix + 60µl TEMED + 60µl 25% (w/v) APS.

Solution B. 7ml 5x mix + 14 $\mu$ l TENED + 14 $\mu$ l 25% (w/v) APS.

5ml of solution A was taken up into a 20ml syringe followed by 6ml of solution B. A gradient was formed in the syringe by drawing 3-5 air bubbles through the solutions. The gradient was poured into the gel casting apparatus and followed by the remaining solution A. The comb was inserted and the gel allowed to polymerise horizontally.

Once set, the gel was placed in a vertical electrophoresis tank with 1xTBE as buffer in the upper and lower reservoirs. The comb was removed and the wells washed out with buffer to remove urea and acrylamide debris. Samples (prepared as in section 2.1.12) were heated to 75°C for 5 minutes, loaded into the gel, and subjected to electrophoresis at a constant 38 W for 1.5-4 hours. The gel was removed from the tank, submerged in a fixing solution (10% (v/v) glacial acetic acid, 10% (v/v) methanol) for 15 minutes and dried onto Whatman paper. The dry gel was exposed to X-ray film at room temperature and the film developed (2.2.5.3b).

#### 2.1.8.4 Visualisation of oligonucleotides

Radioactive oligonucleotides (2.1.7.3) were examined by electrophoresis through 16% (w/v) polyacrylamide / urea gels. A gel mix (80ml 40% (w/v) acrylamide / 2% (w/v) bis-

acrylamide (section 2.1.8.3) + 20ml 10xTBE + 92g urea, made up to 200ml with water) was prepared and stored at 4°C. For each gel, 45ml of the above mix was polymerised by the addition of 90µl TEMED and 90µl 25% (w/v) APS and the gel poured into sequencing gel casting apparatus. 6µl of freshly phosphorylated oligonucleotide was mixed with 6µl formamide loading buffer (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol), heated to 80°C for 15 minutes, loaded onto the gel and subjected to electrophoresis at a constant 38 W with 1xTBE as buffer. The gel was removed from the glass plates, covered with cling film and placed at -80°C next to X-ray film for 10-20 minutes. The film was developed and the integrity and size of the oligonucleotide examined.

#### 2.1.9 Estimation of size and concentration of DNA

##### 2.1.9.1 Estimation of DNA concentration

The concentration of DNA in aqueous solution could be estimated from the optical density at 260nm. At this wavelength the optical density of double-stranded DNA at a concentration of 50µg/ml is 1 absorbance unit, for single-stranded DNA 40µg/ml is equivalent to 1 absorbance unit (Sambrook et al (1989)).

The concentration of linearised DNA fragments could be estimated by visualising the stained DNA after agarose gel electrophoresis (2.1.8.1) and comparing the intensity of the stain to known DNA concentrations on the same gel. Linearised pBR322 DNA of known concentration was prepared by digestion of commercially available pBR322 DNA with the restriction endonuclease EcoR1 for 2 hours at 37°C.

#### 2.1.9.2 Estimation of DNA size

For the estimation of DNA size during electrophoresis (2.1.8.1-2) commercially available DNA was digested with restriction endonucleases to prepare fragments of known size. These DNA samples were loaded into the electrophoresis gel alongside the DNA to be examined.

Two DNA marker populations were prepared :

| <u>EcoRI-HindIII</u>          |            | <u>HinfI</u>                |            |
|-------------------------------|------------|-----------------------------|------------|
| DNA (0.4 $\mu$ g/ $\mu$ l)    | 50 $\mu$ l | DNA (0.5 $\mu$ g/ $\mu$ l)  | 10 $\mu$ l |
| lambda                        |            | pBR322                      |            |
| Buffer                        | 10 $\mu$ l | Buffer                      | 5 $\mu$ l  |
| <u>EcoRI</u> (127U/ $\mu$ l)  | 1 $\mu$ l  | <u>HinfI</u> (12U/ $\mu$ l) | 2 $\mu$ l  |
| <u>HindIII</u> (10U/ $\mu$ l) | 9 $\mu$ l  | Water                       | 33 $\mu$ l |
| Water                         | 30 $\mu$ l |                             |            |

Fragment size (base-pairs) :

|             |           |
|-------------|-----------|
| 21226       | 1632      |
| 5148 / 4973 | 517 / 506 |
| 4277        | 396       |
| 2027        | 344       |
| 1904        | 298       |
| 1584        | 221 / 220 |
| 1330        | 154       |
| 983         | 75        |
| 831         |           |
| 564         |           |

### 2.1.10 Isolation of DNA from agarose gels

#### 2.1.10.1 DES1 method

DES1 paper was prepared by soaking in 2.5M NaCl for 60 minutes, washing in distilled water, then stored in 1mM EDTA at 4°C.

DNA fragments were separated by agarose gel electrophoresis and the position of the fragment to be isolated visualised under UV light. The agarose gel was cut, with a clean razor blade, close to the band to be isolated on the side nearest the positive electrode. A piece of DES1 paper, presoaked in 1xTAE, was inserted into the gel so as to cover the entire DNA band width. Excess tank buffer was removed such that the gel was not submerged. The DNA fragment was run onto the DES1 paper at 60mA, the paper was removed and washed briefly in distilled water before being dried between filter paper. The paper was cut into small pieces and transferred to an eppendorf tube. To this tube was added 400µl elution buffer (20mM Tris-HCl (pH 7.5), 1mM EDTA, 1.5mM NaCl) and vortexed vigorously. The tube was incubated overnight at 37°C to elute the DNA from the paper.

The DNA solution and paper fragments were centrifuged at 8000 xg for 2 minutes through siliconised glass wool. The DNA solution was shaken vigorously with 2-3 volumes of butanol saturated water and the top layer discarded. To

## 2.1.10 Isolation of DNA from agarose gels

### 2.1.10.1 DE81 method

DE81 paper was prepared by soaking in 2.5M NaCl for 60 minutes, washing in distilled water, then stored in 1mM EDTA at 4°C.

DNA fragments were separated by agarose gel electrophoresis and the position of the fragment to be isolated visualised under UV light. The agarose gel was cut, with a clean razor blade, close to the band to be isolated on the side nearest the positive electrode. A piece of DE81 paper, presoaked in 1xTAE, was inserted into the gel so as to cover the entire DNA band width. Excess tank buffer was removed such that the gel was not submerged. The DNA fragment was run onto the DE81 paper at 60mA, the paper was removed and washed briefly in distilled water before being dried between filter paper. The paper was cut into small pieces and transferred to an eppendorf tube. To this tube was added 400µl elution buffer (20mM Tris-HCl (pH 7.5), 1mM EDTA, 1.5mM NaCl) and vortexed vigorously. The tube was incubated overnight at 37°C to elute the DNA from the paper.

The DNA solution and paper fragments were centrifuged at 8000 xg for 2 minutes through siliconised glass wool. The DNA solution was shaken vigorously with 2-3 volumes of butanol saturated water and the top layer discarded. To

the DNA was added 2 volumes of 100% ethanol and precipitated on dry ice for 20 minutes. The DNA was pelleted by centrifugation at 8000 xg for 15 minutes at 4°C, washed with 70% ethanol and dried in vacuo. The pellet was resuspended in T.E.

#### 2.1.10.2 Whatman paper method

DNA fragments were separated by agarose gel electrophoresis and the position of the fragment to be isolated visualised under UV light. The agarose gel was cut, with a clean razor blade, close to the band to be isolated on the side nearest the positive electrode. A piece of Whatman No. 1 filter paper and a slightly larger piece of dialysis tubing (prepared as Sambrook et al. (1989)) were soaked in 1xTAE and placed in the cut agarose gel such that the paper was between the DNA and the dialysis tubing, the dialysis tubing being furthest towards the positive electrode. Excess tank buffer was removed such that the gel was not submerged. The DNA fragment was run onto the paper at 60mA, the current was reversed for 10 seconds and the paper + tubing removed to an eppendorf. The paper was washed at least 3 times with 100µl T.E., the DNA-containing eluent being collected in a 2ml eppendorf tube. The final solution was centrifuged at 8000 xg for 5 minutes to pellet any debris. The supernatant was transferred to a clean tube and the DNA

precipitated as described in section 2.1.6. The DNA was resuspended in T.E..

### 2.1.11 Mutagenesis

#### 2.1.11.1 Preparation of oligonucleotides

Oligonucleotides were designed for efficient hybridisation in order to create specific mutations when used in mutagenesis reactions. The following oligonucleotides were obtained :

E177K 5' 521**TGATTTC**AAAAGCAGCAAG<sup>539</sup> 3'  
E177A 5' 522**GATTTCA**GCTGCAGCAAG<sup>539</sup> 3'  
E177D 5' 523**ATTTCAG**CGCAGCAAG<sup>539</sup> 3'  
SEAAE 5' 552**AATATAT**TGGAA[ ]AATCATTGGAT<sup>514</sup> 3'

The 4 oligonucleotides described above were generous gifts from ICI Pharmaceuticals. Nucleotides indicated in bold type represent mismatches. [ ] represents the residues coding for amino acids Ser176 to Arg180 inclusive that were deleted.

The 4 oligonucleotides detailed overleaf were purchased from Research Genetics (USA)

R29A 5' 73ATCAGAGCTGTTCGCCGGTCGTTAACA<sup>99</sup> 3'  
R180Q 5' 526TCAGAAGCAGCACAATTCCAATATATT<sup>552</sup> 3'  
R180A 5' 526TCAGAAGCAGCAGCAATTCCAATATATT<sup>552</sup> 3'  
R180M 5' 526TCAGAAGCAGCAATGTTCCAATATATT<sup>552</sup> 3'

Nucleotide numbers refer to those given for the prepropricin cDNA (Lamb et al. (1985))- see appendix.

Oligonucleotides were phosphorylated (2.1.7.3) and checked by electrophoresis (2.1.8.4) to determine size and integrity. The concentration of the oligonucleotides was adjusted to 10µg/ml with distilled water.

#### 2.1.11.2 Mutagenesis reactions

Mutagenesis was performed using the Amersham oligonucleotide-directed in vitro mutagenesis system. The theory behind this high efficiency approach is described in Taylor et al. (1985).

Single-stranded M13 template DNA was prepared as described in section 2.1.4.6, the concentration was estimated and approximately 5µg DNA used per mutagenesis reaction. The single-stranded template and the kinased oligonucleotide were annealed by heating to 70°C for 3

minutes then cooling slowly to room temperature (approximately 30 minutes). TG1 cells, made competent by the method described in section 2.1.2.2, were used for transformation (2.1.3.2).

Non-blue plaques were picked for preparation of single-strand DNA for sequencing (2.1.12.1).

#### 2.1.12 DNA sequencing

##### 2.1.12.1 Sequencing of single-stranded M13 DNA

Single-strand M13 DNA was prepared as described in section 2.1.4.6. Sequencing primers were used at 10 $\mu$ g/ml. 1-2 $\mu$ g of single-strand DNA was annealed to 10ng primer in buffer (40mM Tris-HCl (pH 7.5), 20mM MgCl<sub>2</sub>, 50mM NaCl) by heating to 65°C for 2 minutes then allowing the temperature to decrease slowly to below 35°C.

Sequencing reactions were performed using the USB Sequenase system as described by the manufacturer. Sequenase is a modified bacteriophage T7 DNA polymerase (Tabor et al., 1987) and the sequencing system is based on the dideoxynucleotide termination method described by Sanger et al. (1977). 3-5 $\mu$ l of the terminated reactions was loaded onto a denaturing polyacrylamide gel as described in section 2.1.8.3. Sequencing primers are described in the appendix.

### 2.1.12.2 Sequencing of double-stranded plasmid DNA

DNA was prepared as described in section 2.1.4.2. Approximately 8 $\mu$ l (4 $\mu$ g) of denatured plasmid DNA was annealed to 10ng sequencing primer in the manner described above. The DNA was sequenced using the Sequenase system as described.

## 2.2 Protein techniques

### 2.2.1. Estimation of protein concentration

#### 2.2.1.1 Dye-binding assay

Protein concentration was estimated using the Bio-rad protein assay (based on Bradford, 1976). Two assay procedures were used : the standard assay for protein concentrations of 0.2 to 1.4 mg/ml, and the microassay procedure for concentrations of less than 25  $\mu$ g/ml. The standard protein chosen for the assays was bovine gamma globulins Cohn fraction II (approx 99% gamma globulins). Assays of the standard protein and the samples were performed in duplicate and the mean results recorded. A new standard curve was constructed for each set of new samples tested. All measurements were recorded within 60 minutes of addition of the colour reagent, as suggested by the manufacturer.

### 2.2.1.2 Optical density at 260nm + 280nm

For rapid screening of crude protein preparations, or when a rough estimate of concentration was sufficient, the mean absorbance of duplicate samples at 260nm and 280nm was recorded. Concentration was estimated from the formula:

$$\text{mg/ml} = 1.55 \text{ OD}_{280} - 0.76 \text{ OD}_{260}$$

### 2.2.2 Protein precipitation

Protein samples were often prepared for further experiments by precipitation. Three methods of precipitation could be employed.

#### 2.2.2.1 Acetone

For the analysis of proteins on SDS-PAGE (2.2.3), protein samples of low concentration were precipitated with acetone to facilitate increased loading of polypeptides for visualisation (2.2.5). Samples were mixed with an equal volume of 100% acetone (-20°C) and placed at -20°C for at least 30 minutes. The protein was pelleted by centrifugation at 8000 xg for 30 minutes at 4°C. The supernatant was removed and the pellet allowed to dry before resuspending in buffer.

#### 2.2.2.2 Trichloroacetic acid

An additional method to acetone precipitation that was used for preparation of samples for SDS-PAGE was the use of trichloroacetic acid (TCA). Ice-cold TCA (100% (w/v)) was added to the protein sample to a concentration of 10% (w/v), mixed and incubated on ice for 15 minutes. The sample was centrifuged for 15 minutes at 8000 xg at 4°C and the waste TCA solution discarded. The acidic protein pellet was washed twice with 100% (-20°C) acetone by centrifugation at 8000 xg for 3 minutes. The acetone was removed and the pellet dried.

#### 2.2.2.3 Ammonium sulphate

The differential precipitation properties of proteins in ammonium sulphate solutions was exploited during initial protein purification experiments. Since ammonium sulphate precipitation does not denature the proteins (in contrast to the TCA method) it could be used to prepare active enzyme samples. Solid ammonium sulphate (amount determined by reference to tables in Sambrook et al., 1989) was added to the protein solution, with stirring, at 4°C. Protein was pelleted by centrifugation at 8000 xg for 30 minutes at 4°C.

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### 2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analysed on polyacrylamide gels using the discontinuous buffer system originally described by Laemmli (1970). The percentage polyacrylamide chosen was dependent on the size range of the polypeptides to be separated. For routine analysis of protein preparations, small polyacrylamide gels (11.5cm x 16cm) were used with a final acrylamide concentration in the resolving gel of 15% (w/v). The gel mixes for this type of gel were:

| 15% (w/v) acrylamide gel. | ml solution |          |
|---------------------------|-------------|----------|
|                           | Resolving   | Stacking |
| Acrylamide stock          | 10.0        | 2.0      |
| 30% (w/v) acrylamide      |             |          |
| 0.8% (w/v) bis-acrylamide |             |          |
| 0.5M Tris-HCl (pH 6.8)    | --          | 2.5      |
| 3.0M Tris-HCl (pH 8.8)    | 2.5         | --       |
| SDS (10% (w/v))           | 0.2         | 0.1      |
| H <sub>2</sub> O          | 7.0         | 5.0      |

Polymerisation of the resolving gel mix was initiated by the addition of 50 $\mu$ l TENED + 200 $\mu$ l APS (10% (w/v)) with mixing. This mix was quickly poured into the gel casting apparatus and a layer of ethanol placed on the surface of the polymerising resolving gel. Once set, the ethanol was washed off the gel with distilled water, the excess water

was removed, and the stacking gel mixture (polymerisation initiated by the addition of 50 $\mu$ l TEMED and 100 $\mu$ l AFS (10% (w/v)) to the above mixture) poured onto the resolving gel. The gel comb (14 or 21 wells) was inserted carefully. Once set, the comb was removed and the gel placed into a vertical electrophoresis tank with buffer (freshly diluted from 10 x stock) in both top and bottom reservoirs containing 25mM Tris-HCl (pH 8.8), 192mM glycine and SDS (0.1% (w/v)).

Before loading the protein samples the gel was pre-run at 30mA for 5 minutes. Protein samples were mixed with loading buffer and heated to 100°C in a boiling water bath for 5 minutes. Loading buffer was prepared as indicated:

|                        |         |         |
|------------------------|---------|---------|
|                        | 3 x     | 1 x     |
| 0.5M Tris-HCl (pH 6.8) | 3.75ml  | 1.25ml  |
| glycerol               | 5.0 ml  | 2.0 ml  |
| SDS                    | 0.6 g   | 0.2 g   |
| 2-mercaptoethanol      | 1.5 ml  | 0.5 ml  |
| water                  | ---     | 6.25ml  |
| Bromophenol blue       | 30.0 mg | 10.0 mg |

The samples were loaded into the wells of the gel using a Hamilton syringe. Gels were usually run until the dye front had migrated to the bottom of the gel such that 30kD polypeptides were in the centre of the resolving gel (approximately 3 hours at a constant current of 30mA).

Occasionally, larger polyacrylamide gels were prepared (17.5cm x 20cm) with a final polyacrylamide concentration of 10% or 7% in the resolving gel. The solutions required for the construction of such gels is shown below:

|                                | ml solution |          |
|--------------------------------|-------------|----------|
| 10% (w/v) acrylamide gel.      | Resolving   | Stacking |
| Acrylamide stock               | 13.3        | 2.5      |
| 30% (w/v) acrylamide           |             |          |
| 0.8% (w/v) bis-acrylamide      |             |          |
| 0.5M Tris-HCl (pH 6.8)         | --          | 5.0      |
| 3.0M Tris-HCl (pH 8.8)         | 5.0         | --       |
| SDS (10% (w/v))                | 0.4         | 0.2      |
| H <sub>2</sub> O               | 19.3        | 11.3     |
| APS (10% (w/v))                | 0.3         | 0.15     |
| TEMED                          | 20µl        | 15µl     |
| <b>7% (w/v) acrylamide gel</b> |             |          |
| Acrylamide stock               | 10.5        | 2.5      |
| 30% (w/v) acrylamide           |             |          |
| 0.8% (w/v) bis-acrylamide      |             |          |
| 0.5M Tris-HCl (pH 6.8)         | --          | 5.6      |
| 3.0M Tris-HCl (pH 8.8)         | 5.6         | --       |
| SDS (10% (w/v))                | 0.45        | 0.23     |
| H <sub>2</sub> O               | 25.51       | 14.35    |
| APS (10% (w/v))                | 0.15        | 0.15     |
| TEMED                          | 0.15        | 0.15     |

The polypeptides were visualised by staining (2.2.5.1) or by Western blotting (2.2.5.2).

#### 2.2.4 Estimation of protein size

The size of polypeptides was estimated by examining the position of the polypeptide on SDS-PAGE gels (2.2.3) relative to polypeptides of known size loaded on the same gel. Two types of protein markers were used.

##### 2.2.4.1 Pre-stained molecular weight markers

Pre-stained molecular weight markers were used for SDS-PAGE when the gel was to be Western blotted (2.2.5.2) or stained (2.2.5.1) to visualise the protein. The approximate sizes of the marker polypeptides are indicated below.

| Proteins                  | Apparent molecular weight (daltons) |
|---------------------------|-------------------------------------|
| Phosphorylase B           | 106000                              |
| Bovine serum albumin      | 80000                               |
| Ovalbumin                 | 49500                               |
| Carbonic anhydrase        | 32500                               |
| Soybean trypsin inhibitor | 27500                               |
| Lysosyme                  | 18500                               |

Protein markers were prepared as suggested by the manufacturer. 5 $\mu$ l of prepared markers was sufficient for visualising on small SDS-PAGE gels.

#### 2.2.4.2 Radioactive molecular weight markers

5 $\mu$ l (25nCi) of commercially available  $^{14}\text{C}$  methylated proteins was used for SDS-PAGE where the proteins of interest were to be visualised by autoradiography. The solution of marker proteins was mixed with loading buffer (2.2.3) and electrophoresis performed as described. The apparent molecular weights of the proteins are indicated below.

| $^{14}\text{C}$ -methylated proteins | Apparent molecular weight (daltons) |
|--------------------------------------|-------------------------------------|
| Myosin                               | 200000                              |
| Phosphorylase B                      | 97400                               |
| Bovine serum albumin                 | 69000                               |
| Ovalbumin                            | 46000                               |
| Carbonic anhydrase                   | 30000                               |
| Lysozyme                             | 14300                               |

## 2.2.5 Visualization of proteins after SDS-PAGE

### 2.2.5.1. Protein staining techniques

#### 2.2.5.1a Silver staining

Protein samples were analysed by silver staining of polyacrylamide gels (2.2.3). After SDS-PAGE the polyacrylamide gel was soaked in 50% (v/v) methanol for at least 3 hours, with changes. A staining solution was freshly prepared. This consisted of:

Solution A. 0.8g  $\text{AgNO}_3$  dissolved in 4ml distilled water.

Solution B. 21ml 0.36% (w/v) NaOH + 1.4ml concentrated ammonia solution.

Solution A was added dropwise, with stirring, to solution B. To this resulting mix was added 75ml water, with stirring. The gel was removed from the methanol solution and stained in the staining solution for 15 minutes, with shaking. The gel was washed in distilled water 4 times before being left in water for 5 minutes with shaking. During this time the developer was prepared: 500ml water + 2.5ml 1% (w/v) citric acid + 0.3ml 38% formaldehyde. After the washing step, the developer was added to the gel and shaken until the protein bands were visible. To stop colour development the gel was rinsed in water before the addition of a solution containing 45%

(v/v) methanol + 10% (v/v) glacial acetic acid. The gel was dried onto Whatman paper for storage.

#### 2.2.5.1b Coomassie Blue staining

Following SDS-PAGE (2.2.3) the polyacrylamide gel was added to a staining solution (10% (v/v) glacial acetic acid + 45% (v/v) methanol + 0.25% (w/v) coomassie brilliant blue R250) for 60 minutes at room temperature. The gel was then destained by shaking with repeated changes of a destaining solution (30% (v/v) methanol + 10% (v/v) glacial acetic acid). The gel was dried onto Whatman paper for storage.

#### 2.2.5.2 Western blotting

##### 2.2.5.2a Protein transfer

Polypeptides from SDS-PAGE (2.2.3) were transferred to a nitrocellulose filter using the BRL transblot apparatus. 3 litres of transfer buffer was prepared (25mM Tris, 0.2M glycine, 20% (v/v) methanol), in which the filter was soaked before blotting. Transfer was carried out at a constant voltage of 60V for 2 hours. On completion of transfer (as judged by the transfer of prestained molecular weight markers (2.2.4.1) to the filter) the nitrocellulose was placed in a blocking

solution (TBST + 5% Marvel (dried milk powder)). TBST is a solution of Tris buffered saline with Tween 20: 10mM Tris-HCl (pH 8), 15mM NaCl + 0.05% Tween 20. TBS was shown to lead to better colour development (see below) than the alternative phosphate-buffered saline (PBS).

The polyacrylamide gel remaining after protein transfer was placed in 50% (v/v) methanol before silver staining (2.2.5.1)

#### 2.2.5.2b Colour development

Nitrocellulose filters prepared as described above were incubated with 30µg sheep anti-ricin A-chain IgG (purification described in section 2.4) in 10ml TBST + 5% Marvel. The filter was placed in a sealed plastic bag on a shaker, at room temperature for at least 2 hours (usually overnight). The filter was washed for 4 x 5 minutes with 100ml TBST. 10ml of TBST + 5% Marvel was added containing 1.5µl of an antibody conjugate (anti-sheep IgG conjugated to alkaline phosphatase) and the filter incubated in this solution for 60 minutes at room temperature with shaking. The filter was washed twice with 100ml TBST then twice with TBS (i.e. TBST without Tween 20). 10ml of alkaline phosphatase buffer was prepared (100 mM Tris-HCl (pH 9.5) 5 mM MgCl<sub>2</sub>, 100mM NaCl), to which was added 66µl NBT (nitro blue tetrazolium (50mg/ml in 70%(v/v) dimethylformamide)) and 33µl BCIP (5-bromo-4-chloro-5-

indoyl phosphate). The solution was mixed then added to the gently blotted filter and incubated, with shaking, until the protein bands were visible. The reaction was stopped by replacing the developer with a stop solution (20mM Tris-HCl (pH 8.0), 5mM EDTA). The filter was dried before storing at room temperature in a sealed bag.

### 2.2.5.3 Autoradiography

#### 2.2.5.3a Fluorography

Proteins incorporating  $^{35}\text{S}$ -methionine were subjected to electrophoresis as described (2.2.3). The gel was submerged in a fixing solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid) for 1 hour to fix the proteins into the gel and remove unincorporated radioactivity. The fixing solution was replaced with a solution of Amplify (Amersham) and shaken for 15 minutes. The gel was dried onto Whatman 3MM paper and placed in a light-proof cassette next to a piece of X-ray film. The cassette was placed at  $-80^{\circ}\text{C}$  and the film developed as below to visualise the proteins. Marker proteins were used as described in section 2.2.4.2.

### 2.2.5.3b Developing X-ray film

Radioactive samples from SDS-PAGE (2.2.3), sequencing (2.1.8.3) and oligonucleotide phosphorylation (2.1.8.4) were exposed to X-ray film (Fuji BX). The film was developed, in a dark room, using Kodak LX-24 developer and fixed with Kodak FX-40 fixer, both diluted 1:4 with water.

## 2.3 Transcription / translation

### 2.3.1 In vitro transcription

#### 2.3.1.1 Transcription of DNA

All the DNA constructs used in this study had been engineered to be in the correct orientation in pGEN 1 (see appendix) for transcription from the T7 promoter. The following protocol was observed.

The efficiency of transcription could be monitored by measuring the incorporation of  $^{32}\text{P}$ -UTP into the transcript. A transcription premix was prepared as indicated overleaf and stored at  $-70^{\circ}\text{C}$ :

Final concentration  
in transcription reaction

|                         |          |
|-------------------------|----------|
| 10x Transcription salts | 1x       |
| 20mM spermidine         |          |
| 400mM HEPES (pH 7.5)    |          |
| 60mM magnesium acetate  |          |
| BSA                     | 100µg/ml |
| DTT                     | 10.0mM   |
| UTP, ATP, CTP           | 0.5mM    |
| GTP                     | 0.1mM    |

For each transcription reaction the following reagents were assembled at room temperature:

|                                    |        |
|------------------------------------|--------|
| Transcription premix               | 12.0µl |
| H <sub>2</sub> O                   | 1.5µl  |
| RNasin (25U/µl)                    | 0.5µl  |
| CAP ( $m^7G(5')ppp(5')Gm$ ) (16mM) | 1.0µl  |
| <sup>32</sup> P-UTP (0.5µCi)       | 1.0µl  |

2µl (1µg/µl) of linearised DNA and 2µl T7 RNA polymerase (50U/µl) were added and incubated at 40°C for 30 minutes. 1µl 8mM GTP (freshly diluted from 0.5M stock with 20mM HEPES (pH 7.5, KOH)) was added and incubated for a further 30 minutes at 40°C.

The RNA prepared in this way was monitored for

incorporation of  $^{32}\text{P}$ -UTP as described below and stored at  $-70^{\circ}\text{C}$  before use in in vitro translation reactions (2.3.2).

#### 2.3.1.2 Preparation of transcripts for counting

1 $\mu\text{l}$  of RNA prepared as above was diluted to 5 $\mu\text{l}$  with water. 2 $\mu\text{l}$  was spotted, in duplicate, onto DE81 paper and air dried. One sample was untreated, placed in a scintillation vial, and counted as described below. This sample represented the total counts in the transcription reaction. The other sample was washed 5 times for 2 minutes each in 200ml  $\text{Na}_2\text{P}_2\text{O}_7$ , once in water and a final wash in methanol. The paper was dried before counting as below. This sample represented the actual counts incorporated into nucleic acid. Percentage incorporation could thus be calculated.

#### 2.3.1.3 Monitoring radiolabel incorporation

Samples from transcription and translation reactions were monitored for radiolabel incorporation. Paper squares were prepared as described (2.3.1.2, 2.3.2.3), placed in scintillation vials and 4ml 'Optiphase Safe' aqueous / non-aqueous scintillant added. The radioactivity in each sample was counted in an LKB 1219 Rackbeta Liquid

Scintillation Counter for 60 seconds and a counts per minute (cpm) value recorded.

### 2.3.2 In vitro translation

#### 2.3.2.1 Translation in wheatgerm lysate

In vitro translation of transcripts (2.3.1.1) was performed using a wheatgerm translation system as described by Anderson et al. (1983). Active wheatgerm mix was prepared from 12g of commercially available wheatgerm and was a generous gift from Mike Westby. Immediately before use, the wheatgerm was centrifuged at 8000 xg for 4 minutes at 4°C to pellet debris. The pellet was discarded and the supernatant used in the reaction. A translation premix was prepared as indicated .

#### Wheatgerm translation premix.

|                                | µl   |
|--------------------------------|------|
| 1M HEPES (pH 7.6, KOH)         | 350  |
| 0.1M ATP                       | 250  |
| 0.4M creatine phosphate        | 500  |
| 0.1M DTT                       | 425  |
| 1M potassium acetate           | 2100 |
| 10mg/ml creatine phosphokinase | 100  |
| 1.5mg/ml spermine (pH 7.0)     | 500  |

(cont. overleaf)

|                                       |     |
|---------------------------------------|-----|
| 2.0mM GTP/Mg <sup>2+</sup>            | 250 |
| from 40mM GTP/magnesium acetate stock |     |
| 5.0mM each amino acid mix (-Met)      | 125 |
| Distilled water                       | 100 |

The translation reaction was prepared on ice as follows:

|                                       |        |
|---------------------------------------|--------|
| Distilled water                       | 4.4µl  |
| Translation premix                    | 2.3µl  |
| Transcription mix                     | 1.0µl  |
| Wheatgerm                             | 3.75µl |
| <sup>35</sup> S methionine (15µCi/µl) | 1.0µl  |

The reaction was incubated at 30°C for 60 minutes. The polypeptides were analysed by SDS-PAGE (2.2.3) and autoradiography (2.2.5.3). The incorporation of <sup>35</sup>S methionine into protein could be monitored as described in section 2.3.2.3.

#### 2.3.2.2 Translation in rabbit reticulocyte lysate

##### 2.3.2.2a Preparation of the reticulocyte lysate

Rabbit reticulocyte lysate, prepared as described in Clements, (PhD thesis, 1988), was a generous gift from Mike Westby. For translation reactions the lysate was nuclease with micrococcal nuclease from Staphylococcus

sureus to reduce background protein synthesis (see Results section 3.2.5.1). Typically the lysate was nucleased with the following procedure :

|                            |              |
|----------------------------|--------------|
| Rabbit reticulocyte lysate | 1.0ml        |
| Creatine kinase (10mg/ml)  | 5.0µl        |
| 0.1M CaCl <sub>2</sub>     | 10.0µl       |
| Nuclease (7.5U/µl)         | 75-100 units |

The lysate was incubated at 20°C for 15 minutes before the addition of 10µl 0.2M EGTA to stop the nuclease reaction. Lysate prepared in this way was stored at -80°C. Alternatively, nucleased lysate was purchased from Promega and used as directed.

#### 2.3.2.2b Preparation of reaction mix

The efficiency of the reticulocyte lysate translation system can vary with differing potassium and magnesium concentrations. Thus the translation conditions were optimised for each batch of lysate (see Results section 3.2.5.1). However the ratios shown overleaf gave adequate translation :

auraus to reduce background protein synthesis (see Results section 3.2.5.1). Typically the lysate was nucleaseed with the following procedure :

|                            |              |
|----------------------------|--------------|
| Rabbit reticulocyte lysate | 1.0ml        |
| Creatine kinase (10mg/ml)  | 5.0 $\mu$ l  |
| 0.1M CaCl <sub>2</sub>     | 10.0 $\mu$ l |
| Nuclease (7.5U/ $\mu$ l)   | 75-100 units |

The lysate was incubated at 20°C for 15 minutes before the addition of 10 $\mu$ l 0.2M EGTA to stop the nuclease reaction. Lysate prepared in this way was stored at -80°C. Alternatively, nucleaseed lysate was purchased from Promega and used as directed.

#### 2.3.2.2b Preparation of reaction mix

The efficiency of the reticulocyte lysate translation system can vary with differing potassium and magnesium concentrations. Thus the translation conditions were optimised for each batch of lysate (see Results section 3.2.5.1). However the ratios shown overleaf gave adequate translation :

|   |               |
|---|---------------|
| 2M KCl  | 137.5 $\mu$ l |
| 40mM magnesium acetate  | 117.5 $\mu$ l |
| 10mM Tris-HCl (pH 7.4)  | 80.0 $\mu$ l  |
| Distilled water   | 492.5 $\mu$ l |
| Amino acid mix (-methionine)<br>(each at 2mM, (pH 8.3, KOH))                  | 125.0 $\mu$ l |
| Energy mix  | 155.0 $\mu$ l |
| 20mM ATP, 4mM GTP,<br>0.2M Tris-HCl (pH 7.5),<br>8% (w/v) creatine phosphate. |               |

#### 2.3.2.2c Translation reaction

The following reagents were incubated at 30°C for 60 minutes :

|   |              |
|---|--------------|
| Reaction mix                                      | 7.0 $\mu$ l  |
| Nucleased lysate                                  | 10.0 $\mu$ l |
| <sup>35</sup> S methionine (15 $\mu$ Ci/ $\mu$ l) | 2.0 $\mu$ l  |
| Transcription mix (2.3.1.1)                       | 1.0 $\mu$ l  |

The polypeptides were analysed by SDS-PAGE (2.2.3) and autoradiography (2.2.5.3). The incorporation of <sup>35</sup>S methionine into protein could be monitored as described below.

### 2.3.2.3 Monitoring $^{35}\text{S}$ methionine incorporation

To assess the efficiency of translation reactions, 1 $\mu$ l of translation product was spotted onto 1cm<sup>2</sup> squares of Whatman No.1 paper, in duplicate, and air dried for 10 minutes. The paper was washed twice in 100ml ice cold 10% (w/v) TCA for 5 minutes on ice. The waste TCA was discarded and the paper boiled for 15 minutes in 100ml 5% (w/v) TCA to hydrolyse tRNA. The samples were washed twice in distilled water then twice in 100% ethanol before drying. The amount of radioactivity in each sample was assessed as described in section 2.3.1.3.

### 2.3.3 Preparation of ribosomes

#### 2.3.3.1 Preparation of yeast ribosomes

50ml of YPG media was inoculated with 50 $\mu$ l of a Saccharomyces cerevisiae protease deficient yeast strain ABYS 1 (Achstetter et al., 1984) from a frozen (-70°C) stock.

YPG media is prepared as:

- 1% w/v yeast extract
- 2% w/v bacto-peptone
- 2% w/v glucose
- + 1ml 0.2% w/v adenine
- + 1ml 0.2% w/v uracil

This culture was incubated at 30°C / 250rpm overnight. 1ml of culture was used to inoculate a second flask containing 500ml YPG media which was incubated, with shaking (250rpm), at 30°C until the optical density at 600nm was approximately 2. The cells were harvested (2000 xg / 5 minutes / 4°C) and the cell pellet washed twice with 100ml of cold sterile distilled water, thoroughly resuspending the pellet each time with an acid-washed glass rod. The washed pellet was resuspended in 3 pellet volumes of lysis buffer (100mM potassium acetate, 2mM magnesium acetate, 20% (v/v) glycerol, 20mM HEPES-KOH, pH 7.4) + 1/200<sup>th</sup> volume freshly made PMSF (20mg/ml). To the cell suspension was added 1/4 volume of acid-washed 40mesh glass beads. The suspension was vortexed at 4°C for 1 minute periods until at least 50% of the cells were lysed (as observed by light microscopy). The preparation was centrifuged at 500 xg / 4°C for 2 minutes to pellet the glass beads. The supernatant was then centrifuged at 17,500 xg / 4°C / 20 minutes to pellet mitochondria, nuclei etc. The supernatant was carefully transferred to 3ml polycarbonate ultracentrifuge tubes and spun at 100,000rpm for 100 minutes at 4°C in a Beckman TLA-100.3 rotor to pellet the ribosomes.

The supernatant was discarded and the pellet briefly rinsed in a small volume of 1xENDO buffer (25mM Tris-HCl (pH 7.6), 25mM KCl, 5mM MgCl<sub>2</sub>). The pellet was resuspended in a small volume of 1xENDO buffer using an acid-washed

glass rod. The optical density at 260nm of this preparation was measured and the ribosome concentration was estimated (an OD<sub>260</sub> of 25 = 2mg/ml). The ribosomes were stored in aliquots (30-40µg/µl) at -80°C. Typical yield from this method was 40mg ribosomes/l culture.

#### 2.3.3.2 Preparation of reticulocyte ribosomes

1ml untreated rabbit reticulocyte lysate (Promega) was layered onto a 1ml cushion of 1M sucrose in 1xENDO buffer in a 3ml polycarbonate ultracentrifuge tube. The ribosomes were pelleted by centrifugation at 100,000rpm for 40 minutes at 2°C in a Beckman TLA-100.3 rotor. The supernatant was discarded. The pellet was resuspended in 200µl 1xENDO buffer and repelleted for 40 minutes. This washing step was repeated once more. The final ribosome pellet was resuspended in a small volume of 1xENDO buffer and the ribosome concentration estimated by measuring the optical density at 260nm, as above. Typical yield of ribosomes was 750µg/ml.

#### 2.3.3.3 Salt-washing of ribosomes

Ribosomes were washed in 0.5M KCl to remove bound elongation factors and aminoacyl t-RNA (Blobel, 1971). Ribosomes prepared as above were incubated on ice for 1 hour in 50mM Tris-HCl (pH 7.5), 0.5M KCl, 5mM MgCl<sub>2</sub> at a

concentration of 2mg/ml. The ribosomes were pelleted by centrifugation at 100,000rpm for 40 minutes at 2°C in a Beckman TLA-100.3 rotor. The pellet was briefly rinsed in 1xENDO buffer, resuspended in a small volume of buffer and the concentration estimated .

#### 2.3.3.4 Storage of ribosomes

Purified ribosomes (standard preparation and salt-washed) were stored in 1xENDO buffer at -80°C at a concentration of 30-40µg/µl, in aliquots of 20µl.

#### 2.3.4 Assessment of RNA specific N-glycosidase activity

##### 2.3.4.1 During an in vitro translation

To assess the depurination of rabbit reticulocyte ribosomes by an in vitro synthesized ribosome-inactivating protein the following method was used.

A translation mix was prepared as below:

|   |      |
|---|------|
| Translation reaction mix (2.3.2.2b)             | 14µl |
| Rabbit reticulocyte lysate (non-nucleasid) 20ul |      |
| 0.6mM methionine                                | 2µl  |
| Transcript (2.3.1.1)                            | 1µl  |
| Distilled water                                 | 3µl  |

This mix was incubated at 30°C for 60 minutes, after which the ribosomal RNA (rRNA) was extracted. The volume of the translation mix was made up to 100µl with 50µl water and 10µl SDS (10% (w/v)) and mixed vigorously. Two phenol/chloroform extractions (2.1.5.1) were performed to remove contaminating proteins. The aqueous layer was made up to 100µl with water and the RNA was precipitated with 10µl 2M sodium acetate (pH 6.0) + 250µl 100% ethanol, on dry ice for 30 minutes. The precipitated RNA was pelleted by centrifugation at 8000 xg for 30 minutes at 4°C. The pellet was washed in 70% ethanol, dried in vacuo, and resuspended in 10µl distilled water.

1.0µl of the RNA solution was added to 499µl water and the absorbance profile from 190 to 320nm examined. Assuming a value of 1 OD unit at 260nm is equivalent to 40µg RNA/ml, the concentration of rRNA was determined. The RNA was then suitable for treatment with aniline and subsequent electrophoresis as described below.

#### 2.3.4.2 Depurination of yeast ribosomes

The depurination activity of proteins synthesised in vivo could be assessed using yeast ribosomes (prepared as described in section 2.3.3.1).

30µg of yeast ribosomes were incubated at 30°C for 60 minutes with a sample of protein in 1xENDO buffer (25mM Tris-HCl (pH 7.6), 25mM KCl, 5mM MgCl<sub>2</sub>) in a total

reaction volume of 30 $\mu$ l. The RNA was extracted by one of two methods:

a. SDS extraction. To the 30 $\mu$ l reaction mix was added 20 $\mu$ l 10% (w/v) SDS + 150 $\mu$ l water and mixed vigorously.

b. Kirby buffer extraction. To the 30 $\mu$ l reaction mix was added 170 $\mu$ l Kirby buffer (Kirby, 1968) and mixed vigorously.

The 200 $\mu$ l extraction mix was extracted twice with 200 $\mu$ l phenol/chloroform (2.1.5.1) before precipitation of the rRNA with 2M sodium acetate (pH 6.0) as above. The concentration of the RNA was estimated and an aliquot used for aniline treatment (2.3.4.4) and electrophoresis (2.3.4.5)

#### 2.3.4.3 Depurination of rabbit reticulocyte ribosomes

30-40 $\mu$ g of rabbit reticulocyte ribosomes (prepared as in section 2.3.3.2) were used to assess the depurination activity of crude and purified protein samples using the same procedures as outlined in section 2.3.4.2.

#### 2.3.4.4 Aniline treatment of rRNA

To 3-5  $\mu$ g rRNA was added 20 $\mu$ l M aniline (pH 4.8, glacial acetic acid) (D'Alessio, 1982) and incubated at 60°C for 2 minutes in the dark. The rRNA was quickly precipitated by the addition of 2.6 $\mu$ l 7M ammonium acetate

+ 65µl 100% ethanol and 30 minutes on dry ice. The rRNA was pelleted by centrifugation at 8000 xg for 30 minutes at 4°C, washed in 70% ethanol and dried in vacuo. The pellet was resuspended in 20µl 60% (v/v) deionised formamide / 0.1x TPE (3.6mM Tris, 0.2mM EDTA, 3mM sodium dihydrogen orthophosphate). An untreated sample was also prepared using the same quantity of rRNA made up to 20µl with deionised formamide (to 60% (v/v)) and 1x TPE (to 0.1x). Samples were run on a denaturing agarose gel as described below.

#### 2.3.4.5 Electrophoresis of RNA

Samples of rRNA, as prepared above, were heated to 65°C for 5 minutes, after which 4µl of loading buffer (50% (v/v) glycerol in 0.1xTPE) was added to each sample. The samples were loaded into the wells of a denaturing agarose gel (1.2% (w/v) agarose, 50% (v/v) deionised formamide, 0.1x TPE) cast in a 15cm x 15cm gel former, and subjected to horizontal electrophoresis at a constant current of 20mA with 0.1x TPE as buffer. The gel was unsubmerged. The rRNA was visualised by staining the gel in water + ethidium bromide (2µg/ml) for 15 minutes, destaining in water for 30 minutes, then viewed on a UV transilluminator.

## 2.4 Antibody purification

### 2.4.1 Preparation of serum

Serum was obtained from sheep (previously injected with recombinant ricin A-chain) and stored at  $-80^{\circ}\text{C}$ . The serum was heated to  $56^{\circ}\text{C}$  for 30 minutes to inactivate complement, then dialysed against 5mM phosphate buffer (pH 8.0), 10mM NaCl for 36 hours. The serum was filter sterilised before purification of the IgG fraction.

### 2.4.2 Purification by chromatography

The IgG fraction of the serum was separated from other proteins by ion-exchange chromatography using an anion-exchange column matrix. The column chosen was a TSK DEAE 5PW HPLC column on a Beckman 421A HPLC. The column was equilibrated with 5mM phosphate buffer (pH 8.0) + 10mM NaCl. 5ml of serum, prepared as above, was loaded onto the column and washed with buffer until the optical density at 280nm was zero. A linear NaCl gradient was produced from 10mM to 300mM and fractions eluting from the column examined by SDS-PAGE (see overleaf). Sodium aside was added to the collected IgG fraction (to 0.05% (w/v)) before aliquoting into small volumes for storage at  $-80^{\circ}\text{C}$ .

The concentration of antibody was estimated (2.2.1.1) to be  $2\mu\text{g}/\mu\text{l}$ .

#### 2.4.3 Analysis of antibody fractions by SDS-PAGE

Samples of the fractions eluted from the DEAE column described above were analysed by SDS-PAGE (2.2.3) with the following modifications. Samples were run on large protein gels (17.5cm x 20cm) under denaturing but non-reducing conditions through a 7% (w/v) polyacrylamide gel (2.2.3). Loading buffer was prepared as described (2.2.3) without the addition of 2-mercaptoethanol. The gel was stained with Coomassie blue as described (2.2.5.1b). The results of this purification are indicated in section 3.4.1.

#### 2.5 In vivo expression

##### 2.5.1 Large scale protein production

Expression of recombinant ricin A-chain constructs was performed using the pDS5/3 plasmid construct, as described in O'Hare et al. (1987). The procedures for harvesting, and preparation of crude cell extracts were essentially the same as described by O'Hare. However sections of the method were altered, specifically the column purification of ricin A-chain from crude extracts. A description of the expression conditions and the purification scheme is found in the Results sections 3.3 and 3.4 where the reasoning behind the changes is

explained. Expression of toxic proteins was carried out in category II containment facilities.

#### 2.5.2 Concentration of protein after purification

Fractions eluted from the purification column containing recombinant ricin A-chain were sometimes concentrated to more suitable volumes for storage. Concentration was performed using an Amicon ultrafiltration system with filters (Filtron) that had a 10000 dalton exclusion limit. Procedures were carried out in accordance with the manufacturers' instructions.

#### 2.5.3 Small-scale screening of expression

For the rapid analysis of bacterial colonies for ricin A-chain expression, 10ml cultures were inoculated with 1ml of overnight culture and incubated at 30°C / 250rpm until the optical density at 550nm was 0.6-0.8. To the cultures was added 10µl M IPTG (to 1mM final concentration) and the culture incubated for a further 1- 2½ hours. 1ml of culture could be removed for an analysis of whole cell expression, as described below (2.5.4). The induced culture was centrifuged in a Beckman GPE bench centrifuge at 3500rpm for 10 minutes at 4°C. The cell pellet was resuspended in 300µl phosphate-buffered saline (PBS)(4% (w/v) NaCl, 0.2% (w/v) KCl, 1.15% (w/v)

disodium hydrogen phosphate, 0.2% (w/v) potassium dihydrogen phosphate) and incubated with 30 $\mu$ l lysozyme (20mg/ml in PBS) for 5 minutes on ice. The cells were disrupted by sonication for 3x 20 seconds, on ice, in a MSE Soniprep 150.

The sonicated culture was centrifuged for 30 minutes at 8000 xg at 4°C and the pellet discarded. 150 $\mu$ l of supernatant was precipitated with acetone (2.2.2.1) and the protein pellet resuspended in 1x loading buffer (2.2.3). The remaining 150 $\mu$ l of supernatant was centrifuged at 107,000 xg for 60 minutes at 4°C in a Beckman TLA-100.3 rotor to pellet insoluble and aggregated material. The supernatant ('soluble fraction') was precipitated with acetone and the pellet resuspended in 1x loading buffer.

Samples of the soluble, total and whole cell proteins were used in SDS-PAGE and ricin A-chain visualised by Western blotting (2.2.5.2).

#### 2.5.4 Whole cell protein analysis

The protein composition of the whole bacterial cell could be analysed by SDS-PAGE. 1ml of culture was centrifuged at 8000 xg for 3 minutes at room temperature and the supernatant discarded. The cell pellet was resuspended in 60 $\mu$ l 1x reducing/denaturing sample buffer (2.2.3) and heated to 100°C for 3 minutes. Cell debris was

pelleted by centrifugation at 8000 xg for 3 minutes at room temperature and the supernatant used directly for SDS-PAGE (2.2.3).

## 2.6 Chromatography techniques

Various chromatography methods were employed for the purification of ricin A-chain which are discussed at length in the Results section 3.4. Indicated below are details relating to those techniques.

### 2.6.1 Chromatography equipment

#### 2.6.1.1 Columns at 4°C

Equipment for column chromatography was obtained from Pharmacia; P-1 pump, single-channel chart recorder, Frac-100 fraction collector, UV-1 OD detector and was used as directed by the manufacturer.

#### 2.6.1.2 HPLC at room temperature

Equipment for HPLC chromatography was obtained from Beckman; 421 A controller, 114 M solvent delivery modules, 160 absorbance detector. Fractions were collected with a Pharmacia Frac-100.

## 2.6.2 Column matrices and buffers

All buffers detailed below were filter sterilised (0.22µm, Micropore) and degassed before use.

### 2.6.2.1 Chromatofocusing

30ml PBE 94 (Pharmacia) was prepared and packed into a double-walled 20cm x 1.5cm plastic column as described by the manufacturer. The column matrix was equilibrated in 0.025M ethanalamine (pH 9.4, acetic acid). Proteins were eluted with Polybuffer 96 (pH 6.0, acetic acid) at a flow rate of 30ml/hour.

### 2.6.2.2 CM-Sephacose and S-Sephacose

30ml CM-Sephacose CL-6B was prepared by washing three times with 100ml ice cold sterile water then once with 5M phosphate buffer (pH 6.5). pH 6.5 phosphate buffer was produced by mixing 68.5ml 50M  $\text{NaH}_2\text{PO}_4$  + 31.5ml 50M  $\text{Na}_2\text{HPO}_4$  and making to 1 litre with water. The column matrix was packed into a double-walled 20cm x 1.5cm plastic column under pressure (35ml/hour). The matrix was washed with 3 column volumes of 5M phosphate buffer (pH 6.5) + 300mM NaCl then equilibrated with 15 column volumes of 5M phosphate buffer (pH 6.5). Protein samples were loaded and the column was washed (30ml/hour) with 5M

phosphate (pH 6.5) until the optical density at 280nm was reduced to baseline. A linear NaCl gradient was produced from 0 to 300mM NaCl. The matrix was washed with 5 column volumes of 5mM Tris-HCl (pH 8.0) to remove any bound material the re-equilibrated to pH 6.5 with 5M phosphate.

S-Sepharose matrix was prepared in the same way as described for CM-sepharose. Flow rates for this matrix were greater and thus the column was packed at a flow rate of 55ml/hour and run with samples at 50ml/hour.

#### 2.6.2.3 Antibody column construction

Affinity columns were constructed using anti-ricin A-chain antibodies (section 2.4) coupled to an Affi-Gel 10 (a derivatised crosslinked agarose bead support, supplied by Bio-rad). Coupling was performed in a variety of buffers (see Results section 3.4), and remaining active ester residues were blocked after coupling by the addition of 0.1ml M ethanolamine-HCl (pH 8.0) per ml gel. Columns were constructed in 7ml disposable plastic columns. Coupling and packing of the column was performed as described by the manufacturer at 4°C.

#### 2.6.2.4. Anion-exchange HPLC

Protein samples were loaded onto a TSK DEAE-5PW (21.5mm x 150mm) column attached to the HPLC system previously described. Proteins were eluted at a flow rate of 3ml/minute.

*Chapter Three*

**RESULTS**

### 3.1 MUTAGENESIS

#### 3.1.1 Introduction

Mutagenesis of specific residues of ricin A-chain formed the basis of this study and therefore the choice of which residues to mutate was crucial. When the project was in an early stage there were three major areas of background information available. There was information deduced from protein sequence comparisons, structural information from the 3D crystal structure, and limited information from A-chain modification studies. Using a combination of these three sources of information, residues were identified that were potentially important in the functioning of ricin A-chain. The information that was available before mutagenesis reactions were planned is introduced in sections a-c below.

##### a. Sequence comparison

As the class of proteins termed RIPs are examined in greater detail, an increasing number of protein sequences and deduced amino acid sequences have become available. A study of sequence similarity gives an indication of residues that may be potentially important in the mechanism of action or the structural integrity of the protein. It does not unequivocally state that the

conserved residues have the same function to the same degree in different proteins, but rather suggests that the residues may play a role in the functioning of the protein, that has necessitated their conservation. Once homology has been established, it provides a basis for further experimentation and the characterisation of these potentially important residues.

At the beginning of this study, sequence comparisons of ricin A-chain, trichosanthin and abrin had been made which gave a preliminary indication of the conservation of residues within the RIP group. Xeujun & Jiahui (1986) had examined the homology between ricin A-chain and trichosanthin (from Trichosanthes kirilovii) and found 91 identical and 42 conservative residues (totalling 56% of the trichosanthin sequence). This comparison also revealed three pentapeptides of identical sequence, one of which was the sequence SEAAR (residues 176-180 of ricin A-chain) which has been targeted in this study. A similar comparison was performed by Funatsu et al. (1988) with the A-chain of abrin-a from the seeds of Abrus precatorius. Alignment of sequences revealed 106 identical residues (approximately 42% abrin residues), which is considerable since Abrus and Ricinus are not closely related taxonomically. As the project progressed, more RIP sequences became established and these were examined for similarity. In a recent homology alignment (Katsin et al. 1991) of ricin A-chain, abrin A-chain, trichosanthin, MAP

and saporin-6, nine residues were invariant (Table 2). A further 39 residues are conserved amongst these, and other proteins studied. A sequence comparison is presented in Fig. 3.1.

Table 2. Invariant amino acids in a recent sequence alignment (Katrin et al., 1991).

|            |     |     |     |     |     |     |     |     |     |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Position   | 21  | 29  | 80  | 123 | 144 | 177 | 178 | 180 | 211 |
| Amino acid | Tyr | Arg | Tyr | Tyr | Leu | Glu | Ala | Arg | Trp |

Three of these invariant residues (Arg29, Glu177 and Arg180) were chosen for mutagenesis. In addition to conservation, information from the structure and from modification reactions was considered when making this choice, as discussed below.

#### b. Structural information

With the determination of the crystal structure of ricin to 2.8Å (Montfort et al., 1987), information became available as to the positions of the individual residues and the possible relationships between them. Again this technique can only provide ideas which then require testing experimentally, however it is extremely powerful



when used in conjunction with other sources of information. The structure of ricin and ricin A-chain has already been introduced in detail (section 1.7). Examination of the positions of the conserved residues indicates that Glu177 and Arg180 lie in close proximity at the base of the putative active site cleft. These residues are thought to hydrogen bond to a water molecule (designated #323) in the active site (Katsin et al., 1991) and to each other. Arg29 has been shown to be away from the proposed active site in a cleft area bordered by arginine residues. It has been postulated to be involved in RNA binding. Mutagenesis at this locus and subsequent kinetic measurements were designed to examine the role of this invariant Arg29.

c. BYF modification studies

Studies by Watanabe & Funatsu (1986) examined the involvement of arginine residues in protein synthesis inhibition by ricin A-chain. Incubation of the A-chain protein with phenylglyoxal (PGO) or 1,2-cyclohexanedione (CHD) caused a marked decrease in A-chain activity. These reagents react with the arginine side-chain specifically. Chemically reversing the reaction of CHD restored the activity to the A-chain, demonstrating that the inactivation was not due to non-specific effects. It was also shown that greater than 90% of the A-chain activity

was lost with modification of approximately 3 (out of 20) arginine residues and that some of the 6 residues in the N-terminal region of the A-chain reacted with PGO faster than the other arginines. Three of these N-terminal arginines (positions 29, 48 and 56) are conserved amongst RIPs and two of these (Arg48 and 56) were the subject of mutagenesis (May et al., 1989). Arg48 and 56 were mutated to alanine with no loss in A-chain activity. Thus it was proposed that although these residues were conserved, they did not have a crucial role in A-chain action. Arginine 29 appeared to be potentially more important since it was invariant amongst the RIPs whose sequences were known.

The potential involvement of Glu177 in the catalytic mechanism was proposed following studies by Hovde et al. (1988). Glu167 of Shiga-like toxin I (equivalent to Glu177 in ricin A-chain) was mutated to Asp resulting in a 1000 fold decrease in enzymatic activity. This residue had been shown to be conserved (Ready et al., 1988) and was thus proposed to have a key role in catalysis. Later mutagenesis during the course of this project by Schlossman et al. (1989), examined the role of Glu177 by mutagenesis to alanine and aspartic acid, and observed a 15-80 fold reduction in activity. Glu177 was also suggested as being potentially important during studies to isolate random RTA mutants that allowed yeast, bearing the RTA plasmid, to survive. Of the 9 mutants isolated, 2 had an altered amino acid at position 177 (Frankel et al.

1989). Glu177 was selected for this study and mutated to Lys, Ala and Asp for expression in vitro and in vivo. Production of the A177 and D177 mutants were underway when the results of the work by Schlossman et al. was published. However, it was decided to continue with the study of these mutants to examine the curious result of decreased activity of the D177 mutant relative to A177. It was of interest to express these mutants in vivo using the system available in the Warwick laboratory which was substantially different from that used by Schlossman and colleagues.

Mutation of the arginine 180 locus was chosen on the basis of its total conservation throughout all RIP sequences examined and its position in the putative active site cleft relative to the conserved glutamate. The mutants that were created were designed to examine the role of the arginine side-chain. Gln180 was constructed to investigate the requirement of a positive charge at position 180; Ala180 removed both the positive charge and the hydrogen bond potential of the side chain; Met180 removed the positive side-chain but retained a side-chain structure to prevent the residues around the gap, created by the removal of Arg180, from 'collapsing' into the space.

The pentapeptide deletion (SEAAE) from Ser176 to Arg180 inclusive was performed on the A-chain to check a previous observation by May et al. (1989). In this earlier

study the deletion had no effect on the activity of the A-chain and the construct appeared to retain full activity. In the light of the activity measurements of Hovda et al. and Schlossman et al., and the structural and homology data available, this result proved to be interesting. The SEAAE region contains three of the most conserved residues in R1Ps and forms the back wall of the putative active site cleft. This mutagenesis reaction was repeated to check this apparent anomaly.

In order to mutate key residues in the ricin A-chain, appropriate fragments of the ricin cDNA (see appendix) were cloned into suitable vectors for mutagenesis and sequencing. Eight mutants were to be prepared by site-directed mutagenesis as described below:

| Mutant name | Mutation                     |
|-------------|------------------------------|
| K177        | Glu177 mutated to Lys177     |
| A177        | Glu177 mutated to Ala177     |
| D177        | Glu177 mutated to Asp177     |
| Q180        | Arg180 mutated to Gln180     |
| A180        | Arg180 mutated to Ala180     |
| M180        | Arg180 mutated to Met180     |
| A29         | Arg29 mutated to Ala29       |
| SEAAE       | Deletion of Ser176 to Arg180 |

The template for mutagenesis was based on the cDNA sequence as published by Lamb et al. (1985) in all cases but for the mutagenesis of the Arg 180 locus. The latter mutagenesis reactions were carried out on a fragment of DNA obtained from the genomic ricin-containing plasmid pAKG (Halling et al., 1985) with various modifications as discussed below.

The use of M13 vectors for mutagenesis and sequencing reactions is commonplace because of the ease with which one can prepare single-stranded DNA that is suitable for either of the aforementioned reactions. The reader is directed to Yanisch-Perron et al. (1985) and Messing (1991) for detailed information on the theory of the use of M13 vectors.

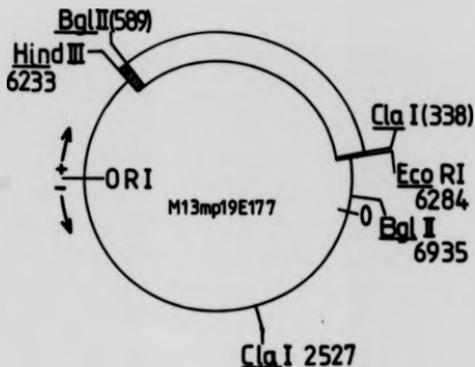
### 3.1.2 Results

#### 3.1.2.1 Template construction

To construct a template for mutagenesis at the Glu177 locus the following procedure was performed. A fragment of the ricin cDNA was excised from the plasmid pUC8RA (containing the entire ricin A-chain coding region from -72 to 852) by digestion with the restriction enzymes ClaI and BglIII. These enzymes cleave the DNA at positions 338 and 589 respectively, to release a 251bp fragment of DNA that codes for the region of interest for mutagenesis

(from amino acid residue Arg114 to Arg197). This fragment was isolated from the remaining plasmid DNA and electrophoresed to check the size. The 251bp ricin A-chain fragment was ligated into the isolated vector arms of a ClaI-BalII cut pIC-20H plasmid (Marsh et al., 1984) to construct pIC-20HRA. This intermediate plasmid was used to allow easy cloning of the RTA fragment into M13 in the correct orientation for mutagenesis.

A fragment of the pIC-20HRA was isolated, following cleavage with the enzymes EcoRI and HindIII, which consisted of the ClaI-BalII RTA fragment enclosed within short 'arms' from the pIC-20H polylinker. This 267bp fragment was ligated into the M13mp19X vector similarly cleaved with EcoRI and HindIII. M13mp19X is a derivative of M13mp19 with the removal of the XbaI site and the addition of a XhoI linker. This alteration has no effect on the cloning strategy discussed here because this region of the polylinker was removed during digestion with EcoRI and HindIII. Clones with the RTA fragment inserted were identified as clear plaques when grown on selective IPTG/X-GAL agar plates. Positive clones were checked by the preparation of miniprep DNA, digestion and electrophoresis. Positive clones were finally checked for possession of the 251bp RTA fragment by sequencing using a ricin-specific primer CL584 (see appendix) and M13 universal primer. A representation of this Glu177 template (M13mp19E177) is presented in Fig. 3.2. Mutated clones



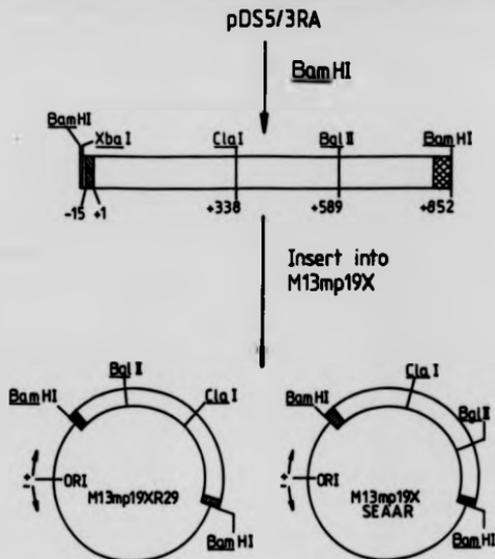
**Fig. 3.2** Template for mutagenesis of glutamate 177.

A 251bp ClaI-BglII fragment (338 to 589) containing the region to be mutated was inserted into pIC20-H and then excised as an EcoRI-HindIII fragment for insertion into M13mp19X cut with EcoRI and HindIII. The resultant M13 + strand from the vector M13mp19E177 was complementary to the oligos detailed in Fig. 3.4. Nucleotide numbers refer to wild-type M13mp19. Numbers in parenthesis refer to ricin cDNA nucleotides.

were then excised from the template by digestion with ClaI+BglII.

A different approach was used for the construction of the template for mutagenesis of A29 and SEAAE. The whole ricin A-chain sequence was excised from the plasmid pDS5/3RA and inserted into an appropriate M13 vector directly. This approach was necessary because of the lack of convenient restriction enzyme sites around the R29 codon. pDS5/3RA was constructed in this laboratory by the fusion of a mutated ricin A-chain DNA sequence with pUC18RA and the isolation of a BamHI fragment from this clone which was ligated into pDS5/3 (Stauber *et al.*, 1984) to form pDS5/3RA (O'Hare *et al.*, 1987).

This BamHI fragment consisted of the full length ricin A-chain DNA sequence with a stop codon introduced after Ile267 and a short region of pUC18 DNA to give a 5' sequence of GATCCTCTAGAGTCGAGGATAACAAC followed by the mature ricin A-chain sequence. Insertion of this BamHI fragment into M13mp19X, cleaved with BamHI, created two possible products depending on the orientation of the insert. These orientations are displayed in Fig. 3.3. Both template orientations were used in this study since the SEAAE oligo had been prepared in the opposite orientation to the published sequence and thus required a different orientation of template. Ordinarily, the template was constructed and the oligos designed such that the products from sequencing could be read off the sequencing gel with



**Fig. 3.3** Template for mutagenesis of Arg29 and SEAAAR.

A 873bp BamHI fragment from pDS5/3RA (O'Hara *et al.*, 1987) was ligated into M13mp19X cleaved with BamHI to create two orientations of A-chain cDNA. The vector labelled M13mp19XR29 produced single-stranded + DNA that was complementary to the R29A oligo detailed in Fig. 3.4, whilst the vector labelled M13mp19XSEAAAR was suitable for mutagenesis of the SEAAAR deletion.

the same sequence as the published sequence.

For the construction of the template for mutagenesis at the Arg180 locus another approach was used (Schlossman et al., 1989). A 893bp BamHI fragment from the genomic ricin-containing plasmid pAKG was subcloned into pUC119 (Vieira & Messing, 1987) and the following mutations were performed by site-directed mutagenesis. The codon for Asn2 was altered from AAC to CCC to create a 5' BamHI site and the codon for Pha267 (TTT) was altered to Leu (TTG) with the following two codons altered from TCTTTG to GATCCG creating a 3' BamHI site. Thus a full length ricin A-chain DNA sequence was prepared by the laboratory of Dr. Frankel which was contained within a 810bp BamHI fragment and this was termed RTA20. This clone possesses viral origins of replication and antibiotic resistance. With the use of a helper phage, single-stranded DNA was prepared for mutagenesis.

#### 3.1.2.2 Oligonucleotides for mutagenesis

Oligonucleotides were commercially prepared for mutagenesis of the DNA templates described in the section above. Oligos were designed with at least 8 bases on either side of the mutated base in order that they would anneal specifically to the templates. See Fig. 3.4 overleaf for a list of the oligo sequences (bold letters indicate mismatches, [ ] indicates a deletion).

Fig. 3.4 Oligonucleotides for mutagenesis.

|       |    |     |  |    |       |
|-------|----|-----|--|----|-------|
| K177K | 5' | 521 | TCATTTCAAAAGCAGCAAG <sup>539</sup>         | 3' | 19mer |
| K177A | 5' | 522 | GATTTCAGCTGCAGCAAG <sup>539</sup>          | 3' | 18mer |
| K177D | 5' | 523 | ATTTCAGAGCCAGCAAG <sup>539</sup>           | 3' | 17mer |
| SEAR  | 5' | 552 | AATATATTGGAA[ ]AATCATTGGAT <sup>514</sup>  | 3' | 24mer |
| E29A  | 5' | 73  | ATCAGAGCTGTTGCCGGTCGTTAACA <sup>99</sup>   | 3' | 27mer |
| E180Q | 5' | 526 | TCAGAAGCAGCACAATTCCAATATATT <sup>2</sup>   | 3' | 27mer |
| E180A | 5' | 526 | TCAGAAGCAGCAGCATTCCAATATATT <sup>552</sup> | 3' | 27mer |
| E180M | 5' | 526 | TCAGAAGCAGCAATGTTCCAATATATT <sup>552</sup> | 3' | 27mer |

Before use, the oligos were phosphorylated at the 5' end to make them suitable for extension. A small aliquot of oligo was phosphorylated with radioactive <sup>32</sup>P (as described in section 2.1.7.3) to check the size and the integrity of the oligo. The results of the phosphorylation of the oligos for SEAR, K177, A177, D177 and E29 are shown in Fig. 3.5. Oligo concentration was estimated by measuring the absorbance at 260nm and adjusted to 10ng/μl for use in the mutagenesis reaction.

OLIGONUCLEOTIDE

LENGTH 1 2 3 4 5 6 7  
(bases)



**Fig. 3.5** Phosphorylation of oligonucleotides for mutagenesis.

Oligos for mutagenesis of E177K, E177A, E177D, R29A and SEAAR were phosphorylated with  $^{32}\text{P}$  at the 5' end. Oligos were visualised by electrophoresis on a 16% polyacrylamide/urea gel and subsequent exposure to film for 20 minutes at  $-80^\circ\text{C}$ . Lanes 1 and 2 represent control oligos of 18 and 22 nucleotides in length respectively. Lanes 3, 4, 5, 6 and 7 represent the mutagenesis oligos SEAAR (24mer), D177 (17mer), K177 (19mer), A177 (18mer) and A29 (27mer) respectively.

### 3.1.2.3 Mutagenesis of the Glu177 locus

Mutagenesis of the template construct (Fig. 3.2) was performed utilizing the oligos detailed in Fig. 3.4 and using the site-directed mutagenesis system described in 2.1.11. The mutagenesis reactions were successful in all cases, as indicated by sequencing of the mutated region. Fig. 3.6 shows sequence information from the sequencing of the single-stranded M13 clones containing the mutated RTA fragment with the ricin specific primer CL584. No additional mutations were observed when the mutated region was sequenced.

### 3.1.2.4 Mutagenesis of the Arg180 locus

Mutagenesis reactions were performed on the pUC119 RTA plasmid 'RTA20' (as detailed previously) with the mutagenesis system described in 2.1.11. These experiments were performed by me in the laboratory of Dr. A. Frankel, Duke University, N. Carolina, USA, who graciously provided the clones and oligos for this work. The mutagenesis reactions successfully produced three mutants at codon position 180 and no additional mutations were found after sequencing. Sequencing reactions and subsequent electrophoresis were performed by Audrey Alexander and Phil Walsh respectively. The sequence data for these mutagenesis reactions is not shown, however plasmid

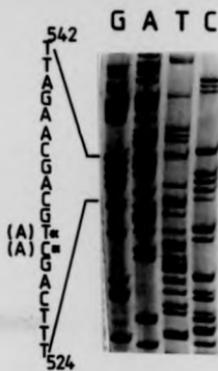
Fig. 3.6 Sequencing of glutamate 177 mutants.

Mutations of Glu177 were created in an M13-derived vector and sequenced using the Sequenase system and primer CL584. Products were separated by electrophoresis at 38W for 2 hours as described (2.1.8.3). Samples were loaded G-A-T-C as shown (referring to the dideoxynucleotide used for termination of DNA chain extension).

Alongside each figure is the nucleotide sequence as read (numbering according to Lamb et al., 1985). Mutated nucleotides are highlighted with an asterisk and the wild-type nucleotide is included in parenthesis.



E177K



E177A



E177D

sequencing of the mutant expression constructs has been carried out and is presented in section 3.2.2.1d.

#### 3.1.2.5 Mutagenesis of Arg29 and SEAAR

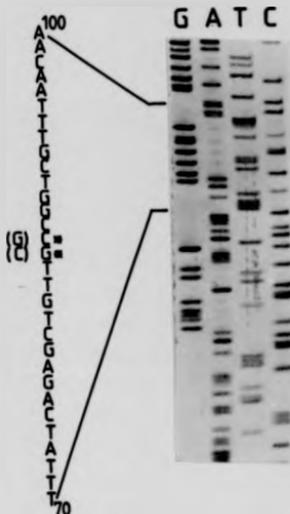
Mutagenesis of Arg29 was carried out by myself at Duke University as above, however the resulting mutant construct was not suitable for cloning into the in vivo and in vitro expression vectors available in the Warwick laboratory. Thus the mutagenesis was repeated using the full length RTA BamHI fragment inserted into M13mp19X as detailed in section 3.1.2.1. Mutagenesis of the arginine codon (CGC) to that coding for alanine (GCC) was performed successfully. The sequence was checked using primer CL582 and also the M13 universal primer (sequence of the mutated area is shown in Fig. 3.7). This mutation was additionally verified by restriction modification of the double-stranded mutant with the enzyme MspI. This was feasible because of the creation of a CCGG restriction site during mutagenesis. An additional cleavage product was observed (data not shown).

The synthesis of the SEAAR oligo determined that the RTA fragment was inserted into M13mp19X in the reverse orientation. Mutagenesis to introduce this deletion proved to be more difficult than the previous mutations, however eventually a deletion was produced which was checked by sequencing using reverse orientation primers. The sequence

of the deleted mutant ricin A-chain is shown in Fig. 3.8 alongside the wild-type sequence for comparison.

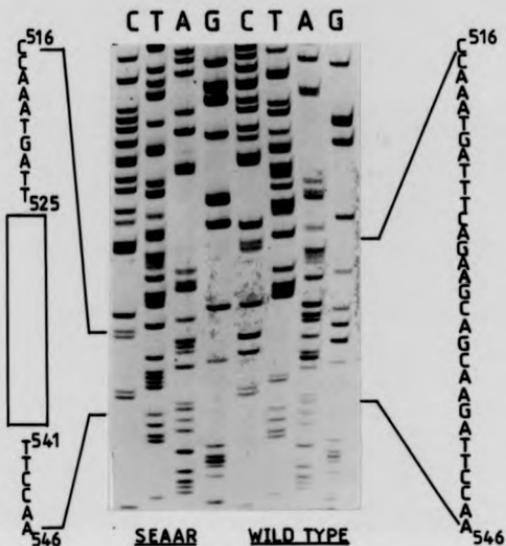
#### 3.1.2.6 Summary of template construction and mutagenesis

Templates for mutagenesis were constructed and site-specific mutagenesis reactions were performed to introduce specific changes into the ricin A-chain cDNA. Mutated regions were confirmed by sequencing and, in some cases, restriction enzyme digestion. Three A-chain residues were targeted for study (R29, E177, R180) and a deletion (S176 to R180) was also prepared.



**Fig. 3.7** Sequencing of the Ala29 mutation.

The mutagenesis of Arg29 to Ala was performed using a M13-derived vector as described in the text. The sequence above was generated using M13 universal primer. G, A, T and C refer to the dideoxy-termination mixes used. The sequence around the mutated area is shown. Mutated nucleotides are highlighted with an asterisk and the replaced wild-type nucleotide is included in parenthesis.



**Fig. 3.8** Sequencing of the SEAAR mutation.

Sequence data was generated using primer CL294. Due to the reverse orientation of the SEAAR template, the figure presented above produces a nucleotide sequence from the 3' to the 5' of the published sequence when the gel is read from bottom to top. The nucleotides removed to create the SEAAR deletion are indicated by a box between T525 and T541 when compared to the wild-type sequence.

## 3.2 IN VITRO EXPRESSION

### 3.2.1 Introduction

The properties of mutant A-chain proteins were first examined using in vitro methods for determining enzymatic activity. Mutated DNA fragments were cloned into an in vitro expression vector pGEM1 (Promega), which is structured to allow transcription of DNA inserts from a T7 or SP6 RNA polymerase promoter (see appendix). All the clones used in this study were orientated for T7 RNA polymerase transcription.

Transcripts were translated in a translation system prepared from wheatgerm lysate or rabbit reticulocyte lysate. The components of each translation system and their use is described in section 2.3.2. Expression of constructs in wheatgerm was used to confirm the size of in vitro translated polypeptides. The feasibility of this approach is due to observations that ribosomes from wheatgerm lysate are relatively insensitive to ricin A-chain action, when compared to ribosomes from rabbit reticulocyte lysate. Observations by Osborn (1991), using the aniline assay first described by Endo et al. (1987), demonstrated that wheatgerm ribosomes were 1000 fold less sensitive than rabbit reticulocyte ribosomes under the same conditions of depurination.

Reasons for this difference in sensitivity were

proposed, including the fact that there were different concentrations of elongation factors present in the preparations (Olsnes et al., 1975; Fernandez-Puentes et al., 1976b). The author examined the effects of salt-washing the ribosomes to remove bound elongation factors and aminoacyl tRNA. It was shown that the removal of bound factors increased the susceptibility of both populations of ribosomes however an appreciable difference remained. This unexplained difference has continued to be one of the most interesting dogmas within this field of research. This lower sensitivity of wheatgerm lysate ribosomes is exploited while expressing in vitro translation products that are potentially toxic, since the lysate can support translation of the toxin for extended periods. Products can then be visualised on SDS-PAGE and the size of the mutant constructs checked against wild-type A-chain. A toxic protein inactivates a rabbit reticulocyte lysate translation to such a degree that it is extremely difficult to visualise such a band using this translation system.

The second in vitro translation experiments exploit the ribosome-inactivating properties of ricin A-chain in the form of two sensitive methods to assess activity of the toxin. In vitro prepared transcripts were translated in one of two preparations of rabbit reticulocyte lysate as described in section 2.3.2.2-2.3.4.3. The first of these methods is termed the 'double translation' method

and was first reported by May et al. (1989). It is based on a rabbit reticulocyte lysate translation system that has been inactivated by toxic molecules during translation of a toxin-encoding transcript, and thus cannot support the translation of a second 'reporter' transcript added later. After a one hour incubation of the ricin A-chain transcript in the presence of <sup>35</sup>S methionine, a second transcript (coding for yeast preproalpha factor) is added to the translation mix and incubated for a further 30 minutes. Analysis of the products of translation with SDS-PAGE and autoradiography allows the visualisation of the 'reporter' protein and non-quantitative estimates can be made of the activity of the in vitro product.

In addition to this method, A-chain transcripts were translated in a second, non-nuclease treated rabbit reticulocyte lysate and the rRNA was extracted for use in the aniline assay as previously described (2.3.4). The aniline assay is the definitive assay of RIP activity since it measures the actual result of RIP activity i.e. a specific depurination of the ribosomal RNA. Experimental treatment of the depurinated rRNA with 1M aniline (pH 4.5) leads to preferential cleavage of the phosphodiester backbone at this site and the release of a 390b fragment that is then visualised by electrophoresis.

This chapter illustrates the results obtained from cloning mutant ricin A-chain constructs into in vitro

expression vectors, their transcription, translation, and an assessment of their biochemical properties in vitro.

### 3.2.2 Results

#### 3.2.2.1 Preparation of in vitro mutant expression constructs

##### 3.2.2.1a Construction of pGEM K177 mutants

pGEM1RA was chosen as the in vitro expression vector into which the mutated DNA fragments were inserted. pGEM1RA was constructed from the preprorin cDNA (Lamb et al., 1985) into which a translation stop codon had been introduced immediately after the codon for Phe267 of mature A-chain (O'Hara et al., 1987). The DNA fragment containing the entire RTA coding sequence was excised from an earlier clone (pUC8RA) as a XhoI-SalI fragment and ligated into the SalI site of pGEM1 to give pGEM1RA (see appendix). pGEM1RA was cleaved with the enzymes ClaI and BalII and the vector fragment isolated.

The three mutations of the Glu177 codon were excised from double-stranded M13 mutagenised constructs using the restriction enzyme sites of ClaI (338) and BalII (389). The 251bp fragments released after digestion were isolated from 1.2% agarose gels using the Whatman paper method (2.1.10.2). DNA fragments were resuspended in T.E. and

their concentration checked by visualisation on agarose gels against DNA of known concentration. Approximately 100ng of vector fragment and 50ng of 251bp insert were ligated overnight and aliquots transformed into competent TG1 cells. Digestion of the resulting miniprep DNA with a variety of restriction enzymes identified potentially correct clones. A single fragment insertion was verified by digestion with ClaI and in some cases multiple fragment insertion was observed. The presence of the mutated region was confirmed by plasmid sequencing. Large scale DNA preparations were produced using the alkaline lysis method (2.1.4.3) and approximately 100µg of DNA was recovered which was suitable for in vitro transcription reactions.

#### 3.2.2.1b. Construction of pGEM29

The Ala29 mutation was initially prepared using the RTA20 template, as previously indicated, but this proved to be unsuitable for cloning into the pGEM vector chosen for this study. Thus the A29 mutation was recreated using the full length RTA sequence derived from pDS5/3RA (O'Hare et al., 1987). Cloning of the mutated DNA into the pGEM vector required some modification of the vector. The first modification was the removal of the unique BamHI restriction site from pGEM1. This was achieved by digestion of pGEM1 with BamHI for 4 hours at 37°C, heat inactivation of the enzyme by incubation of the digestion

at 70°C for 15 minutes, and subsequent electrophoresis of an aliquot for verification of linear DNA. All the DNA appeared to be linearised. The cohesive termini were removed by digestion of approx. 2µg of linearised DNA with 6 units of T4 DNA polymerase in the absence of dNTPs for 5 minutes at 37°C. The 3' to 5' exonuclease activity of the enzyme removes single-stranded DNA to create blunt ends (Sambrook et al., 1989). To ensure that there were no overlapping ends created by inefficient or excessive exonuclease activity, complementary strand synthesis was initiated by the addition of dNTPs (to 200µM) and the mix incubated for a further hour at 37°C before heat inactivation. Ligation of the blunt ends was performed to create pGEMidBam. Digestion of miniprep DNA with BamHI verified the removal of the BamHI site.

The pGEMidBam construct was then digested with SalI and into this site was ligated the XhoI-SalI fragment from pUC8RA containing the entire wild-type ricin A-chain sequence (from the ATG at -72 to the BamHI site at +852) to create pGEMidBamRA. This construct was cleaved with BamHI to remove the wild-type ricin A-chain coding region (-41 to +852) and the vector fragment isolated. Into the vector was cloned the 873bp fragment from the double-stranded M13 preparation, containing the A29 mutation, released after digestion with BamHI. Since the vector was not treated with CIP (calf intestinal alkaline phosphatase) to remove the 5' phosphate, a proportion of

the clones recovered from the ligation were religated vector. However some colonies were isolated possessing the A-chain fragment in a single insert (checked by ClaI digestion) and in the correct orientation (determined by digestion with EcoRI + ClaI). DNA was prepared by alkaline lysis and sequenced to verify. The A29 construct thus possessed a 5' region encoding a 19 amino acid N-terminal extension in the pGEM expression vector which would have the sequence NYAVATWLCFGSSRVEDNN. This is in contrast to the 24 amino acid N-terminal sequence found in the pGEM Glu177 mutants (Fig. 3.9).

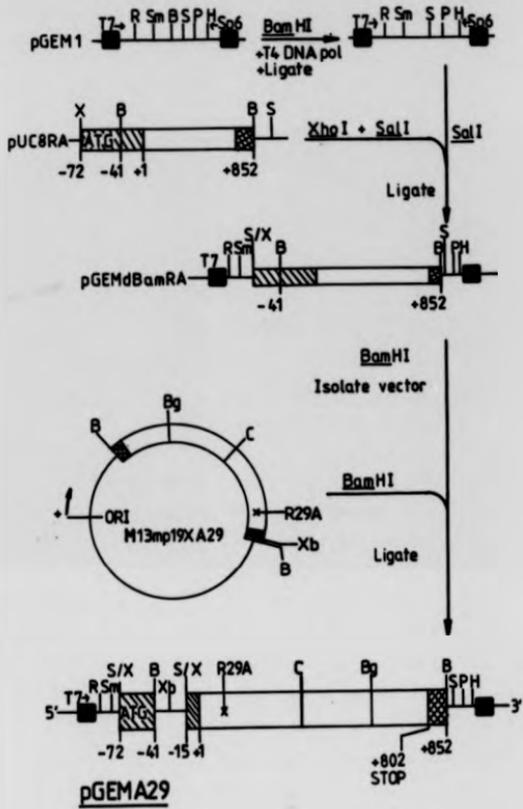
#### 3.2.2.1c. Construction of pGEMSEAAR

As with the cloning of the mutations at the Glu177 locus, the SEAAR deletion is contained within the 251bp fragment that could be excised from the M13 clone using the restriction enzymes ClaI and BalII. Thus construction of pGEMSEAAR followed a similar procedure to that previously described for the Glu177 constructs in 3.2.2.1a. The 251bp fragment was isolated and ligated into pGEMIRA ClaI-BalII vector arms. Analysis of miniprep DNA by restriction enzyme digestion indicated clones that were potentially correct. The presence of the deletion was confirmed by restriction digestion of the mutant with HinfI since creation of the deletion removes a HinfI site at 539. As HinfI is a common site in pGEM1 (9 sites),

**Fig. 3.9** Construction of the vector pGEMA29.

For in vitro expression of Ala29, the mutation was cloned into pGEM1. pGEM1 was altered to facilitate cloning of a mutated BamHI fragment from M13mp19IR29, as described in the text. The final pGEMA29 construct possessed the -72 ATG from pUC8BA in-frame for translation, following in vitro transcription from the T7 RNA polymerase promoter. The N-terminal amino acid sequence encoded by this construct was 5 amino acids shorter than for the remaining pGEM mutant constructs and had the sequence:

M Y A V A T W L C F G S S R V E D N N-mature ricin A-chain



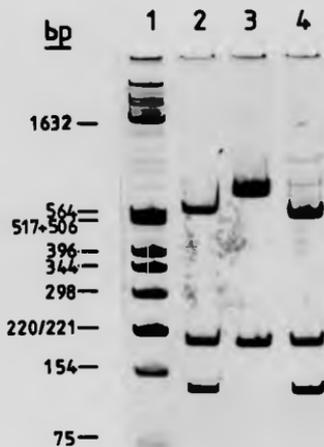
pGEMSEAAR was first digested with BamHI to release the A-chain and a 893bp fragment was isolated. Two control DNA samples (pGEM1RA and pGEM1A29) were similarly prepared. Approximately 500ng of the isolated BamHI fragments were digested for 2 hours at 37°C with 1µl (12 units) HinfI before loading onto a 10% vertical polyacrylamide gel and electrophoresis at 45mA for 1.5 hours. The stained product is shown in Fig 3.10. As is shown below, loss of the HinfI site as a result of the creation of the deletion ([ ]), is observed as two cleavage products compared to three from the control samples (pGEM1RA and pGEM1A29 in Fig. 3.10).

|         | <u>Bam</u> HI | <u>Hin</u> fI | <u>Hin</u> fI | <u>Bam</u> HI |
|---------|---------------|---------------|---------------|---------------|
|         |               |               |               |               |
| Control | -----         |               |               |               |
|         | -41           | 539           | 658           | 852           |

Fragments predicted (bp): 580, 194, 119.

|       | <u>Bam</u> HI | <u>Hin</u> fI | <u>Hin</u> fI | <u>Bam</u> HI |
|-------|---------------|---------------|---------------|---------------|
|       |               |               |               |               |
| SEAAR | -----[ ]----- |               |               |               |
|       | -41           | 525           | 541 658       | 852           |

Fragments predicted (bp): 683, 194.



**Fig. 3.10** Confirmation of the deletion in pGEMSEAAAR.

A 893bp BamHI fragment was digested with HinFI and the products analysed on a 10% polyacrylamide gel and stained with ethidium bromide. Lane 1 indicates the pBR322/HinFI size markers. Lanes 2 and 4 represent digests of control BamHI fragments from pGENRA and pGEMA29 respectively. Lane 3 indicates the digestion products for the SEAAAR mutant.

Examination of the digestion products in Fig. 3.10 revealed that these fragments were produced as predicted, confirming the presence of the deletion.

3.2.2.1d. Construction of pGEM 180 mutants and the discovery of a second mutation

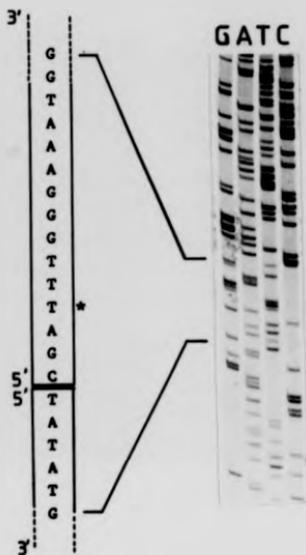
Mutations of the arginine 180 residue were created using the RTA20 template as described previously (3.1.3.4). The initial strategy for cloning the mutated region into the pGEM1 vector was similar to that described for the mutations at Glu177 and the SEAAR deletion. Thus a ClaI-BglII fragment was excised from the template construct, isolated and ligated into the ClaI-BglII cut pGEM1RA. Clones of apparently the correct size were analysed by restriction digestion and large scale amounts of DNA was prepared. Plasmid sequencing of the constructs was performed in order to confirm that the mutated fragment had been correctly integrated into the in vitro vector.

However, incorrect sequencing patterns were obtained for some pGEMQ180 and pGEMA180 constructs when the sequence was generated using primer CL584. Sequences were generated which related to the sequence of the corresponding strand from 3' to 5'. Further sequencing using the primer CL583 revealed that there was an inversion of a segment of cDNA from 340 to 412. The

sequence of pGEMQ180 with primer CL583 is shown in Fig. 3.11. Since the primer CL584 anneals to DNA within this segment, inversion would create the sequencing patterns observed.

Sequencing also revealed a single nucleotide mutation at position 414 creating a thymidine instead of a guanosine. This mutation unfortunately created a ClaI site at 409 in the A-chain, and in addition to altering the DNA sequence, this mutation also caused an amino acid change at position 138 of a glutamate (GAG) to an aspartate (GAT). The mutation was not reported in the original publication of the ricin A-chain containing pAKG plasmid, from which the RTA20 template was derived (Helling et al., 1985). Thus the mutation had been introduced during construction of the template or during the mutagenesis reactions presented in this study. Since the mutation was present in more than one of the B180 mutants, it was likely that this single base change was inherent in the template for mutagenesis. Sequencing of the pUC119 'RTA20' template with the primer CL583 confirmed that the mutation was present in the template (Fig. 3.12).

Therefore during construction of the pGEMR180 clones for in vitro expression, the ligation mix of fragment and vector was a heterogeneous population since both ClaI sites would be cleaved. It would appear that there was carry through of the ClaI (338)-ClaI (409) fragment during gel isolation of the mutated B180 fragment. The presence



**Fig. 3.11** Sequencing of the initial pGEMQ180 construct.

Sequencing with primer CL583 revealed a DNA inversion of position 340 to 412. This was caused by a mutation of G414 to T (marked with an asterix). 5' and 3' notation refers to the orientation of the DNA in the wild-type ricin A-chain (as described by Lamb et al., 1985).

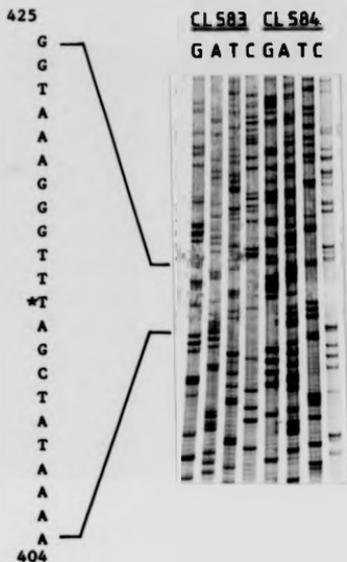
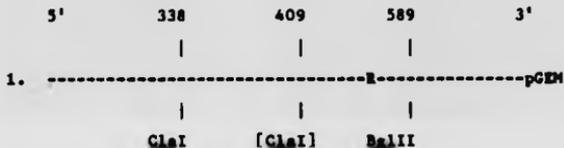


Fig. 3.12 Sequencing of pUC119 RTA20.

To demonstrate the presence of the mutation G414T in the mutagenesis template. Using the primer CL583 the mutation was discovered (highlighted with an asterix). The template was shown to be 'wild-type' around the active site using primer CL584.

of this fragment complicated the ligation and many slightly different sized ligation products were produced. Reviewing the original miniprep digests confirms this. Theoretically three different populations of ligation were possible even without multiple insertions due to the presence of this 'new' ClaI site (indicated as [ClaI] in the schematic diagram below where R represents the location of the R180 codon relative to the positions of the restriction sites and <<<<<< represents the inversion of the reading frame).

**Possible ligation permutations:**



The correct construct.



Loss of the small ClaI fragment.



partial digestion of the mutated pUC119 DNA was employed.

In order to perform these digestion experiments the conditions of digestion (i.e. temperature, time, enzyme concentration, DNA concentration etc.) needed to be ascertained. It was noted that partial digestion could be achieved by decreasing the time of reaction or by increasing the enzyme:substrate ratio by decreasing the concentration of the enzyme.

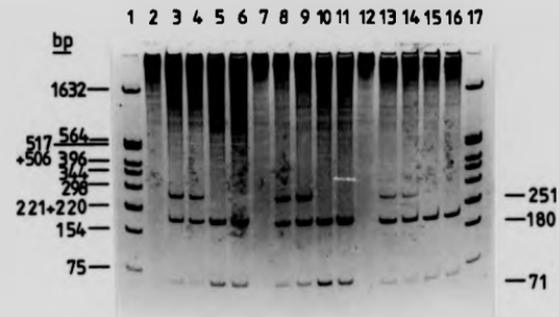
The DNA was digested with an excess of BglII (calculated to be 0.5pl (5U) per 400ng pUC119) to linearise the plasmid. The reaction was not terminated because the BglII site and the ClaI sites are not in close proximity and therefore there would be no steric interference between the enzymes. The DNA was digested with 6 units ClaI for periods of 30 seconds, 1 minute, 5 minutes and 20 minutes (complete digestion) before heat inactivation at 75°C for 15 minutes. The products were electrophoresed on a 10% vertical polyacrylamide gel and stained with ethidium bromide. The results of this experiment are indicated in Fig. 3.13. It was shown that complete digestion of the linearised DNA was achieved between 1 and 5 minutes. However, samples removed at 30 and 60 seconds (e.g. lanes 3 and 4 in Fig. 3.13) retained a proportion of the complete 251bp fragment.

In order to isolate the required 251bp fragment, the reaction quantities were increased to 4µg of BglII linearised DNA cut with 30 units ClaI for 30 seconds at

37°C. The cleavage products were subjected to electrophoresis on a 1.6% agarose/TBE gel and the 251bp fragments isolated. The isolated fragments were ligated into ClaI-BglII cut pGEN1RA vector and DNA minipreps verified for fragment insertion. Possible clones were sequenced by plasmid sequencing (with primers CL584 and CL583) to verify the integrity and orientation of the mutant insert and demonstrate the presence of the mutation. Fig. 3.14 presents the results from sequencing reactions using primer CL584. A control "wild-type" A-chain was constructed possessing the additional E138D mutation to act as a control for the Arg180 clones, as described below.

#### 3.2.2.1 Construction of pGENp+

It was unknown whether the mutation of Glu138 to Asp, in the Arg180 mutants, would affect the functioning of the A-chain. Therefore a 'pseudo-positive' (p+) clone was constructed to generate a ricin A-chain polypeptide that was equivalent to the A-chain used in this study but possessing the mutation E138D. This clone was constructed using a partial digest of the A29 mutant in pUC119 with the enzymes ClaI and BglII as used for the construction of the Arg180 mutants above. The 251bp fragment was thus wild-type apart from the E138D mutation, since the Ala29 mutation is outside the ClaI-BglII fragment. Plasmid

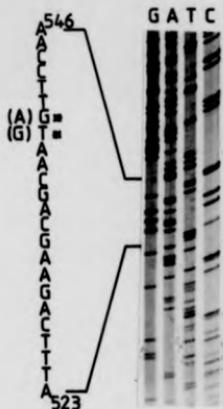
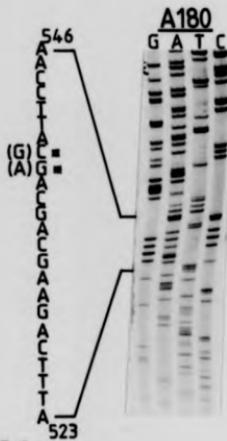
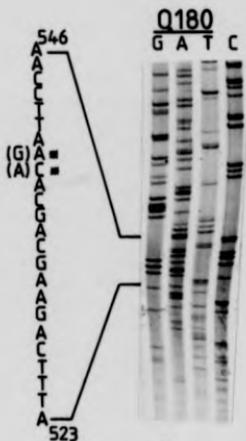


**Fig. 3.13** Partial digestion of mutated pUC119 DNA.

BglII linearised pUC119 DNA was digested with ClaI in order to isolate a 251bp ClaI-BglII fragment. Products of digestion were electrophoresed in a 10% polyacrylamide gel and stained with ethidium bromide. Lanes 1 and 17 are pBR322/HinfI markers with fragment sizes indicated (bp). The remaining lanes represent Q180 (2-6), A180 (7-11) and M180 (12-16). The first lane of each mutant represents BglII linearised DNA, followed by ClaI digestion for 0.5, 1, 5 and 20 minutes respectively.

Fig. 3.14 Plasmid sequencing of Arg180 mutants.

Mutants constructed in pGEM1 using partial digestion of the mutated pUC119 template were plasmid sequenced as described previously using primer CL584. Mutated nucleotides are highlighted with an asterisk and the wild-type residue is indicated in parenthesis. Nucleotide numbers refer to Lamb et al. (1985).

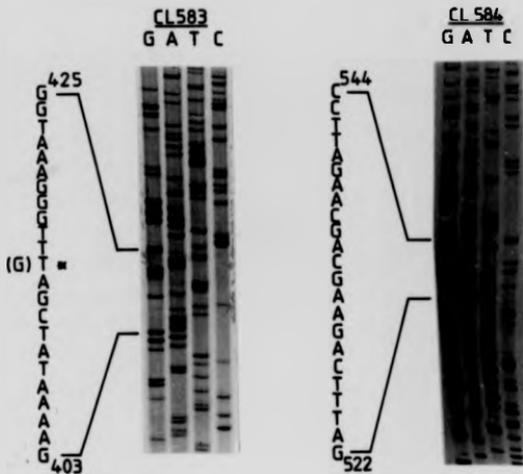


**M180**

sequencing was used to verify the E138D mutation and a single base change of G to T was observed at position 414 (Fig. 3.15).

### 3.2.2.2 Construction of pGEM1ppoF

Experiments in vitro to assess A-chain activity in rabbit reticulocyte lysate are described in a later section (3.2.5). This method requires the addition of a transcript encoding a non-toxic protein to be used as a 'reporter' polypeptide during A-chain activity measurements. The reporter transcript of choice is yeast preproalpha factor, for which a clone was available in our laboratory. However the clone was in an orientation for SP6 polymerase transcription. For simplicity, the coding region was transferred into pGEM1 in the correct orientation for transcription using T7 RNA polymerase. This was achieved by digesting pGEM2ppoF with EcoRI and SalI and ligating the isolated 610bp fragment into a similarly cut pGEM1 vector. The construct was checked by restriction enzyme digestion of miniprep DNA before a large scale preparation was made. See appendix for plasmid map.



**Fig. 3.13** Sequencing of pGEMp+.

To confirm the presence of the mutation G414T, pGEMp+ was sequenced with primer CL583. To confirm the sequence around the active site, pGEMp+ was sequenced with primer CL584. The nucleotide sequence as read is indicated, with the mutation highlighted with an asterix (wild-type nucleotide in parenthesis).

### 3.2.3 In vitro transcription

All clones for in vitro transcription were constructed for transcription using T7 RNA polymerase. Transcription was performed using DNA that had been prepared by the alkaline lysis method (2.1.4.2) using the method outlined in 2.3.1. pGEM1 constructs were linearised prior to transcription with the restriction enzyme HindIII and the concentration adjusted to 1µg/µl. pGEM1ppαF was linearised with Sall. Transcription efficiency was monitored and RNA concentration estimated in order to add equivalent amounts of mutant RNA to the translation reactions. Transcription reactions were between 40-80% efficient as measured by incorporation of <sup>3</sup>H-UTP or <sup>32</sup>P-UTP.

### 3.2.4 Translation in wheatgerm lysate

In vitro produced transcripts were translated in the wheatgerm lysate system (Anderson et al., 1983) as described in section 2.3.2.1. After each transcription reaction the transcripts were translated in wheatgerm to confirm that the transcription reaction had been successful. The size of the mutant products were compared to wild-type A-chain and radioactive markers. Major polypeptide bands were visualised at the correct size for

a protein of approx. 30kDa (Fig. 3.16). Two further bands of lower molecular weight were often seen and it is thought that these polypeptides represent initiation of translation at methionine residues away from the N-terminal Met. There are two Met residues in the ETA sequence (at 175 and 188) and 3 in the Met180 mutant. With the exception of this mutant, translation of the ETA clones could theoretically produce products of 267, 92 and 79 amino acids with approximate sizes of 30kDa, 10.3kDa and 8.8kDa respectively. These molecular weights would appear to be in accordance with the bands visualised on SDS-PAGE (Fig. 3.16). No dimerisation of the polypeptides was observed under these reducing/denaturing conditions. The polypeptides produced by in vitro translation of pGEMppoF and pGEMpreB are also shown in Fig. 3.16.

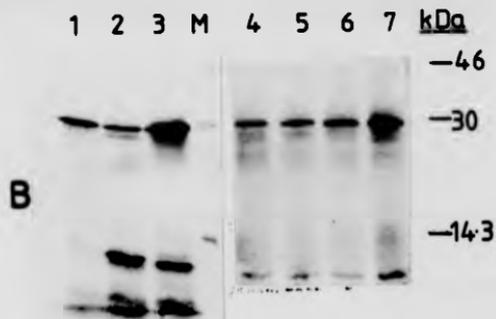
It is important to be able to visualise the products of in vitro transcription/translation reactions at this stage because during translation of the transcripts in rabbit reticulocyte, as described in the next section, active A-chain molecules will inactivate the translation system rapidly such that often a product cannot be visualised.

Fig. 3.16 In vitro expression of constructs in wheatgerm lysate.

DNA constructs (ricin A-chain, A-chain mutants, controls) were linearised and transcribed with T7 RNA polymerase. Transcripts were translated in wheatgerm lysate as described (2.3.2.1). Aliquots of the translation mix were analysed by SDS-PAGE and autoradiography.

In figure A, lanes 1, 5, 6 and 7 represent ricin A-chain, pseudopositive, preproalpha factor and ricin B-chain respectively. The Arg 180 mutants Q, A and M are shown in lanes 2, 3 and 4 respectively.

In figure B, lanes 1 and 7 represent wild-type ricin A-chain. Lanes 2 and 3 refer to A29 and SEAAR, whilst lanes 4, 5 and 6 refer to K177, A177, and D177 respectively. Lane M represents the molecular weight markers.



3.2.5 Translation of transcripts in rabbit reticulocyte lysate and assessment of in vitro N-glycosidase activity

3.2.5.1 Optimisation of reaction conditions

The efficiency of translation in rabbit reticulocyte lysate is dependent on a number of factors which can be altered experimentally. Lysate was treated with micrococcal nuclease (section 2.3.2.2a) to remove endogenous mRNA, otherwise polypeptides would be produced which would complicate the interpretation of the SDS-PAGE gels. However a balance must be achieved between removing unwanted RNA and maintaining an efficient translation system since non-specific cleavage of other RNA species can occur. After a series of experiments it was decided to use 75U of nuclease per ml of lysate since this removed the majority of the background (principally B-globin), but conserved the translation properties of the lysate (data not shown).

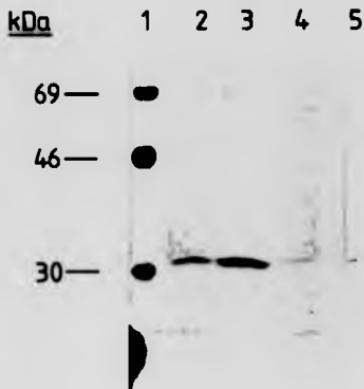
The concentration of magnesium and potassium ions have also been proposed to affect the efficiency of translation. It is suggested that the effect is specific for different preparations of transcript and thus the efficiency of translation was investigated using transcripts made as in 2.3.1. Altering the magnesium concentration in the range of 0-5mM led to an observable

difference in A-chain translation with an optimum at approximately 1.5mM (Fig. 3.17). In addition the optimum potassium concentration was shown to be in the region of 55-65mM. It has recently been reported that the use of KCl in the translation mix leads to greater efficiency and more accurate translation when compared to using potassium acetate (Jackson, 1991).

### 3.2.5.2 In vitro activity of mutations of Glu177

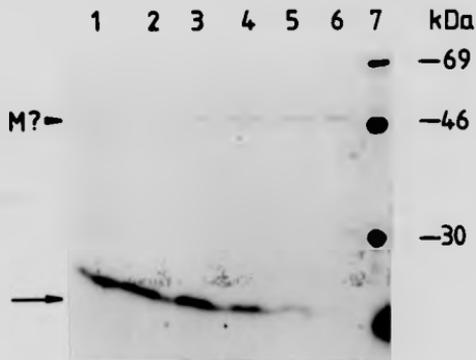
As a preliminary experiment, the rate of inactivation of the reticulocyte translation system was assessed. By visualising the disappearance of the reporter protein after pre-incubation of the translation mix with an RTA transcript, it was demonstrated that in vitro translated RTA inactivated the system within 20-30 minutes such that at 30 minutes no reporter protein was visible (Fig. 3.18). No ricin A-chain was visible. Only when RTA transcripts were translated in reticulocyte lysate derived from Promega was an A-chain band visible after 60 minutes translation, albeit faint (data not shown).

Equivalent amounts of in vitro transcript coding for the mutation K177, A177 and D177 were translated in nuclease rabbit reticulocyte lysate as described in section 2.3.2.2 for 60 minutes at 30°C. After this period 1 $\mu$ l of pGEMppc~~W~~ transcript was added and incubated for a further 30 minutes to assess the residual protein



**Fig. 3.17** Effect of magnesium concentration on the efficiency of in vitro translation in rabbit reticulocyte lysate.

Identical quantities of pGENQ180 transcript were translated in nucleated rabbit reticulocyte lysate with differing concentrations of magnesium in the reaction mix. Molecular weight markers are shown in lane 1. Final concentrations of  $Mg^{2+}$  in the reaction mix are 0.5, 1.5, 2.5 and 5.0mM in lanes 2, 3, 4 and 5 respectively.



**Fig. 3.18** Inactivation of rabbit reticulocyte lysate by in vitro transcribed ricin A-chain.

A-chain transcripts were added to rabbit reticulocyte lysate for defined periods of time. A-chain activity was stopped by the addition of rabbit anti-RTA antibodies and the residual translation ability assessed by visualising translation of the added reporter transcript (arrowed) which was incubated for 30 minutes. Lane 1 represents zero minutes incubation with the A-chain transcript, lanes 2, 3, 4, 5 and 6 represent 5, 10, 15, 20 and 30 minutes of A-chain incubation. Lane 7 represents the molecular weight markers. The polypeptide highlighted by M? refers to a putative methionine-binding protein.

synthesis capability of the translation system. Half the translation mix (10 $\mu$ l) was subjected to electrophoresis on a 15% polyacrylamide gel and subsequent autoradiography. Polypeptide bands were usually visible after 16 hours.

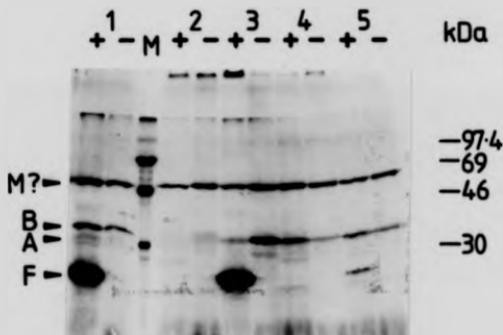
The results of one such experiment are presented in Fig. 3.19. The non-toxic primary transcript (B chain) is visualised as a strong band of 32kDa and the reporter ppof polypeptide is also visible (lane 1). The toxic primary transcript control (wild-type ricin A-chain) polypeptide cannot be visualised and neither can the reporter protein (lane 2). This indicates that the A-chain had inactivated the translation system after 60 minutes and no further translation was possible. In fact the inactivation was probably a lot faster than this since the ETA band itself is not visible, suggesting that the few molecules of ETA produced during the early stages of translation inactivated the system immediately before enough molecules of ETA could be produced for visualisation by autoradiography.

The results for the Glu177 mutants are shown in lanes 3 to 5 of Fig. 3.19. K177 is visible as a polypeptide of 30kDa (lane 3) and the reporter protein is visualised with an intensity equivalent to that in the B chain translation (lane 1). This suggests that the mutant K177 does not retain ribosome-inactivating properties that can be measured using this qualitative method. Mutants A177 (lane 4) and D177 (lane 5) would appear to possess partial

toxicity towards the translation system. A-chain polypeptides are visible in both double translation experiments suggesting that they are less toxic than wild-type A-chain. The preproalpha factor protein is only visible to a small degree in the D177 lane indicating that a fraction of the protein synthesising ability of the translation system remained. No ppof is present after incubation with A177 suggesting that the mutant protein has retained the majority of its toxic properties. However, it is proposed that it is less toxic than wild-type ricin A-chain because the A-chain polypeptide itself can be visualised.

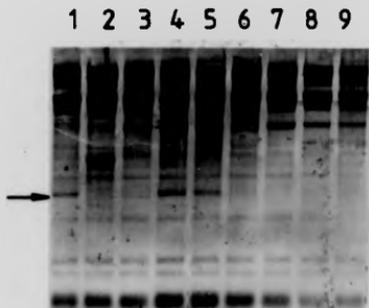
Glul77 transcripts were also translated in non-nucleated reticulocyte lysate to assess N-glycosidase activity using the aniline reagent. This experiment was used to confirm the results obtained from the double translation experiments above. The principle of the approach has been introduced in section 1.9 and by Endo et al. (1987). 4µg of ricin A-chain treated rRNA extracted from ribosomes was treated with aniline at acidic pH and electrophoresed in a denaturing 1.2% agarose/ 50% formamide gel as previously described (2.3.4.4). The gel was stained with ethidium bromide for visualisation and is shown in Fig. 3.20.

Lane 1 of Fig. 3.20 represents the toxic wild-type ricin A-chain control and the 390b aniline fragment is clearly seen (indicated by an arrow). To demonstrate that



**Fig. 3.19** In vitro activity of Glu177 mutants.

Activity of Glu177 mutants was assessed by the double translation method (the principle of which is described in the text). Lane 1 represents the activity of the non-toxic control, ricin B-chain. Ricin A-chain activity is indicated in lane 2. The mutations K177, A177 and D177 are described in lanes 3, 4 and 5 respectively. Experiments were performed in pairs: + indicates the reporter transcript (yeast preproalpha factor) was added, - indicates no addition of the second (reporter) transcript. Lane M refers to the molecular weight markers. Polypeptides highlighted A, B, F and M? indicate RTA, B-chain, reporter protein and the putative methionine binding protein respectively.



**Fig. 3.20** N-glycosidase activity of Glu177 mutants.

Total RNA from a reticulocyte translation system was extracted following translation of added transcripts, as previously described. The rRNA was treated with aniline (lanes 1 to 5) and electrophoresed in a denaturing agarose gel and stained with ethidium bromide. This allowed any cleavage products (indicated with an arrow) to be visualised.

Lane 1 (RTA transcript) clearly shows the aniline fragment (characteristic of N-glycosidase action) when compared to the non-aniline treated sample (lane 6). A negative control (preproalpha factor) shows no cleavage product (lane 2). Mutants K177, A177 and D177 (lanes 3, 4 and 5 respectively) display variable cleavage patterns when compared to the non-aniline treated samples (lanes 7, 8 and 9 respectively).

the cleavage product was not an artefact of the experiment, a non-toxic transcript (preproalpha factor) was translated and the rRNA treated in the same way. No cleavage product was observed (lane 2). Mutant K177 (lane 3) also showed no cleavage product, indicating that, under the conditions used in this experiment, no N-glycosidase activity remained. However, A177 (lane 4) produced a clear cleavage product when compared to the non-aniline treated sample (lane 8), indicating that A177 was still extremely toxic. Mutant D177 (lane 5) appears to exhibit reduced activity as suggested by the lower intensity of the 390b product.

The results generated by the aniline method would therefore appear to be in agreement with those estimated from the double translation method. This consistency gives confidence to the observations.

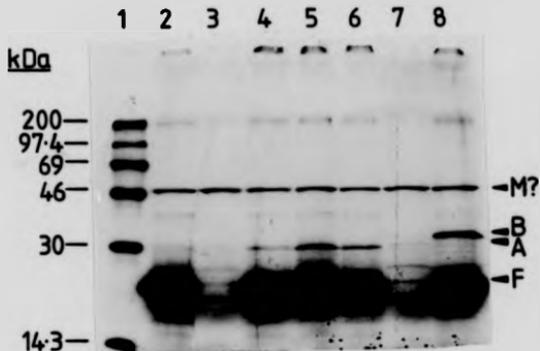
In summary it has been indicated by in vitro assessments of activity that K177 appeared to be inactive, A177 seemed to have retained the majority of its activity but D177 was only be weakly active towards ribosomes.

### 3.2.5.3 In vitro activity of mutations of Arg180

The results for the double translation experiment with the three mutations at Arg180 are shown in Fig. 3.21. Wild-type ricin A-chain and mutant p+ appeared to be of equal activity since no reporter protein could be

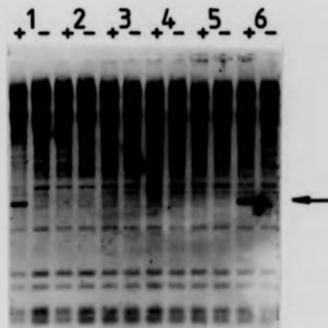
visualised in either case (lanes 3 and 7 respectively). For this experiment, the negative (non-toxic) control was ricin B-chain and comparison of lane 8 (B-chain + reporter) with lane 2 (reporter alone) demonstrates that the translation of B-chain had no effect on the visualisation of the reporter. Mutated Arg180 A-chain polypeptides were visualised (lanes 4 to 6), although they were weak. However the ppcf polypeptides translated after the mutant ETA transcripts were of equal intensity to that of the B chain control. Although the mutant polypeptides apparently poorly translated, the fact that there was no reduction in the reporter protein to any visible degree suggested that these mutants were inactive when assessed in this system and protein synthesis was able to continue after mutant A-chain expression. Appearance of visible ETA would itself suggest the production of inactive proteins.

To confirm this result the mutants were tested using the aniline assay as presented in Fig. 3.22. Examination of this gel confirmed the observation that the Arg180 mutants are non-toxic. No aniline cleavage fragments were observed in lanes 2 to 4, nor in the negative ppcf control translation (lane 5), whereas the ETA and the p+ transcripts gave rise to a cleavage fragment (lanes 1 and 6) and appeared to be equally active (judged by calculating the I depurination by scanning densitometry of the gel). Thus the mutation of E138D within the Arg180 mutants had little or no effect on the activity when



**Fig. 3.21** In vitro activity of Arg180 mutants.

Activity was assessed by the double translation method as previously described. Lane 1 represents the molecular weight markers. The primary transcripts for the remaining lanes were, lane 2-preproalpha factor; lane 3-wild-type ricin A-chain; lane 4-Q180; lane 5-A180; lane 6-M180; lane 7-pseudopositive; lane 8-ricin B-chain (non-toxic). Lanes 3 to 8 had an aliquot of preproalpha factor added after the initial incubation of transcripts for 1 hour at 30°C. B, A, F and M? indicate the positions of ricin B-chain, A-chain, yeast preproalpha factor and the putative methionine-binding protein respectively.



**Fig. 3.22** N-glycosidase activity of Arg180 mutants.

Assessment of the activity of Arg180 mutants using the aniline method. Lanes labelled + are aniline-treated rRNA samples, those labelled - are untreated samples. The fragment released on aniline treatment is indicated with an arrow.

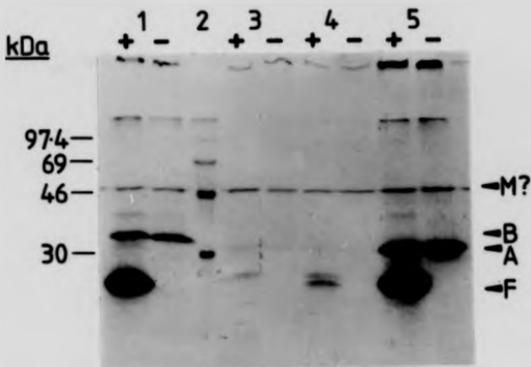
Lane 1 represents wild-type ricin A-chain and lane 5 (the negative control) is preproalpha factor. Lane 6 represents the pseudopositive control and a cleavage fragment is clearly seen. Lanes 2, 3 and 4 refer to the mutations Q180, A180 and M180 respectively.

assessed by this method and was not responsible for the lack of activity of mutants Q, A and M180. It has been shown, using nuclease-treated reticulocyte lysate, that these constructs are translated in this system (Fig. 3.21 and elsewhere (data not shown)). Therefore the lack of an observed cleavage fragment is probably a direct result of an inactive A-chain protein.

#### 3.2.5.4 In vitro activity of A29 and SEAAR

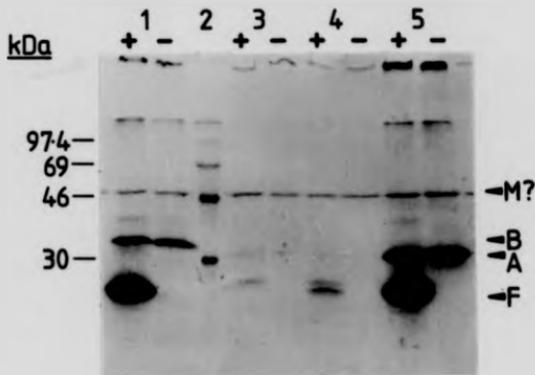
Mutants A29 and SEAAR were analysed with the same in vitro methods as discussed above. Fig. 3.23 shows the results from the double translation experiments using A29 and SEAAR transcripts. Lane 5 shows a SEAAR A-chain product together with a strong ppoF band, indicating that the SEAAR mutant was non-toxic in this system. However the A29 mutant appears almost fully toxic with only a faint ppoF product visible in lane 4. The RTA (lane 3) and B chain (lane 1) controls appear normal.

Examination of the mutants for depurination activity confirmed the results generated from the double translation experiment (as shown in Fig. 3.24). Lane 2 clearly demonstrates the appearance of a cleavage fragment when the translation system was treated with A29 transcripts. A29 would appear to have decreased activity relative to wild-type ricin A-chain, although the apparently large difference in intensity of the cleavage



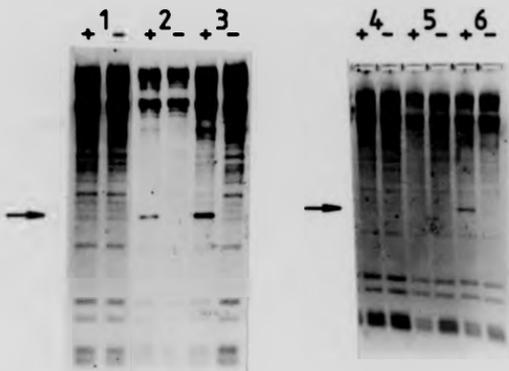
**Fig. 3.23** In vitro activity of Ala29 and SEAAR.

Assessment of activity by the double translation experiment. Lanes indicated + had preproalpha factor transcript added to the translation system after incubation of the primary transcript for 1 hour. Lane 1 refers to the non-toxic ricin B-chain control. Lane 2 indicates the molecular weight markers. Lane 3 represents the wild type ricin A-chain sample, whilst lanes 4 and 5 refer to A29 and SEAAR respectively. B, A, F and M? represent ricin B-chain, RTA, preproalpha factor and the putative methionine binding protein respectively.



**Fig. 3.23** In vitro activity of Ala29 and SEAAR.

Assessment of activity by the double translation experiment. Lanes indicated + had preproalpha factor transcript added to the translation system after incubation of the primary transcript for 1 hour. Lane 1 refers to the non-toxic ricin B-chain control. Lane 2 indicates the molecular weight markers. Lane 3 represents the wild type ricin A-chain sample, whilst lanes 4 and 5 refer to A29 and SEAAR respectively. B, A, F and M? represent ricin B-chain, RTA, preproalpha factor and the putative methionine binding protein respectively.



**Fig. 3.24** N-glycosidase activity of mutants A29 and SEAAR.

Assessment of activity using the aniline method. Lanes labelled + are aniline-treated rRNA samples, those labelled - are untreated samples. The fragment released on aniline treatment is indicated with an arrow.

Lanes 3 and 6 refer to wild-type ricin A-chain transcripts. Lanes 1 and 4 indicate the results following translation of non-toxic primary transcripts (preproalpha factor and B-chain respectively). Lanes 2 and 5 indicate the results of A29 and SEAAR respectively.

product is partly due to differences in rRNA loading.

Lane 5 of Fig. 3.24 indicates the results for the assessment of the N-glycosidase activity of SEAAAR. No cleavage product was observed, indicating that the SEAAAR construct was non-toxic. Since a SEAAAR polypeptide had previously been observed (Fig. 3.23), it is unlikely that the apparent lack of toxicity was due to a problem with the translation of the mutant.

#### 3.2.5.5 Summary of in vitro activity data

Use of the wheatgerm and rabbit reticulocyte translation systems has enabled the in vitro expression and the in vitro activity of the mutant ricin A-chain species to be qualitatively assessed. Translation in wheatgerm verified that the constructs were suitable for reticulocyte lysate in vitro translation experiments and allowed the polypeptides to be visualised. Double translation experiments examined the inhibition of protein synthesis by the A-chain constructs and, in particular, demonstrated those mutants that were inactive. In addition, the depurination of rRNA was assessed using the aniline assay which generated data that was particularly suitable for demonstrating those mutants which retained activity towards ribosomes. A summary table is shown in Fig. 3.25 overleaf.

Fig. 3.25 Summary of in vitro activity of rRTA mutants

| Mutant | Double translation | Aniline |
|--------|--------------------|---------|
| K177   | NT                 | NM      |
| A177   | T                  | M       |
| D177   | PT                 | PM      |
| Q180   | NT                 | NM      |
| A180   | NT                 | NM      |
| M180   | NT                 | NM      |
| A29    | PT                 | PM      |
| SEAAR  | NT                 | NM      |
| p+     | T                  | M       |

T- Toxic

M- Modifying

PT- Partial Toxicity

PM- Partial modification

NT- Non toxic

NM- Non modifying

3.2.6 The solubility/ribosome binding of rRTA is an in vitro system

A criticism of the in vitro translation experiments could be that apparently inactive toxins could lack ribosome-inactivation ability because they are produced in an insoluble form. It is plausible that the mutations created in this study may cause structural changes in the polypeptide as it is synthesised that cannot be accommodated into the folding process. This may lead to

aggregated, inactive proteins that are non-functional but are still translated. Thus polypeptides will be visualised by SDS-PAGE during the double translation experiments and assumed to be inactive because of removal of a crucial residue. However, the microenvironment and complexity of translation systems is poorly understood, as are the processes involved in protein folding. This limited knowledge means that prediction of the folding of mutant proteins is difficult and one has to assess this experimentally. Additionally, one cannot directly correlate the solubility properties of proteins produced in two heterologous expression systems in order to claim that a protein is 'soluble'.

In order to address this potential criticism, a preliminary experiment to investigate the ability of ricin A-chain and ricin A-chain mutants to be retained in the 'soluble' supernatant of a rabbit reticulocyte translation was carried out. In this experiment, and throughout the thesis, the criterion for solubility was retention of the protein in the supernatant following centrifugation at 100,000 xg for 60 minutes at 4°C.

It is important to state at this stage that there are difficulties in analysing the results of this experiment using this criterion of solubility. In a reticulocyte lysate translation system the concentration and total amount of rRTA produced is likely to be extremely low, hence the need to visualise the products with

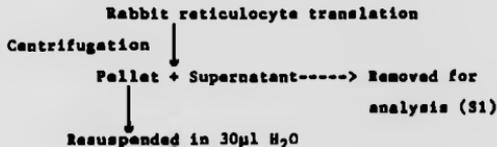
radioactivity. Since aggregation of insoluble proteins is dependent on concentration, it is possible that the insoluble aggregates formed in this study will be extremely small. If this was the case, aggregated (and therefore inactive) products may not pellet by centrifugation and would be incorrectly determined to be soluble.

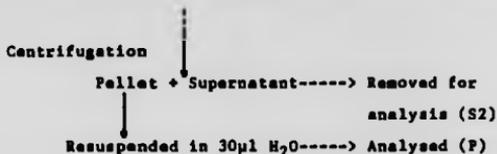
Initially the experiment involved centrifuging a reticulocyte lysate to examine the proportion of product in the supernatant and assess solubility. It was later decided to include several washing steps in an attempt to recover 'soluble' rRTA that was bound to, or associated with, the ribosomes during centrifugation. Under these conditions of centrifugation the ribosomes would be pelleted. Since the amount of rRTA was likely to be low, and since rRTA has an affinity for ribosomes, it was expected that the fraction of protein bound to the ribosomes would be high. This bound ricin A-chain might be incorrectly designated 'insoluble' by the above criterion of centrifugation alone. The first experiment to examine the solubility of the Glu177 mutants (Fig. 3.26a) used distilled water to wash the proteins from the ribosome. It was assumed that placing the ribosomes in a solution of unbuffered water would lead to alterations in the conformation of the ribosomes and the release of loosely bound proteins. With hindsight, this was perhaps a poor assumption since the very structural destruction of the

ribosome that was hoped for would likely affect the structure of rRTA and maybe induce aggregation.

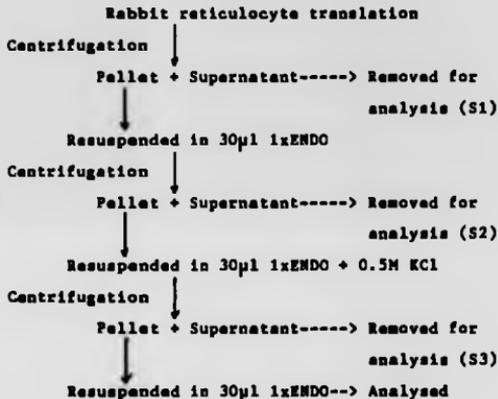
Subsequent experiments to assess the solubility of the R180, p+, A29 and SEAAR mutants used ixENDO (25mM Tris-HCl (pH 7.6), 25mM KCl and 5mM MgCl<sub>2</sub>) to wash (and resuspend) the ribosomes. This is considered to be a more physiologically favourable environment for ribosome stability. Washing the ribosomes in this buffer should release only loosely associated proteins, yet retain ribosome structure. An additional washing step was included in an attempt to remove all bound proteins from the ribosome using 0.5M KCl, as used in the preparation of salt-washed ribosomes (2.3.3.3). Thus any proteins pelleted after washing with 0.5M KCl were either aggregated (because of their structure or because of the experimental procedure) or very tightly bound to the ribosomes.

The results for the Glu177 mutants are shown in Fig. 3.26a. The abbreviations S1, S2 and P are explained in the experimental procedure below:





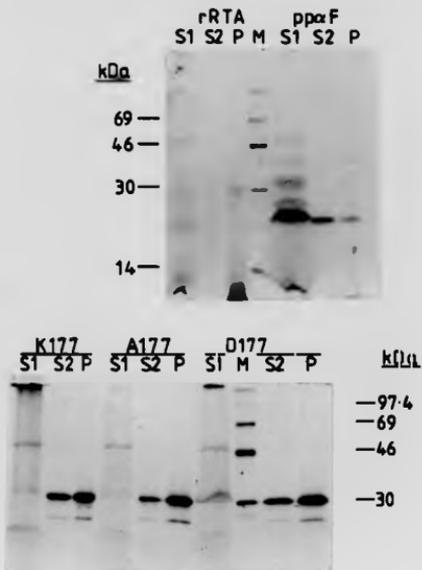
The results for the B180, p+, A29 and SEAAE mutants are described in Fig. 3.26b. The experimental procedure for this experiment was as follows:



Analysing the results presented in Fig. 3.26a allows several observations to be made. Firstly, the intensity of the products recovered was extremely low when the

transcript was wild-type ricin A-chain but greater for the mutant A-chains. This was to be expected since rRTA would inactivate the translation system within the period of the incubation (60 minutes). It can be seen however, that the majority of ricin A-chain species (wild-type and mutant) were found in the pelleted fractions after washing. This could be due to insoluble material although, in the main, this is thought to be unlikely. Examining the preproalpha factor control demonstrates that some alpha factor was to be found in the pellet, suggesting a small fraction of protein could have aggregated. The most likely explanation for the abundance of ricin A-chain in the pellet fraction is that the rRTA protein has bound to the ribosomes with a high affinity, and have thus been pelleted. The high-affinity binding has been observed previously during experiments with yeast ribosomes (M. R. Hartley, unpublished observations), and may explain the results here.

It will be shown later that mutagenesis of Glu177 to Asp had a negligible effect on the  $K_m$  of the N-glycosidase reaction. Extrapolating this result to here suggests that the interaction between the ribosomes and the ricin A-chain protein may be strong, even with these Glu177 mutants i.e. although their catalytic activities may vary markedly, their ribosome interaction properties may be similar to wild-type ricin A-chain. It is suggested therefore, that these pellet fractions (P) consist



**Fig. 3.26a** In vitro solubility assessment for Glu177 mutants.

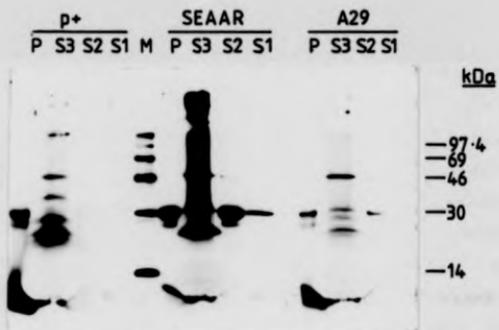
See text for experimental details. S1 refers to the supernatant recovered from a 100,000 xg centrifugation of the translation mixture. S2 refers to the supernatant from a subsequent centrifugation step as a result of resuspending the pellet from S1 in water. P refers to the contents of the pellet after removal of S2.

predominantly of active, soluble rRTA that has retained its capability of binding to the ribosomes.

With this view in mind, it is interesting to assess the solubility of the remaining mutants (shown in Fig. 3.26b). No mutant p+ was seen in the initial wash fraction (S1), indicating that the majority of the A-chain was bound to the ribosomes. Even after resuspending in 0.5M KCl (although without incubation), the p+ polypeptide eluted in the pellet fraction. Generally, the overall translation was lower for p+ and A29, as one would expect in light of the previous in vitro results. A small portion of A29 was found in supernatant S2 but none in S1. However, for those mutants shown to be inactive (Q180, A180, M180 and SEAAR), SDS-PAGE showed that a portion of the protein did not bind to, and was not pelleted with, the ribosomes. A much greater proportion of these mutants were visualised in S1 and S2. It had been shown in Fig. 3.26a that other proteins that do not bind to the ribosomes (e.g. alpha factor,  $\beta$ -globin and the putative methionine binding protein) were found in the first supernatant. This may suggest that the inactive mutants have poorer ribosome-binding properties and are released from the ribosome by simply washing in buffer. Note how the supernatant from the first centrifugation step (S1) contains substantially less mutant protein than the supernatant from the second centrifugation step (S2). Does this S2 supernatant contain soluble, low-binding-activity

Fig. 3.26b In vitro solubility assessment of Arg180 mutants, A29 and SEAAR.

See text for experimental details. S1 refers to the supernatant recovered from a 100,000 xg centrifugation of the translation mixture. S2 refers to the supernatant from a subsequent centrifugation step as a result of resuspending the pellet from S1 in 1xENDO buffer. S3 refers to the supernatant recovered following centrifugation of the pellet from S2 resuspended in 1xENDO + 0.5M KCl. P refers to the pellet recovered from centrifugation of S3.



mutants or does it contain insoluble, small aggregates? I would suggest the former, because if the latter were true then one would expect a greater proportion of rRTA mutant in S1. Some protein was observed in S3. Since it is unlikely that one would have a heterogeneous population of binding affinities, I would suggest that this pellet does in fact represent insoluble material.

In conclusion, it would appear that this type of experiment is affected by the nature of the proteins under test i.e. their ribosome-binding properties, which complicates the interpretation of their solubility. Accurate interpretation is also complicated because of not repeating these experiments, particularly with the wild-type ricin A-chain. Since I have previously shown A177 to be almost as active as rRTA, the fact that the pellet from A177 contains a much greater amount of translation product than the equivalent experiment with rRTA is an apparent anomaly. The potentially poor translation of the wild-type rRTA may explain this observation. However, given these problems with the experimental procedure, one view would be that the Glu177 mutants, created in this study, bind ribosomes with a similar affinity to wild-type ricin A-chain (and p+ and A29), however, the Arg180 mutants and SEAR would appear to bind with lower affinity, which may partly explain their lower activity towards ribosomes.

### 3.3 IN VIVO EXPRESSION

#### 3.3.1. Introduction

In order to produce mutant ricin A-chain that could be purified in quantities suitable for kinetic experiments, ricin A-chain was expressed the cytoplasm of Escherichia coli. E. coli was chosen as host for this expression work because it could easily be manipulated in large volumes, E. coli ribosomes have been shown to be insensitive to ricin A-chain (Endo & Tsurugi, 1988), and it had been previously shown to be a suitable system for ricin A-chain expression (O'Hare et al., 1987). Other expression systems will be discussed at a later stage, however the yield and purity of the protein product using the method described by O'Hare et al. was equivalent to other previously published methods and was chosen as a starting point. The strategies used, and the results obtained from the protein purification experiments are presented in section 3.4.2.

It is the purpose of this section to examine the expression of ricin A-chain and ricin A-chain mutants in E. coli. from construction of suitable expression plasmids, expression conditions, harvesting the protein, to the solubility and properties of the mutants when expressed in a bacterial system.

### 3.3.2 Construction of in vivo expression plasmids

The expression system used for this study was based on the use of the vector pDS5/3 (Stauber et al., 1984) The vector contains the strong coliphage T5 promoter P<sub>M25</sub> fused to the E. coli lac operator, with a ribosome binding site and initiation codon immediately upstream from a unique BamHI site. Downstream of this site are sequences for efficient transcription termination. Into this BamHI site had been inserted a 873bp ricin A-chain BamHI fragment containing the 5' XhoI site (at -15) and a stop codon after the last codon of rRTA. The fusion protein produced when this construct is expressed consists of a 10 amino acid N-terminal extension (MGSSRVEDNN), the first residue being derived from the vector, 5 from the pUC18 polylinker and 4 from the A-chain N-terminal leader, followed by the mature ricin A-chain sequence. The calculated molecular mass of this construct is 30925Da.

Mutated fragments of ricin A-chain were cloned into this pDS5/3RA vector using a similar procedure to that previously described for the construction of the in vitro expression vectors (Section 3.2.2.1). Mutations of Glu177 and the SEAAK deletion were excised from the mutated M13 DNA preparation as a ClaI-BglII fragment of 251bp. These isolated fragments were ligated into similarly digested and isolated pDS5/3RA vector arms, and miniprep DNA produced. Potential clones were screened by restriction

enzyme digestion and correct clones were confirmed by plasmid sequencing. Since the original A-chain template for A29 mutagenesis was derived from pDS5/3RA, mutant A29 was cloned into pDS5/3RA as a BamHI fragment. As described in section 3.2.2.1d, the presence of an additional ClaI site at position 409 in the A-chain complicated the cloning strategy of the Arg180 mutants. Partial digestions of the mutated pUC119 released the 251bp ClaI-BglII fragment which was isolated from agarose gels. The same fragment used for cloning into the in vitro expression vector was used for ligating into the pDS5/3RA DNA digested with ClaI and BglII. Also as before, a control clone was prepared using the ClaI-BglII fragment from the A29 mutant to produce a pseudo-positive (p+) wild-type clone which possessed the E138D mutation present in the R180 constructs. This control ricin A-chain was required for the Arg180 mutants to be sure that the additional mutation at position 138 did not alone affect expression.

### 3.3.3 Expression of wild-type ricin A-chain

Before expression of mutant proteins was attempted, the expression system was examined using wild-type ricin A-chain. Ricin A-chain and A-chain mutants cultured for expression experiments were grown under Category II containment facilities. Initial starting conditions were as described in O'Hare et al. (1987) and in the methods

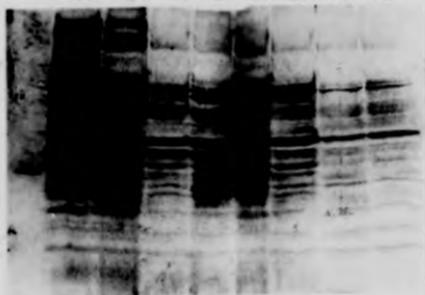
section 2.5. Initial expression experiments were performed in the E. coli strain 71.18 at 30°C in L-broth (L-B). Protein was harvested by treating the cells with lysozyme followed by disruption by sonication. Each stage of this protocol was examined in an attempt to improve the yield of soluble, functional ricin A-chain. The results are described below.

The Escherichia coli strain 71.18 (Yanisch-Perron et al., 1985) had been used previously for ricin A-chain expression. This strain was used because it is reported to produce high levels of the lac repressor and is thus suitable for inducible expression of genes under the control of the lac operator system. Other host strains could be used for the expression and some of these were examined in a direct comparison with 71.18. In one experiment, the total A-chain expression (i.e soluble + insoluble) using E. coli strains TG1, JM101 and JM105 transformed with pDS5/3BA were compared to a similarly grown 71.18 strain. 10ml cell cultures in L-B were grown to mid-log phase, induced with 1mM IPTG, and incubated to OD<sub>550</sub> = 0.9, before harvesting by treatment with lysozyme and cell disruption by sonication. Total expression was determined by electrophoresis of an aliquot of the cell suspension supernatant on SDS-PAGE and Western blotting. The results of such an experiment are shown in Fig. 3.27. No attempt was made to identify the relative levels of soluble A-chain. Of the duplicate samples analysed, the

Fig. 3.27 Comparison of rRTA expression in four E. coli strains.

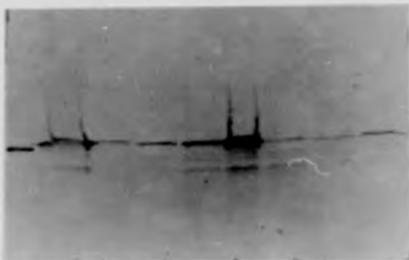
Four strains of E. coli were transformed with pDS5/3BA and the expression of ricin A-chain assessed. Expression is shown of 2 colonies (A & B) of each strain. Protein samples were derived from total cell lysis by boiling in loading buffer. The silver-stained polyacrylamide gel is shown in figure A and the corresponding Western blot in figure B. Lane 1 represents 400ng rRTA. Lanes 2, 3, 4 & 5 refer to the bacterial strains TC1, 71.18, JM101 and JM105 respectively.

1 2 3 4 5  
A B A B A B A B



A

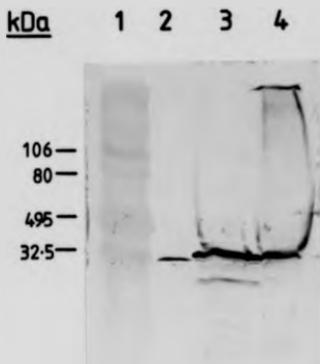
1 2 3 4 5  
A B A B A B A B



B

71.18 strain gave the most consistent A-chain expression although from this experiment it did not produce the highest levels of protein from individual cultures. Subsequent experiments showed that soluble rETA protein could be recovered from TG1 and JM101 cultures grown in L-B (data not shown). In a similar experiment to examine A-chain expression in JM109 in minimal media, the amount of expression of soluble A-chain was greater in the 71.18 strain under these conditions. Thus ricin A-chain could be expressed in a variety of E. coli strains to equivalent levels with no strain being more suited than 71.18.

The bacterial strain XL1-B (Bullock et al., 1987) was also examined for its ability to express the pDS5/3RA plasmid. This strain was capable of expressing soluble ricin A-chain in an relatively undegraded form that was suitable for large scale protein purification (Fig. 3.28). Approximately 1.5mg of pure ricin A-chain was recovered per litre of XL1-B culture as is described in the next section. However, because this strain offered no real advantage over the original 71.18 strain under these conditions, it was decided to maintain 71.18 as the strain of choice. Thus, having tested 5 strains of E. coli for expression of ricin A-chain when transformed with pDS5/3RA, none were found to be particularly more suited for expression than the original 71.18 cells chosen by O'Hara et al. (1987). It was decided to maintain 71.18 cells as the strain of choice, with the option of trying



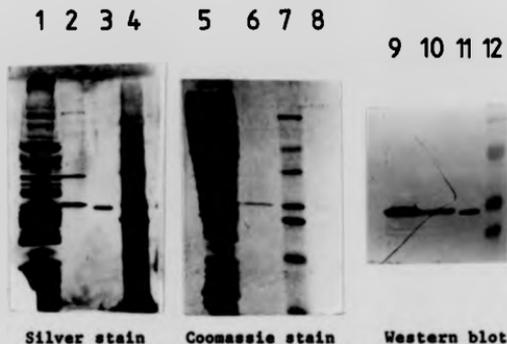
**Fig. 3.28** Expression of ricin A-chain in the *E. coli* strain XLI-B.

Lane 1 indicates the molecular weight markers and lane 2 represents 800ng rRTA. Lane 3 indicates that the RTA produced by XLI-B is retained in the supernatant of a 40% ammonium sulphate precipitation after centrifugation. Lane 4 refers to the supernatant following a 100,000 xg centrifugation, indicating that the vast majority of the RTA was soluble.

another strain (e.g. TG1) for the expression of any problematic mutants.

Often, a distinct A-chain band was difficult to visualise by staining crude E. coli preparations with silver or coomassie blue, indicating that the expressed protein was either not in great abundance or that ricin A-chain stained poorly relative to the background. To investigate this, samples of rRTA were separated by SDS-PAGE and stained with Coomassie blue and silver. It was observed that the staining with coomassie blue highlighted many more E. coli proteins than silver staining (Fig. 3.29). However, staining with silver was more sensitive for ricin A-chain as can be seen by examining the visualisation of the CM-Sepharose purified rRTA and the control rRTA (ICI). Thus the poor visualisation of ricin A-chain in crude E. coli extracts was probably due to the low abundance of the protein.

It is generally regarded that induction of expression of a foreign protein in bacteria should be rapid and be maintained only until sufficient expression levels have been attained. This minimises any host response to the foreign protein and reduces the likelihood of aggregation and production of insoluble, inactive protein. For this reason, cells were cultured to mid-log phase (OD<sub>550</sub> of approx. 0.6) before expression was induced. The pDS5/3RA plasmid has the promoter fused to the lac operator and thus induction of expression is achieved by culturing in



**Fig. 3.29** Comparison of the staining of ricin A-chain by coomassie blue and silver following SDS-PAGE.

The detection of *in vivo* produced ricin A-chain was investigated. Samples were separated by SDS-PAGE using a 15% polyacrylamide gel before staining as previously described (2.2.5).

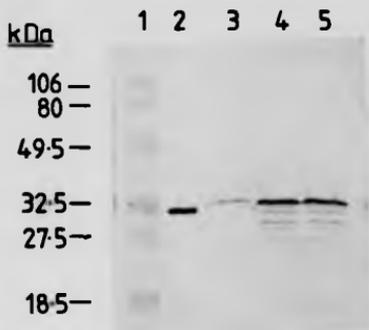
Lanes 1, 5 and 9 represent 200 $\mu$ g of a 40% ammonium sulphate supernatant of a crude *E. coli* sonicate containing ricin A-chain. Lanes 2, 6 and 10 represent approximately 1 $\mu$ g of CM-Sepharose purified A-chain (see Fig. 3.47). Lanes 3, 8 and 11 represent 1 $\mu$ g of rRTA (ICI), whilst lanes 4, 7 and 12 represent molecular weight markers.

the presence of IPTG. Cultures were generally maintained in the presence of the inducer to an OD<sub>550</sub> of 1.0-1.2 before the protein was harvested. It was interesting therefore when an overnight expression culture was assessed for the presence of rRTA. Ricin A-chain was found in a soluble form even after 16 hours at 30°C (data not shown). Unfortunately the activity of this preparation was not assayed therefore one cannot conclude that this preparation would have the same enzymatic activity as rRTA prepared under standard conditions.

It was also observed that there was a certain amount of unregulated protein expression from cultures that were grown in the absence of IPTG. This suggested that the vector was 'leaky' to a certain degree which could have a negative effect on the production of mutant protein, whose potentially strained folded structure may be more easily recognised by the bacterium. It is in this situation in particular when it is advantageous to express the protein for as short a time as possible. During all expression experiments a 1ml sample of bacterial culture was removed before the culture was induced. The total protein content of the cells before induction was examined by SDS-PAGE for cross-reactivity with A-chain antibodies (previously shown not to cross-react with E. coli polypeptides). The appearance of a 30kDa band confirmed this 'pre-induction' (Fig. 3.30). However the expression of rRTA was definitely increased on addition of the inducer as checked by SDS-

PAGE (Fig. 3.30). It was thought that there may be an analogue of the inducer in the culture medium (L-B). To test this suggestion, expression cultures were grown in minimal media (see section 2.1.1.2) with the addition of casamino acids to 1% (reduced expression was observed without the addition of casamino acids). Soluble, undegraded A-chain was shown to be expressed in minimal media to a level equivalent to expression in L-B (data not shown). However, even under these conditions, an amount of pre-induction was visualised although expression increased on addition of IPTG to 1mM. Although experiments were not designed to test this, a second explanation for the observed pre-induction could be insufficient levels of the lac repressor produced by the 71.18 strain used for these studies. This would seem to be the most likely explanation for these observations.

It had previously been noted by O'Hare et al. (1987) that growth of bacteria expressing ricin A-chain at 37°C led to aggregated rRTA with greatly reduced activity. Expression at 30°C was more satisfactory and has also been used by Shire et al. (1990). Other workers have shown that growth at elevated temperatures causes some heterologous proteins to be rapidly degraded or packaged into insoluble protein bodies (Schein & Notaburn, 1988). In contrast however, Piatak et al. (1988) expressed rRTA at 37°C, using a different promoter, to produce soluble, active product. Observations during this study showed that



**Fig. 3.30** Pre-induction of rRTA expression.

During an ricin A-chain expression experiment using *E. coli* 71.18 cells, aliquots of culture were removed and assayed for the presence of ricin A-chain protein. Lane 3 represents an aliquot of cell culture removed before the culture was induced with IPTG. Lanes 4 and 5 indicate the expression of A-chain after 2 and 2.5 hours of induction with 1mM IPTG respectively. Lane 1 indicates the molecular weight markers whilst lane 2 represents 1µg of rRTA (ICI).

soluble ricin A-chain could be produced at 30°C and 37°C, though the yield was slightly greater at 30°C. The expression temperature was thus maintained at 30°C.

The conditions of cell disruption were also examined for their effect on the yield of ricin A-chain. During extraction of the protein from the cells, the protease inhibitor PMSF was present to minimise degradation due to serine proteases. Extraction according to the method of O'Hare et al. utilised the properties of lysozyme and sonication. Cell pellets from 500ml expression cultures were resuspended in 2ml PBS and treated with lysozyme (to 2mg/ml) on ice for 10 minutes to substantially weaken the cell wall. The cells were then lysed by sonication (MSE Soniprap) with 3 x 20 second bursts (amplitude of 24). After a number of experiments it was felt that the lysozyme treatment of the cell suspension was an unnecessary step of the procedure. During multiple sonication experiments, cell suspensions were occasionally treated with the enzyme for longer than the suggested 5 minutes and this led to degradation of the cellular proteins to low molecular weight products. In order to minimise degradation of the rRTA product, the lysozyme step was removed from the protocol.

The sonication buffer was also changed to one consisting of 100mM Tris-HCl (pH 8.5) + 5mM EDTA. The more alkaline pH was thought to be beneficial for this class of proteins and had previously been successfully used during

purification of abrin A-chain (K. Wood, PhD thesis, 1991). There is much debate as to the ideal concentration of cells for sonication. O'Hare resuspended the cell pellet from a 500ml culture in 2ml of buffer which produces a cell suspension of approximately 3ml (i.e. approx. 170 OD units/ml). Other workers have resuspended the cells to 25 OD units/ml and found that this gave satisfactory sonication. Based on a procedure given in Harlow & Lane (1988), most sonication experiments in this study were carried out by the addition of 10 pellet volumes of buffer. It was assumed that the pellet volume of a 500ml culture of  $OD_{550}^{-1}$  was 1ml. Sonication was performed on ice with 8 x 20 second bursts (with 10 second rest periods on ice) being sufficient. When using this method of cell disruption here, approximately 150-200mg of protein was released into the supernatant fraction per litre of culture (as measured by the Biorad protein estimation method).

The use of ammonium sulphate precipitation was examined as a method of increasing the proportion of ricin A-chain in the sample before purification. A preparation of A-chain was subjected to step-wise increments in ammonium sulphate concentration by the addition of solid, from 40% to 80%, with stirring at 4°C. The precipitated protein was pelleted by centrifugation (31,000 xg/15 minutes/4°C), the supernatant decanted into a clean tube, more ammonium sulphate added, and the process repeated.

The protein pellets were resuspended in PBS and the protein precipitated using acetone (2.2.2.1). An aliquot of this precipitated protein was visualised by SDS-PAGE and western blotting (Fig. 3.31). It can be seen that some A-chain precipitated at the lowest salt concentration (40%) but the majority of A-chain was found in the 50%, 60% and 70% pellets. This experiment demonstrated that enrichment of a protein preparation for ricin A-chain from a crude E. coli extract could be achieved using an ammonium sulphate precipitation within the range of 40-70%, i.e. discarding the 40% pellet and retaining the subsequent 70% pellet. As will be described later however, the final purification strategy employed made the use of an ammonium sulphate precipitation stage unnecessary.

#### 3.3.4 Expression of ricin A-chain mutants

It was appreciated that some of the A-chain mutants would have properties that would make them difficult to express in the cytoplasm of Escherichia coli. Expression of ricin A-chain mutants by other workers has shown that often the yield of soluble protein was poor (Frankel et al., 1990; Ready et al., 1991). Different strategies have been employed by groups to express ricin A-chain mutants to reasonable levels, often without a great deal of success. Using the pDS vector system described above, it was hoped that some protein could be isolated that would

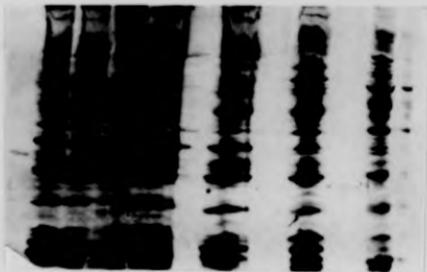
Fig. 3.31 Ammonium sulphate precipitation of ricin A-chain.

A sample of crude E. coli sonicate, shown to express ricin A-chain, was subjected to ammonium sulphate precipitation with stepwise increases in concentration. The concentration of ammonium sulphate was achieved by the slow addition of solid to the rapidly stirring A-chain preparation. Insoluble protein was pelleted by centrifugation. More solid was then added to increase the salt concentration.

Samples were removed and visualized by SDS-PAGE (A) and Western blotting (B). Lane 1=rRTA (300ng); lane 2=crude A-chain preparation before addition of salt; lane 3=untransformed E. coli; lane 4=40% pellet; lane 5=50% pellet; lane 6=50% supernatant; lane 7=60% pellet; lane 8=60% supernatant; lane 9=70% pellet; lane 10=70% supernatant; lane 11=80% pellet; lane 12=80% supernatant.

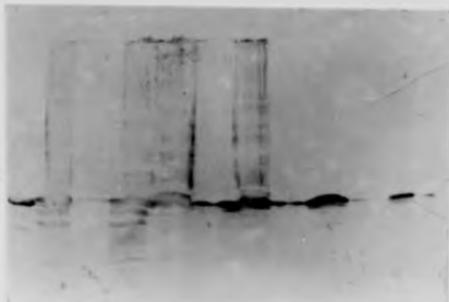
1 2 3 4 5 6 7 8 9 10 11 12

**A**



1 2 3 4 5 6 7 8 9 10 11 12

**B**



be useful for kinetic analysis.

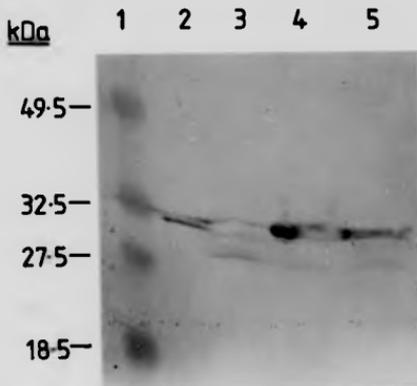
With both the plasmid sequencing and in vitro data available for these mutants it was possible to state that: the mutations were present in the in vivo expression vectors; translation of the mutant-fragment-containing DNA species produced polypeptides of the correct size; soluble, and sometimes active mutant polypeptides had been produced in a reticulocyte lysate translation system therefore correct folding did appear to be possible under the appropriate conditions. This aspect of expression will be discussed in Chapter 4.

The three mutations at the glutamate 177 codon all appeared to give an expression product in L-broth in large scale cultures. Solubility of the mutants was assessed by the usual criterion i.e. retention in the supernatant after a 100,000 xg centrifugation step (60 minutes/4°C). Mutant K177 was shown to be partially soluble in E. coli (the supernatant from a 100,000 xg spin is shown in Fig. 3.32) and additionally was retained in the supernatant following a 40% ammonium precipitation step (data not shown). An aliquot of soluble K177 was tested for N-glycosidase activity using reticulocyte ribosomes, and was shown to be inactive with the amounts used (see section 3.5). Mutant A177 was also shown to be expressed in L-broth in a soluble form that was later used for purification and analysis (Fig. 3.32). Mutant D177 was also expressed at high levels in 71.18 cells and the

be useful for kinetic analysis.

With both the plasmid sequencing and in vitro data available for these mutants it was possible to state that: the mutations were present in the in vivo expression vectors; translation of the mutant-fragment-containing DNA species produced polypeptides of the correct size; soluble, and sometimes active mutant polypeptides had been produced in a reticulocyte lysate translation system therefore correct folding did appear to be possible under the appropriate conditions. This aspect of expression will be discussed in Chapter 4.

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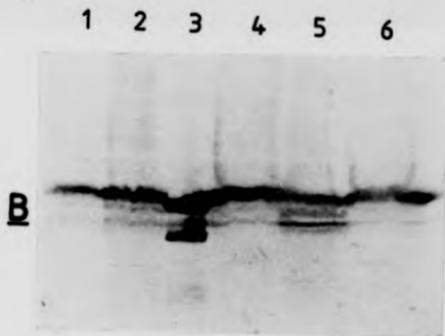
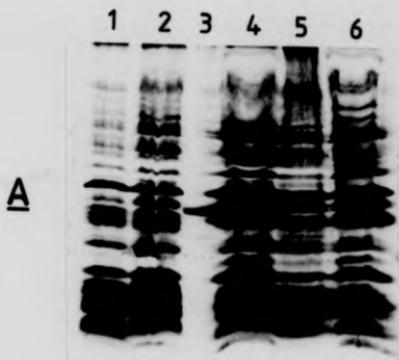
**Fig. 3.32** Expression of soluble glutamate 177 RTA mutants in *E. coli*.

Three 100ml cultures of K, A and D177 were harvested after 4 hours growth following the addition of IPTG. Insoluble protein was pelleted by centrifugation at 100,000  $\times$ g/60 minutes/4°C. 50 $\mu$ l of the supernatant fraction (equivalent to 5ml of cell culture before harvesting) was loaded onto a 15% SDS-PAGE gel and Western blotted. The Western blot is shown above. Lane 1 represents the molecular weight markers, lane 2 represents 1 $\mu$ g rRTA, lanes 3, 4 and 5 refer to aliquots of 'soluble' K, A and D177 respectively (i.e. the supernatants from a 100,000  $\times$ g spin for 60 minutes at 4°C.

Fig. 3.33 Expression of D177 RTA mutant in E. coli 71.18.

Samples of bacterial culture and isolated bacterial protein were assessed for the presence of mutant ricin A-chain. The silver stained gel from SDS-PAGE is shown in figure A, whilst the corresponding Western blot is shown in figure B.

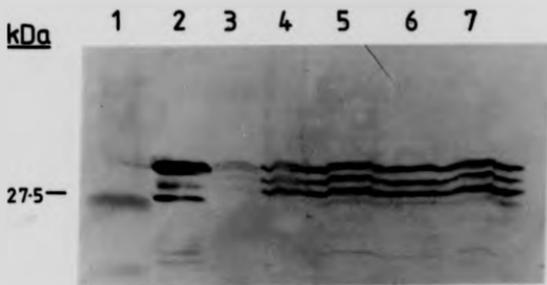
Lane 1 refers to a sample of total cell protein removed prior to the addition of IPTG. Lane 2 indicates A-chain released into the soluble phase after boiling a sample of bacterial culture in loading buffer prior to sonication. Lane 3 represents 800ng rRTA. Lane 4 indicates the supernatant obtained after sonication and low speed centrifugation. Lane 5 refers to the pellet obtained after centrifugation at 100,000 xg for 1 hour, whilst lane 6 indicates the corresponding supernatant.



majority was soluble (Fig. 3.32 and 3.33). This latter mutant was expressed in L-broth and harvested without an ammonium sulphate precipitation step for protein purification and kinetic analysis (section 3.4.2.6).

Expression of the SEAR mutant was unsuccessful under all conditions, with no A-chain cross-reactive band being visualised after screening a number of colonies for expression. This result was not unexpected since it is likely that this pentapeptide deletion may cause gross structural changes in the folding of the A-chain which may increase its susceptibility to degradation by the bacterial host.

Expression of the A29 mutant was observed but at a lower level than wild-type rRTA. Soluble protein could be recovered which appeared to consist of at least two additional putative degradation products (Fig. 3.34). It would appear from the Western blot in Fig. 3.34 that the recombinant rRTA control (ICI) also contained degradation products. These were often visualised when the Western blot developed particularly well. However, the A29 degradation products are clearly a great proportion of the total rRTA expressed. Over a number of experiments, these products were always visualised, suggesting that this protein has an increased susceptibility to degradation. It had been shown in vitro that soluble active product could be produced. Whether this mutant was active towards the E. coli ribosomes or simply more toxic to the bacteria

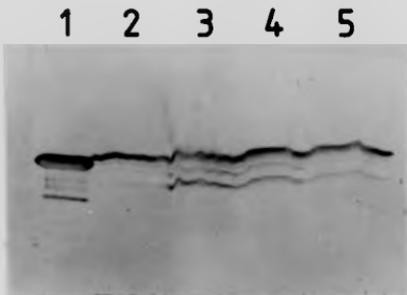


**Fig. 3.34** Expression of mutant A29 in *E. coli* strain 71.18.

Samples of culture and protein were assessed for the presence of the mutant ricin A-chain. In the Western blot above lane 1 refers to molecular weight markers and lane 2 represents 1 $\mu$ g rRTA. Lane 3 indicates a sample of the expression culture before the addition of IPTG, whilst lane 4 represents a total protein sample from the bacterial culture, after induction and before sonication. Lane 5 indicates the supernatant after low speed centrifugation of the sonicated preparation. Lane 6 refers to the pellet after centrifugation of the crude sonicate at 100,000  $\times$ g for 1 hour, whilst lane 7 refers to the corresponding supernatant.

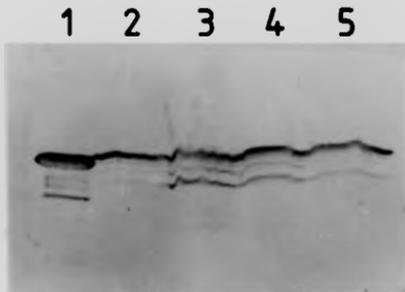
was unknown, however analysis of the growth curve of the A29 mutant in 71.18 cells showed no observable difference to that of wild-type A-chain expression (data not shown). When A29 was harvested at OD<sub>550</sub> =1.2, the cell growth was still in log-phase. Approximately 550mg of protein was recovered from the 1 litre culture which is substantially greater than normally achieved during rRTA expression. 65% of this protein was retained in the soluble fraction. A small amount of pre-induction was observed. The solubility of this protein may be a problem since after 4 weeks at 4°C all the A29 was present in the pellet fraction after a 100,000 xg spin (data not shown).

Expression of the Arg180 mutants in Escherichia coli was unsuccessful. Expression conditions were altered to include expression in minimal media, short and long periods of incubation in the presence of IPTG, and different E. coli strains (XL1-B, TG1). In all cases, no expression, soluble or insoluble, was achieved. It was thought that the extra mutation (E138D) within this polypeptide may in some way be involved in the poor expression. However, expression of the pDSp+ clone in E. coli 71.18 cells, in LB media, showed this not to be the case. The Western blot from the expression of pDSp+ is shown in Fig. 3.35. It was shown that there was an amount of pre-induction of expression (lane 2) as seen in previous experiments. Soluble protein (lane 5) was recovered following sonication of the cell pellet from a



**Fig. 3.35** Recovery of soluble protein from E. coli transformed with pDSp+.

100ml of an E. coli strain 71.18, transformed with pDSp+, was induced to express rRTA by the addition of IPTG to 1mM. After 2.5 hours incubation at 30°C the cells were harvested, subjected to sonication, and centrifuged at 13000 xg/30 minutes/4°C to pellet cell debris. The supernatant was centrifuged at 100,000 xg/60 minutes/4°C to pellet insoluble material. Aliquots of protein were removed at various stages for analysis by Western blotting. Lane 1 represents 1µg of rRTA (ICI). Lanes 2 and 3 represent a sample of cell culture removed before and after induction respectively. Lanes 4 and 5 represent a sample of E. coli sonicate before and from the supernatant after centrifugation of aggregated material at 100,000 xg.



**Fig. 3.35** Recovery of soluble protein from E. coli transformed with pDSp+.

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100ml culture.

It is difficult to rationalise the total lack of expression of the Arg180 mutants in light of the expression of the Glu177 mutants and the pseudo-positive. It is possible that the polypeptides produced were particularly sensitive to degradation by the E. coli host, although no major degradation products were observed. This may indicate that the amino acid chosen for mutagenesis (i.e. Arg180) is crucial to the structural integrity of the protein.

The Arg180 mutants presented here have also been expressed using the method of Schlossman et al. (1989). Expression was achieved using this method, but no soluble protein was recovered (Frankel et al., 1990). At present, soluble, mutated Arg180 protein has only been obtained for R180K and R180H (Frankel et al., 1990).

### 3.4 PURIFICATION OF PROTEINS

This section presents the results obtained from the various protein purification strategies used during the production and identification of ricin A-chain mutants from E. coli. The section begins with a description of the purification of anti-ricin A-chain antibodies that were later used for visualisation of rRTA during purification and for construction of an anti-ricin A-chain column. Various purification strategies were then employed in an attempt to purify ricin A-chain and ricin A-chain mutants for kinetic activity studies. These are presented in section 3.4.2.

#### 3.4.1 Purification of anti-ricin A-chain antibodies

Antibodies were raised against recombinant ricin A-chain by injection of purified protein (produced by ICI) into sheep. Serum was isolated and stored at -70°C until required. Using the method outlined in section 2.4, a protein fraction was prepared enriched in IgG. No selection was made for anti-ricin A-chain IgG. After heat-inactivation of serum complement, 1ml of serum was dialysed in 5mM phosphate buffer (pH 8.0) + 10mM NaCl. A precipitate was observed which was likely to be the IgM fraction of the serum. The dialysed, filter sterilised supernatant was applied to a TSK DEAE 5PW preparative HPLC

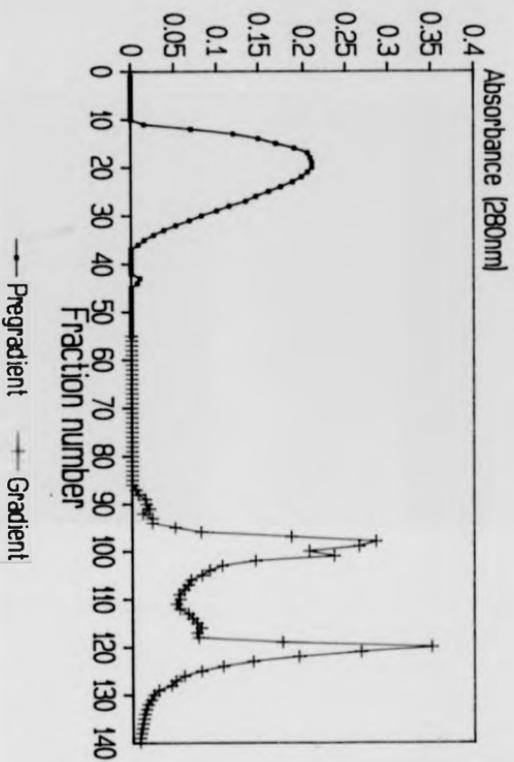
column. The sample was washed with this low salt buffer until the OD 280nm was at baseline (approx. 200 ml buffer). A 360ml linear gradient of 10mM NaCl to 300mM NaCl was used to separate the IgG fraction from the remaining serum proteins. 4.5ml fractions were collected, to which was added sodium azide to 0.05%, and stored in the elution buffer at -70°C. The elution profile is shown in Fig. 3.36.

Having tested the procedure with 1ml of sheep serum, the experiment was repeated with 5ml of dialysed serum. A similar elution profile was obtained. 5µl samples from this large-scale purification were analysed on a 7% denaturing / non-reducing polyacrylamide gel and coomassie blue stained to visualise, as shown in Fig 3.37. The IgG fraction was eluted from the column at the beginning of the salt gradient and fractions 37 to 40 (Fig. 3.37) were used as the IgG fraction for further experiments.

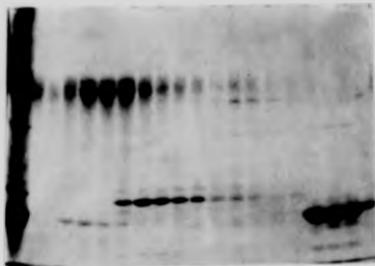
IgG concentration was estimated using the Biorad protein estimation reagent with gamma globulin for the standard curve. Concentration was estimated to be approximately 2mg/ml and therefore the antibodies did not require concentration before storage. Estimation of the concentration was verified using the equation  $OD_{280} 10\text{mg/ml} = 13.6$  OD units. Approximately 70mg IgG fraction was obtained from 5ml sheep serum. 15µl of this IgG enriched fraction was used for each Western blot in later experiments.

Fig. 3.36 Elution profile from ion-exchange chromatography of sheep serum to purify IgG.

1ml of heat-treated sheep serum was loaded onto a TSK DEAE-5PW HPLC column at a flow rate of 3ml/min and washed with 5mM phosphate buffer (pH 8.0) + 10mM NaCl and fractions collected (pragradient fractions). A 360ml linear gradient from 10mM to 300mM NaCl was initiated and 4.5ml fractions collected (gradient fractions).



S 16 38 40 42 44 49 51 53 63  
14 37 39 41 43 46 50 52 62 70



**Fig. 3.37** Purification of anti-ricin A-chain antibodies.

Sheep serum was subjected to DEAE-anion exchange chromatography as described in the text. Aliquots of eluted protein were visualised by electrophoresis on a 7% denaturing/non-reducing polyacrylamide gel and stained with coomassie blue.

Lane S refers to an aliquot of the crude serum before chromatography, the remaining lane numbers refer to the fraction number of elution from the column.

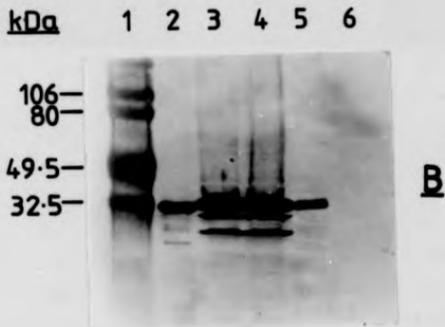
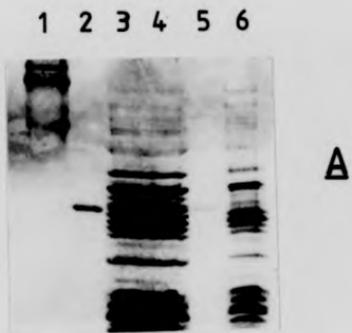
The IgG fraction was shown to be specific for a 30kD polypeptide that was present in E. coli cultures expressing ricin A-chain, and there was no cross-reactivity with E. coli polypeptides (Fig. 3.38). It can be seen in Fig. 3.38 that the rRTA produced by E. coli expressing the pDS5/3RA construct (lanes 3 and 4) was larger than the ICI recombinant ricin A-chain control (lane 2). This is due to the 10 amino acid N-terminal extension that the pDS5/3 translation product possesses relative to the 267 amino acids of native ricin A-chain. When visualised with the anti-sheep IgG-alkaline phosphatase conjugate and the alkaline phosphatase colour development system (2.2.5.2), these antibodies were shown to be sensitive to 1ng ricin A-chain on a dot blot, or 10ng on a 15% polyacrylamide gel (data not shown).

#### 3.4.2 Wild-type recombinant ricin A-chain purification

Crude preparations of recombinant ricin A-chain expressed in E. coli by the method described by O'Hara et al. (1987) were purified by chromatofocusing. The protein produced using this method was enriched for ricin A-chain which was soluble, active and could reassociate with ricin B-chain. However it was not totally pure and was thus unsuitable for kinetic measurements. Various methods have been utilised by other workers to purify rRTA. The choice of method often depends upon the choice of rRTA expression

Fig. 3.38 Specificity of anti-ricin A-chain antibodies.

The IgG-enriched fraction, prepared by DEAE-chromatography of sheep serum, was tested for its specificity for ricin A-chain in a crude bacterial cell extract. Lane 1 refers to the molecular weight markers, whilst lane 2 represents 400ng rRTA. Lanes 3 and 4 represent two independent preparations of 71.18 cells transformed with pDS5/3RA and induced to express ricin A-chain (20 $\mu$ l of each which is equivalent to 2ml of culture). Lane 5 represents approximately 400ng of an alternative ricin A-chain preparation (O'Hare). Lane 6 refers to an aliquot of 71.18 cells transformed with the vector pDS5/3. Figure A is the silver stained polyacrylamide gel and figure B is the corresponding Western blot.



vector. A full discussion of ricin A-chain purification methods is found in Chapter 4. In pursuit of pure rRTA various approaches were taken and represent an investigation into the problems of purifying soluble, active RIPs. The approaches and results below are organised in chronological order.

#### 3.4.2.1 Chromatofocusing

Initial experiments to purify ricin A-chain investigated the chromatofocusing protocol of O'Hare et al. (1987). Chromatofocusing separates polypeptides on the basis of their pI and the theory of the technique is explained in Giri (1990). Crude preparations of E. coli sonicates were applied to a chromatofocusing column equilibrated with ethanalamine (pH 9.4) and eluted with polybuffer 96. This initiated a pH gradient within the column such that the ricin A-chain should elute at its pI. The predicted pI of ricin A-chain is 7.5. The pH of eluted fractions was checked and the OD<sub>280</sub> measured. Fractions were visualised by Western blotting and silver staining of polyacrylamide gels. Ricin A-chain protein was visualised at an elution pH of approx. 7.6 to 7.4. The most encouraging elution profile is seen in Fig. 3.39. Unfortunately, the elution peak was broad and even in this experiment, fractions were found to contain more than one protein species (as shown in the Western blot and silver

Fig. 3.39 Elution profile determined from chromatofocusing of an RTA transformed E. coli culture.

Approximately 200mg of an E. coli sonicate (dialysed against 25mM ethanolamine (pH 9.4)) was loaded onto a chromatofocusing column prepared with PBE 94 (60ml bed volume) and equilibrated with the same buffer. The flow rate was set at 30ml/hour. A pH gradient was initiated with polybuffer 96 and 5ml fractions collected. Eluted fractions were assayed for OD<sub>280</sub> and the pH was checked at every 10 fractions as shown.

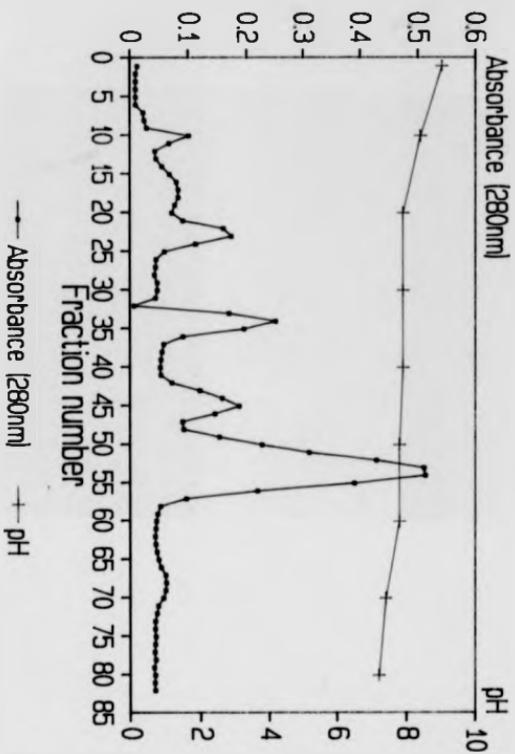


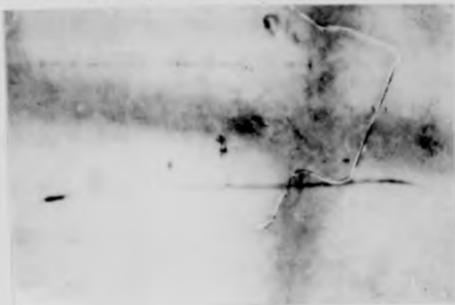
Fig. 3.40 Analysis of fractions from the partial purification of ricin A-chain by chromatofocusing.

Approximately 200mg of an E. coli strain 71.18, expressing active ricin A-chain, was partially purified by chromatofocusing using a pH gradient induced in the column by eluting proteins with polybuffer 96. 200 $\mu$ l of selected 5ml fractions was analysed by 10% SDS-PAGE (silver stain in A) and Western blotting (B). Ricin A-chain samples were identified in the blot using the enriched sheep anti-ricin A-chain IgG and anti-sheep horse-radish peroxidase.

Lane 1 represents 200ng of rRTA (ICI), whilst lane M represents the molecular weight markers. The remaining lanes represent fractions eluted from the chromatofocusing column as indicated in Fig. 3.39.

1 45 53 65 67 68 69 70 M

**A**



1 45 53 65 67 68 69 70 M kDa

**B**



stained gel from SDS-PAGE (Fig. 3.40). After several attempts to repeat this purification without success the procedure was discarded. There was little scope for alteration of conditions and the theoretically superior technique of immunoaffinity chromatography was possible following receipt of sheep serum containing anti-ricin A-chain IgG molecules. Therefore attention focussed on the construction of an affinity column.

#### 3.4.2.2 Anti-ricin A-chain immunoaffinity column

In theory, the immunoaffinity column represents the ideal purification strategy since the protein of interest (i.e. ricin A-chain) is selectively removed from the background proteins by using antibodies specific to that protein. The anti-ricin A-chain antibodies have already been shown to be specific for ricin A-chain and are not cross-reactive with E. coli proteins (Fig. 3.38). The matrix Affi-Gel 10 (Biorad) was chosen to bind the antibodies and prepare an affinity column. Affi-Gel was convenient to use since it spontaneously binds ligands that possess a primary amino group in aqueous solution. Coupling of the ligand (antibody) and column manipulations were performed at 4°C to minimise loss of antibody reactivity.

Antibodies were prepared by the IgG enrichment protocol described in section 3.4.1. Some of these

fractions contained a small amount of contaminating proteins which would also couple to the Affi-gel matrix. To avoid this, it would be possible to immunoabsorb the enriched IgG fraction on an rETA column and elute off only rETA-binding IgG. However, since the contamination of the fractions was low, it was decided to use the IgG enriched fraction directly. Additionally, the contamination was predominantly albumins which would couple to the matrix inefficiently at the coupling pH chosen. It was decided that for 1mg of ricin A-chain approximately 40mg of IgG would be required for efficient purification. According to the manufacturer, efficient coupling of globin was achieved at pH 7.5 (0.1M MOPS) with greater than 30mg ligand per ml gel (and at least 0.5ml ligand/ml gel) for 4 hours at 4°C. Antibodies were available at 2mg/ml.

A column was constructed with a lower than ideal antibody:matrix ratio but with sufficient IgG to purify at least 0.5mg rETA. The column was tested by passing a sample of ricin holotoxin through the matrix. No ricin flowed through the column as measured by the OD<sub>280</sub>. Ricin could not be eluted with 0.5M NaCl, nor with buffer at pH 5.0. At pH 2.0 the majority of the ricin was released from the column without any noticeable loss of IgG from the matrix. This would suggest that the anti-ricin A-chain antibodies recognise the ricin A-chain domain when it is in association with the B-chain. Since ricin A-chain is thought to undergo a conformational change when released

from the B-chain, it is interesting that the binding of the ricin to the column was so great.

The column was subsequently tested with a crude E. coli preparation containing rRTA. rRTA was found in the pre-low pH wash with 100mM Tris-HCl (pH 7.5). Changing the buffer to glycine-HCl (pH 2.15) + 25mM NaCl eluted a 30kDa polypeptide (with no degradation products) which was shown to be ricin A-chain by Western blotting. It can be seen in Fig. 3.41 that a portion of the A-chain passed through the column with the loading buffer (lanes 3 to 6). When the buffer was changed to glycine-HCl (pH 2.15), ricin A-chain was released (lanes 8 and 9), which was shown to retain N-glycosidase activity (data not shown). Unfortunately other polypeptides were visualised by silver stain in the pH 2.15 samples. There was no observable degradation of the matrix-antibody species at pH 2.15, as is apparent by comparing the pH 2.15 lanes (lanes 7 to 10) to lane 12 which contains an aliquot of serum.

There are many reasons why some rRTA may pass through the column. Since the initial preparation was not centrifuged at 100,000 xg before loading, this rRTA may represent poorly soluble, aggregated protein. It is also possible that there was an excess of rRTA present in the preparation to saturate all the IgG binding sites. However, it is unlikely that such a great proportion of A-chain would flow through the column since the matrix was sufficient to purify 0.5mg. This would suggest that either

the IgG used to prepare the column has a low titre of anti-ricin A-chain IgG, or alternatively it is possible that the previous pH 2.0 wash had a detrimental effect on the antibodies, reducing their affinity for rRTA. Since the IgG fraction had been tested to ing rRTA on a dot blot, the former suggestion is unlikely. It was also possible that the flow rate of buffer during loading of the rRTA may have been too rapid for efficient binding. The flow rate was altered by means of placing a syringe needle on the outlet tubing from the column. The bore of the needle adjusted the flow rate such that use of a 21G x 1½ needle achieved a flow rate of approximately 0.1ml/minute. Lowering the flow rates was examined without much success.

A new column matrix was prepared which was mixed with the crude rRTA preparation in a vessel and tumbled for 1 hour at 4°C. The theory of this approach is that the matrix will have a greater chance of binding the A-chain efficiently if it is mixed with the crude preparation over a period of time. The matrix was allowed to settle and the majority of the contaminating protein removed. The matrix was washed two times with 0.1M MOPS (pH 7.5) before it was packed into a column and washed to OD<sub>280</sub> = 0. Protein was eluted at pH 2.0 which was identified as ricin A-chain on SDS-PAGE with some polypeptide contamination visualised by silver stain (data not shown). Again rRTA was found in the washes before the low pH buffer was added.

1 2 3 4 5 6 7 8 9 10 11 12



**Fig. 3.41** Western blot showing the elution of ricin A-chain from an immunosorbent column with low pH buffer.

Approximately 150mg of protein from an E. coli strain expressing ricin A-chain was loaded onto an anti-ricin A-chain column constructed as described previously.

Lane 1 refers to molecular weight markers and lane 2 represents 250ng rITA. Lanes 3 to 6 refer to A-chain samples collected from washing the column with pH 7.5 buffer. Lanes 7 to 10 refer to protein collected after the pH of the column was reduced to pH 2.15. Lane 11 represents an aliquot of unpurified crude E. coli extract, whilst lane 12 represents an aliquot of sheep serum.

An additional reason for the apparent low level of binding of rRTA to the column may be too low a coupling efficiency of the antibody to the matrix. The coupling was assessed using three buffers; 0.1M MOPS (pH 7.5), 0.1M NaHCO<sub>3</sub> (pH 8.5) and 0.1M HEPES (pH 8.0). The same quantity of IgG was coupled to the same volume of matrix and equilibrated with the coupling buffer. Ricin A-chain was passed down each column 4 times before washing to baseline and then elution with glycine/HCl buffer (pH 2.15). Analysis of the amount of protein (using the Biorad assay) in the pre-wash and the low pH wash gave similar results for all columns, however the column constructed in HEPES buffer appeared to give the best retention of rRTA and therefore possibly the best IgG:matrix ratio.

The stability of the A-chain at pH 2.15 was examined by incubation of dilutions of a known concentration of ICI rRTA (1000ng to 1ng) in low pH buffer for 30 minutes on ice. The low pH samples were neutralised by the addition of 1.5µl 0.25M NaOH and the N-glycosidase activity of the rRTA assayed using yeast ribosomes (30°C / 20 minutes). Analysis of the cleavage products from the aniline assay showed that the lowest concentration of rRTA to give a visible cleavage product was 100ng in the neutralised low pH sample but only 10ng in the water-incubated control (Fig. 3.42). Thus it was concluded, from this relatively crude experiment, that the conditions of low pH and/or neutralisation did have a detrimental effect on the

1 2 3 4 5 6 7 8 9 10 11 12

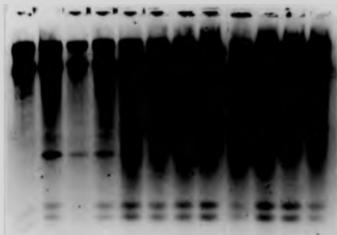


Fig. 3.42 Effect of low pH on RTA activity.

Dilutions of a known amount of rRTA (ICI) were prepared in glycine-HCl buffer (pH 2.15) or water and samples incubated for 30 minutes on ice before neutralisation and assay for N-glycosidase activity using yeast ribosomes. rRNA was extracted as described previously, treated with aniline and electrophoresed on a denaturing agarose/formamide gel.

Lane 1 represents the non-aniline treated control for the samples in lane 2. Lanes 2, 3, 4, 5, 6 and 7 represent 1000, 500, 250, 100, 10 and 1ng respectively of rRTA incubated at low pH and lanes 8, 9, 10, 11 and 12 refer to 1000, 250, 100, 10 and 1 ng rRTA incubated in water.

activity of ricin A-chain, even when compared to the water incubation which represents conditions that are not ideal for its stability. It was noted, after these experiments had been performed, that neutralisation of the acidic pH by addition of alkali was unusual and perhaps detrimental because of the likelihood of subjecting the protein to harsh changes in pH. It was accepted that neutralisation by the addition of high strength buffer at a neutral pH would have been more appropriate.

In addition to the apparent column run through, and the consequent losses of protein, the impact of low pH on the activity of rRTA samples made this immunoaffinity approach, under the conditions described here, an unsuitable method for purifying ricin A-chain for the determination of kinetic parameters. However the potential of this method is great, and since relatively active highly enriched rRTA was recovered from this system, I am confident that given more time a suitable methodology would have been determined. Using polyclonal sera, it is a particularly appropriate technique for the purification of mutant proteins since it is based on the characteristics of structure and not chemical properties that may alter as a result of mutagenesis (such as hydrophobicity). It can thus serve a secondary role of screening for ricin A-chain mutants with the correct structure.

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### 3.4.2.3 Anion-exchange chromatography

Most proteins from Escherichia coli have an isoelectric point (pI) of 6 or less, whereas ricin A-chain has a pI of 7.5. This difference can be exploited for protein purification using ion-exchange chromatography. An anion-exchange column will bind proteins with a net negative charge at a pH greater than the pI of the protein. Experiments were performed using a DEAE-MPLC column at pH 8.0 and pH 7.5 (5mM phosphate buffer). A-chain samples were dialysed against 5mM phosphate buffer + 25mM NaCl at the appropriate pH, filter sterilised, and then washed through the column at 3ml/min with the low salt buffer. When the OD<sub>280</sub> was at baseline, a linear salt gradient was constructed from 25mM to 500mM NaCl over a period of 2 hours and 6ml fractions collected. Fractions for assay were precipitated from the dilute solutions by TCA precipitation (2.2.2.2). Analysis of the pH 8.0 column indicated rRTA in the prgradient wash (Fig. 3.43). Relating this to the profile (Fig. 3.44) showed that the ricin A-chain was to be found in the first column washes. This ricin A-chain preparation had not been checked for solubility and may thus represent aggregated A-chain, whose behaviour on an ion-exchange matrix may be non-representative of soluble A-chain. However N-glycosidase activity of these initial fractions was assessed and active protein was found (data not shown).

**Fig. 3.43** Elution of recombinant rTA in the prgradient of a DEAE-column.

15mg of a crude E. coli sonicate was subjected to ion-exchange chromatography as described in the text. 1ml aliquots of selected 6ml fractions were precipitated with TCA and BSA (to 5µg/ml). Samples were assayed by SDS-PAGE (silver stain - A) and Western blotting (B). Lanes P, M, B, RA and C represent prestained molecular weight markers, protein markers, BSA alone, 250ng rRTA and 200µg of crude, unpurified sample respectively. The remaining lane numbers refer to the prgradient (10 to 46) and gradient (72 to 127) fractions from the elution with salt (see Fig. 3.44).

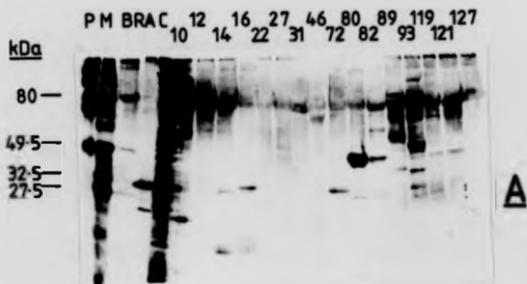
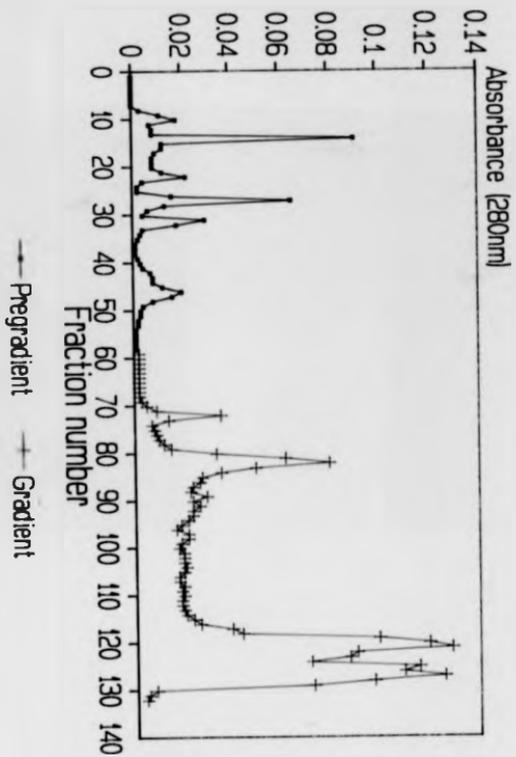


Fig. 3.44 Elution profile of DEAE-chromatography of E. coli extracts containing recombinant RTA.

15mg of crude E. coli sonicate was loaded onto a TSK DEAE 5PW ion-exchange column in 5mM phosphate buffer (pH 8) + 25mM NaCl. Unbound proteins were removed by washing the column with 350ml of the same buffer at 3ml/min. After collection of fraction 58, a 360ml linear salt gradient of 25 to 500mM NaCl (in 5mM phosphate (pH 8)) was initiated and 6ml fractions collected for assay (gradient fractions).



It was unexpected to find recombinant ricin A-chain in the pregradient wash since at pH 8.0 the protein should possess a net negative charge and thus bind to the column. Ricin A-chain at pH 7.5 bound to the column strongly and was eluted in a sample containing many other protein species. A repeat of the pH 8.0 column with a new rETA preparation showed a similar OD<sub>280</sub> recovery profile. However when proteins were precipitated with 10% TCA (with the addition of BSA (5ug/ml) as a 'carrier' protein), A-chain was recovered from the pregradient wash that was not 100% pure (Fig. 3.43). Problems of lack of homogeneity, room temperature purification and HPLC breakdown prompted a change in procedure.

It had been shown that rETA was not retained by this anion-exchange column at pH 8.0 whereas most of the contaminating proteins bound to the matrix. By using a cation-exchange matrix at a lower pH, it was hoped to achieve a more efficient purification of recombinant ricin A-chain using a more 'classical' approach of elution of the protein of interest within a salt gradient.

#### 3.4.2.4 Cation-exchange chromatography

Using the data generated from the DEAE- experiments described above, a cation-exchange column was constructed at 4°C using the matrix CN-Sepharose 6LB (Pharmacia). The matrix was equilibrated to pH 6.5 since this was

approximately 1 pH unit below the predicted pI of ricin A-chain, thus the protein would have a net positive charge and elution should be achieved using a salt gradient of 0 to 300mM NaCl.

The initial purification experiment used the dialysed 40% ammonium sulphate supernatant from an *E. coli* XL1-B rRTA-expressing strain. A sample was loaded onto a CM-Sephacrose column and washed to baseline with 5mM phosphate (pH 6.5) at 30ml/hour. No A-chain was seen to elute in the no salt wash (Fig. 3.45). A linear gradient of 0 to 300mM NaCl was constructed with 300ml of each buffer. rRTA was found to elute at approximately 0.1M-0.15M NaCl in a defined peak and was shown to be almost pure by silver staining (Fig. 3.45). Although not particularly easily visualised in this experiment because of poor gel quality, a polypeptide of greater size usually copurified with the rRTA and did not cross-react with the anti-A-chain antibodies. A-chain migrated at an apparently larger size than the control rRTA (ICI-also unglycosylated) due to the 10 amino acid N-terminal extension. Some degradation of the rRTA was visualised. This was probably due to non-specific degradation by contaminating proteases on storage of the crude sample for 10 days at 4°C before purification. rRTA was estimated to be produced at 1.5mg/litre culture and was shown to be active towards yeast ribosomes (data not shown).

Fig. 3.45 Purification of recombinant ricin A-chain from E. coli XL1-B.

34 mg of supernatant from a 40% ammonium sulphate precipitation (dialysed to 5mM phosphate (pH 6.5)) was loaded onto a CM-Sephacose cation-exchange column (matrix volume approximately 30ml). Unbound proteins were eluted with 5mM phosphate (pH 6.5) and the column washed thoroughly (400ml). A gradient was initiated from 0 to 300mM NaCl (in 5mM phosphate (pH 6.5)) and fractions collected for analysis. Figure A refers to the silver stained gel from SDS-PAGE and figure B represents the corresponding Western blot.

Lane M refers to the molecular weight markers, lane B represents 400ng rRTA and lane C indicates the protein content of the crude A-chain sample before column purification. Lanes 1 and 3 refer to two pregradient fractions, whilst the remaining lanes (7 to 67) refer to selected gradient fractions (prepared by precipitation of 0.6ml of each fraction with acetone).

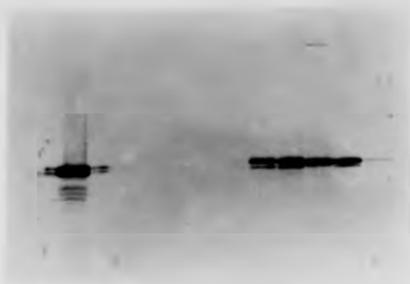
kDa M R C 1 3 7 16 20 24 29 31 35 37 43 67

97.4—  
57.5—  
40—



**A**

M R C 1 3 7 16 20 24 29 31 35 37 43 67



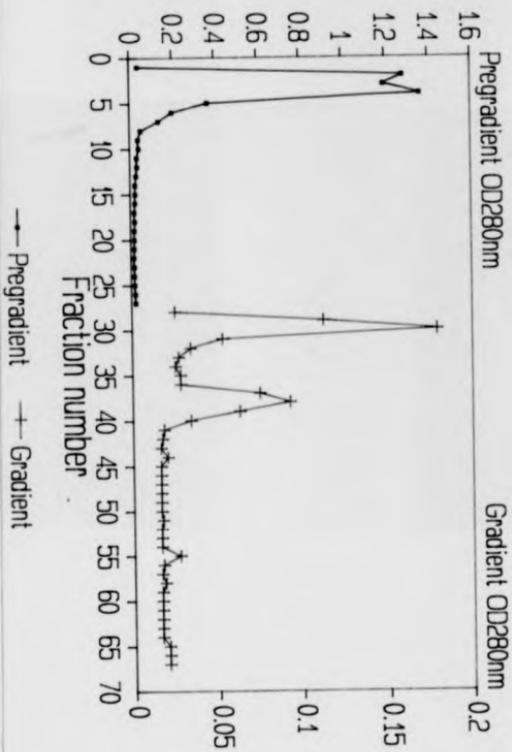
**B**

This procedure formed the basis of the purification strategy used for the various ricin A-chain species produced in this study. It proved to be a reproducible method for semi-purifying rRTA (to at least 50%, with only one contaminant). Before attempting to purify an rRTA, samples of protein were removed during production of the crude preparation for analysis of expression level and solubility (and in some cases in vitro activity towards eukaryotic ribosomes).

Wild-type recombinant ricin A-chain was purified for kinetic activity measurements from Escherichia coli 71.18 cells transformed with pDS5/3RA. It was found that the ammonium sulphate precipitation was not essential for enriching the preparation for ion-exchange chromatography, thus the crude sonicate supernatant was loaded directly onto the CM-sepharose column. A typical elution profile is shown in Fig. 3.46. Unbound proteins were eluted from the matrix at 30ml/hour with 5mM phosphate buffer (pH 6.5). A salt gradient of 0 to 300mM was initiated after collection of fraction 27 and fractions collected for assay. Eluted proteins were visualised by SDS-PAGE and Western blotting and the fractions examined for the presence of rRTA. A typical Western blot and corresponding silver stained gel is shown in Fig. 3.47. A-chain eluted over approximately 10 fractions (i.e 100ml). rRTA fractions were pooled and stored temporarily at 4°C.

Fig. 3.46 Elution profile for CM-Sepharose chromatography of E. coli extracts containing recombinant ricin A-chain.

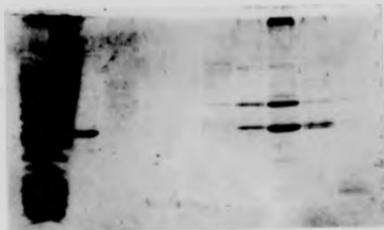
Approximately 40mg of soluble E. coli sonicate was applied to a CM-Sepharose column (matrix volume of 30ml) and washed with 5mM phosphate buffer (pH 6.5) to remove unbound proteins (pragradient fractions). After collection of fraction 27, a 0 to 300 mM NaCl gradient (in 5mM phosphate (pH 6.5)) was initiated. 10ml fractions were collected and the optical density at 280nm recorded (Gradient OD280nm). Selected fractions were assayed for N-glycosidase activity and immunoreactivity with anti-ricin A-chain antibodies.



**Fig. 3.47** Purification of recombinant ricin A-chain by CN-Sepharose chromatography.

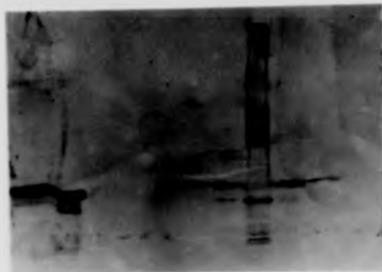
Experimental details are described in the text. 0.7ml of selected fractions (10ml) were precipitated with acetone and visualised by SDS-PAGE and Western blotting. Lanes 1 and 2 refer to a crude sample before loading onto the column and 1 $\mu$ g rRTA respectively. The remaining lane numbers refer to the column fraction of the elution profile (see Fig. 3.46). Figure A indicates the silver stained polyacrylamide gel of the corresponding Western blot (figure B).

1 2 4 29 30 31 36 38 40 55



**A**

1 2 4 29 30 31 36 38 40 55



**B**

It was noted that recombinant ricin A-chain preparations derived from expression in 71.18 cells contained a contaminating protein (Fig. 3.47) that had been seen previously after concentration of the fractions from the purification from E. coli strain XL1-B. It was decided to apply this semi-pure preparation to a second cation-exchange matrix. The theory behind this approach is that proteins interact differently when in the presence of a weak or a strong ion-exchange matrix, and it was hoped that these two proteins (ricin A-chain and the contaminant) would have different characteristics under these conditions.

A strong cation-exchanger, S-Sepharose, was used to construct a column which was equilibrated with 5mM phosphate (pH 6.5) at 50ml/hour at 4°C. The pooled semi-pure rRTA was applied to the column, washed with 5mM phosphate (pH 6.5), and eluted with a linear gradient of 350ml 0mM NaCl + 350ml 300mM NaCl. Fractions of 10ml were collected for assay. The elution profile is shown in Fig 3.48. Fractions analysed by SDS-PAGE and Western blotting (Fig. 3.49) indicated that the contaminating protein had eluted from the column during the low salt wash (peak at fraction 9 in Fig. 3.48) leaving the now pure rRTA to elute with the gradient in 5 fractions at approximately 0.15M NaCl. Thus this purification step also served to further concentrate the rRTA sample. This preparation of rRTA was shown to have N-glycosidase activity and was

suitable for kinetic analysis (section 3.5).

Estimation of the protein concentration revealed that the pooled CM fraction consisted of 4.8mg protein/litre of culture which in turn yielded approximately 2mg/litre of pure rRTA after separation by S-Sepharose chromatography. The total amount of soluble protein isolated from the bacterial culture was 100mg therefore the pure, soluble rRTA represented 2% of the total soluble E. coli proteins (or 1% of the total E. coli proteins).

It was interesting to examine if the use of the CM-sepharose column could be bypassed and A-chain loaded directly onto the S-Sepharose column. A crude A-chain preparation was applied to the S-Sepharose matrix using the same procedure as described above, and fractions eluted as shown in Fig. 3.50. A-chain was recovered in the salt gradient (at approx. 0.1M NaCl) but there were many contaminating proteins present as shown in Fig. 3.51. This demonstrated that S-Sepharose alone cannot suffice as a one-step purification procedure. Comparison of Figs. 3.51 and 3.49 demonstrated the differences in behaviour of proteins in the presence of different strength ion-exchange matrices in the same buffer system.

Fig. 3.48 Elution profile of CM-Sepharose-purified ricin A-chain applied to a S-Sepharose column.

Recombinant ricin A-chain expressed in E. coli 71.18 cells was semi-purified by cation-exchange chromatography on a CM-Sepharose column (Fig. 3.47). Pooled fractions containing ricin A-chain were applied to a S-Sepharose column (matrix volume of 60ml) and washed with 250ml 5M phosphate (pH 6.5). A linear gradient of 0 to 300mM NaCl (in 5mM phosphate (pH 6.5)) was initiated after collection of fraction 25, and 10ml fractions collected for analysis (Fig. 3.49).

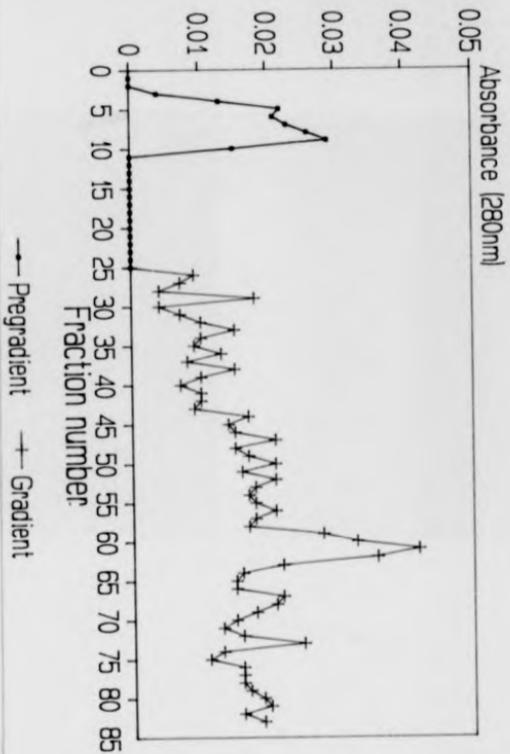


Fig. 3.49 Analysis of fractions from S-Sepharose chromatography of CM-Sepharose-purified recombinant ricin A-chain.

70ml of semi-purified ricin A-chain was applied to an S-Sepharose column and washed with 5mM phosphate buffer (pH 6.5) to remove unbound proteins. A linear gradient was initiated and fractions collected for analysis. 0.7ml of each selected fraction was precipitated with acetone for analysis by silver staining following SDS-PAGE (figure A) or Western blotting (figure B).

Lane 1 indicates the molecular weight markers and lane 2 represents an aliquot of the crude E. coli sonicate before CM- or S-Sepharose chromatography. Lane 3 represents 800ng rRTA and lane 4 refers to an aliquot of pooled CM-Sepharose purified RTA prior to chromatography on S-Sepharose. The remaining lane numbers refer to the fraction numbers shown in Fig. 3.48

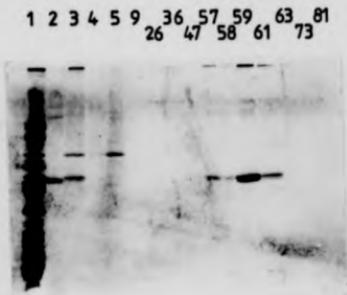


Fig. 3.50 Elution profile determined from S-Sepharose chromatography of E. coli extracts containing recombinant ricin A-chain.

50mg of a crude protein preparation from the supernatant of a 40X ammonium sulphate precipitation from a 0.5 litre culture of E. coli was loaded onto an S-Sepharose column (matrix volume of 60ml). The column was washed with 400ml 5mM phosphate buffer (pH 6.5) at 50ml/hour (pregradient fractions).

A linear gradient from 0mM to 300mM NaCl (in 5mM phosphate (pH 6.5)) was constructed and fractions collected for absorbance measurement (gradient fractions) and SDS-PAGE (Fig. 3.51)

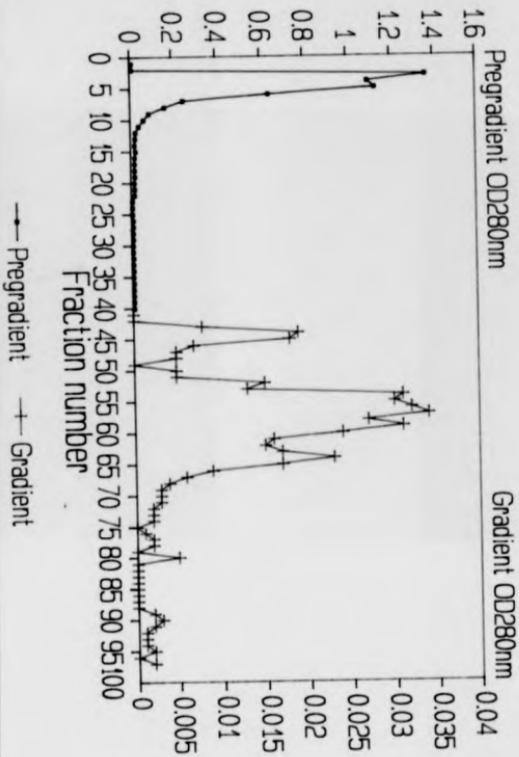
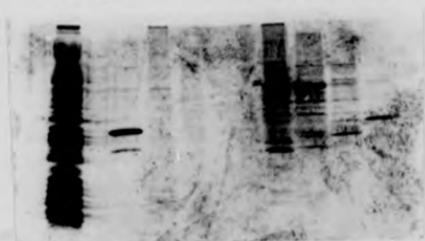


Fig. 3.51 Eluted fractions from a recombinant ricin A-chain preparation applied directly to a S-Sepharose column.

Experimental conditions were as for Fig. 3.49 but for using a crude ricin A-chain-containing bacterial sonicate as starting material. 0.7ml of each selected fraction was precipitated with acetone and the products analysed by SDS-PAGE. The silver stained gel is shown in figure A and the corresponding Western blot in figure B.

Lane M refers to the molecular weight markers. Lane 1 represents an aliquot of crude A-chain preparation prior to chromatography, and lane 3 represents 1µg of rRTA. The remaining lane numbers refer to fractions eluted from the S-Sepharose column (refer to Fig. 3.50).

M 1 2 3 5 45 52 54 57 60 64



**A**

**kDa**  
M 1 2 3 5 45 52 54 57 60 64

80 —  
49.5 —  
32.5 —  
27.5 —  
18.5 —



**B**

### 3.4.2.5 Summary of wild-type rRTA purification strategy

Having examined the production of soluble, active recombinant ricin A-chain as part of this study, the following procedure has been determined for the preparation of wild-type rRTA.

1. The E. coli strain 71.18 was transformed with the A-chain expression vector pDS5/3RA.
2. A 500ml expression culture was prepared in L-broth at 30°C at 250rpm.
3. The bacteria were cultured to an OD<sub>550</sub> of 0.6 to 0.8 before A-chain expression was induced by the addition of IPTG to 1mM.
4. The culture was maintained in the presence of inducer to an OD<sub>550</sub> of 1.0 to 1.2. The cell pellet was harvested by centrifugation at 3000 xg/4°C/10 minutes and the supernatant discarded.
5. The cells were resuspended in 10 pellet volumes of ice-cold sonication buffer (100mM Tris-HCl (pH 8.5) + 5mM EDTA).
6. The cell suspension was sonicated on ice with 8 x 20 second bursts (10 second intervals) at an amplitude of 24. The cells were pelleted by centrifugation (17500 xg/30 minutes/4°C) and discarded.
7. The resultant supernatant was known as the crude A-chain preparation. It was dialysed against 5mM phosphate

buffer (pH 6.5) for 2 x 12 hours at 4°C.

8. The protein solution was applied to a CM-Sepharose cation-exchange column at 30ml/hour at 4°C. After washing, a 600ml salt gradient was initiated from 0 to 300mM NaCl in 5mM phosphate. 10ml fractions were collected and analysed by SDS-PAGE and Western blotting. Recombinant ricin A-chain eluted at approx. 0.1M NaCl.

9. Ricin A-chain fractions were pooled and applied, without re-equilibration, to an S-Sepharose cation-exchange column. A gradient was initiated from 0 to 300mM NaCl and 10ml fractions were collected. Recombinant ricin A-chain eluted at approximately 0.15M NaCl.

10. rRTA fractions could be stably stored at -70°C in 15% glycerol in the elution buffer from the column.

#### 3.4.2.6 Purification of recombinant ricin A-chain mutants from Escherichia coli

Since soluble recombinant ricin A-chain had only been produced in vivo in preliminary experiments for the A-chain constructs K177, A177, D177 and A29, only these mutants were available for purification experiments.

A177 was the first mutant protein to be purified. The mutation of a single amino acid from glutamate to alanine removed a negative charge from the protein. Thus at pH 6.5 the mutant will be more positively charged than the wild-type construct (predicted pI = 7.51) and should therefore

elute at a greater salt concentration from the CM-Sephacrose column. A preparation of A177 was produced which was shown to contain a proportion which was soluble. 60mg of dialysed crude protein was loaded onto a CM-Sephacrose column and washed with 250ml 5mM phosphate buffer (pH 6.5). The flow rate was set at 30ml/hour and 10ml fractions were collected. A linear gradient of 0 to 300mM NaCl was produced (300ml + 300ml). The change in OD<sub>280</sub> for the elution profile is shown in Fig. 3.52.

Selected fractions were analysed by SDS-PAGE and Western blotting for the presence of A177 and contaminating proteins. The purification profile of A177 is shown in Fig. 3.53. Fractions 41-45 were pooled (approx. 0.1M NaCl) and the concentration of protein estimated to be 3.1µg/ml, i.e. approximately 400µg of soluble A177 per litre of initial culture. This is five times lower than for wild-type recombinant ricin A-chain and probably reflects the increased production of insoluble, aggregated A177. The activity of this pure A177 was assayed in vitro and activity was detected. See section 3.5.1 for a detailed comparison of the activity of A177 with wild-type.

Mutant D177 (predicted pI = 6.94) was also purified using the CM-Sephacrose column. A 500ml culture of D177 was prepared in L-broth and grown to an OD<sub>550</sub> of 1.2 before harvesting. The conservative change of Glu to Asp would be expected to have little effect on the purification

Fig. 3.52 Elution profile determined from CM-Sapharose chromatography of recombinant ricin A-chain mutant A177.

60mg of a crude preparation of E. coli 71.18 cells expressing A177 was applied to a CM-Sapharose column (matrix volume of 30ml) and washed with 250ml of 5mM phosphate (pH 6.5) buffer to remove unbound proteins. A linear gradient of 0 to 300mM NaCl (in 5mM phosphate (pH 6.5)) was initiated and 10ml fractions collected. Samples were analysed for OD280nm and then later, as shown in Fig. 3.53, by SDS-PAGE.

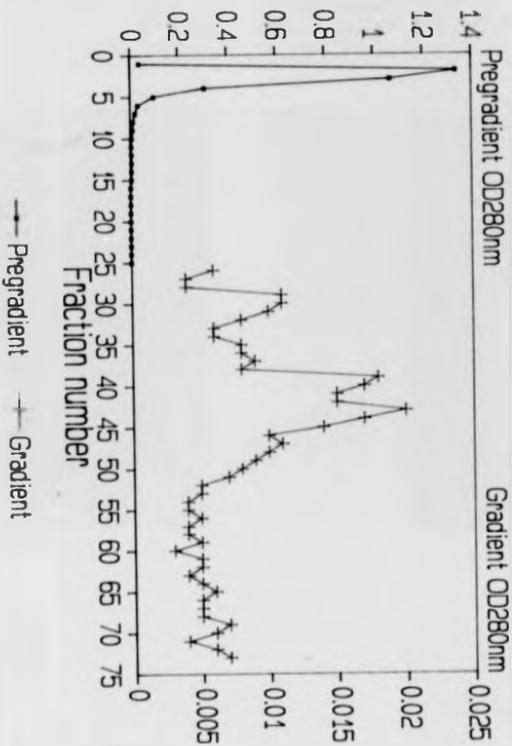
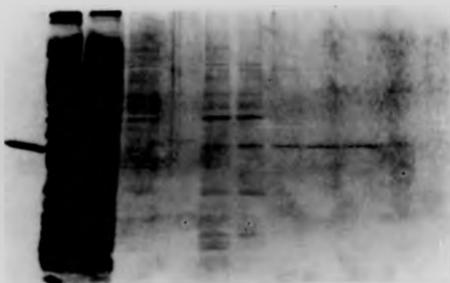


Fig. 3.53 Analysis of fractions from CM-Sepharose chromatography of E. coli extracts containing mutant A177.

A177 fractions were eluted from a CM-Sepharose column using a 600ml linear salt gradient (0mM to 300mM NaCl in 5mM phosphate (pH 6.5)). 0.7ml of each fraction was precipitated with acetone and analysed by SDS-PAGE. The silver stained gel is shown in A and the corresponding Western blot is shown in B

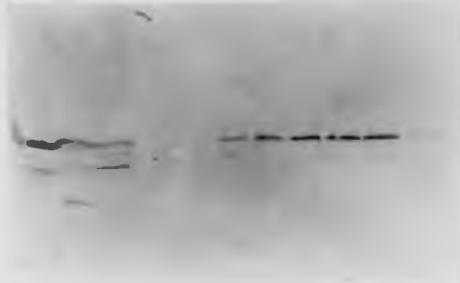
Lane 1 refers to 800ng rRTA and lane C represents an aliquot of crude E. coli extract before purification. The remaining lanes represent column fractions assayed (see Fig. 3.52).

1 C 2 26 30 39 40 41 43 44 47 49



**A**

1 C 2 26 30 39 40 41 43 44 47 49



**B**

properties of D177 and thus a similar strategy was followed for the production of this mutant. 40mg of E. coli sonicate was applied to the CM-Sepharose column and unbound proteins removed by washing with 250ml 5mM phosphate buffer (pH 6.5). In this case a 250ml + 250ml salt gradient was used (0mM to 300mM NaCl in 5mM phosphate buffer (pH 6.5)). The elution profile is shown in Fig. 3.54. Samples were analysed and ricin A-chain was found in the salt gradient (at approx. 0.1M NaCl) as expected.

Fortunately, the D177 appeared to come off the gradient at a slightly greater salt concentration and thus a number of D177-containing fractions were devoid of the usual contaminating polypeptide. The silver stain gel and the Western blot are shown in Fig. 3.55. A small amount of D177 was found in the fractions from the wash (lane 2 in Fig. 3.55). Since this protein preparation had not been checked for solubility before loading on the column, it was thought that this may represent aggregated D177.

To investigate this, a sample of the prgradient wash (fraction 2) and a sample of the pooled gradient fractions (40-42) were centrifuged at 100,000 xg for 1 hour at 4°C and the supernatant and pellet analysed. Fig 3.56 demonstrates the results obtained from this experiment. As can be seen, the pooled gradient fractions contain D177 that is totally soluble (lanes 5 and 6), whereas fraction 2 contains predominantly aggregated A-chain (lane 3). Thus this preparation of D177 required no further purification.

Fig. 3.54 Elution profile of E. coli extracts containing D177 subjected to CM-Sephadex chromatography.

40mg of a crude E. coli extract (including insoluble, aggregated material) was applied to a CM-Sephadex column in 5mM phosphate buffer (pH 6.5) and washed with 250ml of the same buffer to remove unbound protein (pregradient fractions). A linear gradient of 0mM to 300mM NaCl (in 5mM phosphate (pH 6.5)) was initiated and 10ml fractions collected. The optical density at 280nm was measured (gradient OD280nm).

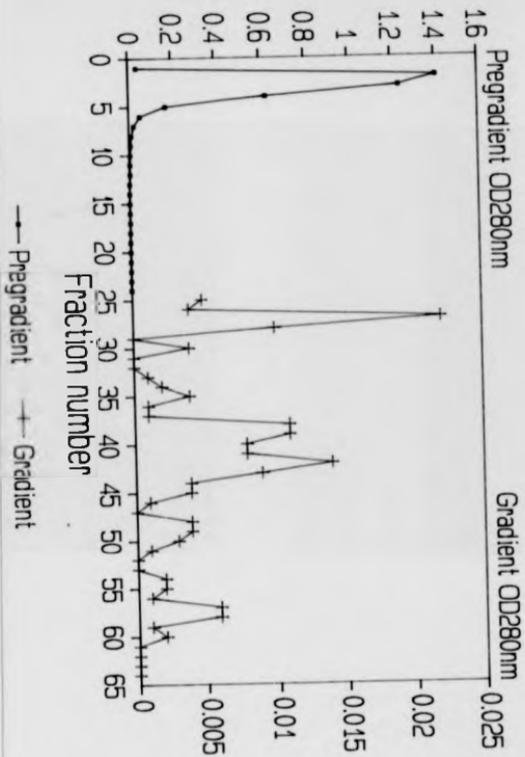
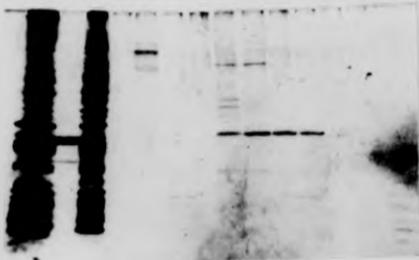


Fig. 3.53 Analysis of fractions from CM-Sepharose chromatography of E. coli extracts containing mutant D177.

Mutant D177 was purified with a similar procedure to that used for wild-type recombinant ricin A-chain as previously described. In this case D177 was eluted with a 500ml linear salt gradient (0 to 300mM NaCl in 5mM phosphate buffer (pH 6.5)). 0.7ml of the 10 ml fractions was precipitated with acetone and analysed by SDS-PAGE, as shown by figure A and the Western blot (figure B).

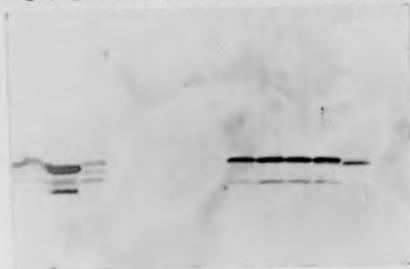
Lane C refers to approximately 150µg of crude E. coli sonicate. Lane 1 represents 800ng rRTA, whilst the remaining lane numbers refer to eluted fractions from the CM-Sepharose column (see Fig. 3.54).

C 1 2 4 27 30 35 38 39 40 42 44 49 57

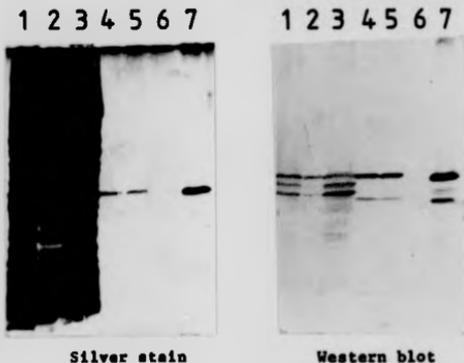


**A**

C 1 2 4 27 30 35 38 39 40 42 44 49 57



**B**



**Fig 3.56** Solubility of CM-Sepharose purified D177.

Samples of fraction 2 and pooled fractions 40 to 42 were centrifuged at 100,000  $\times g$  for 1 hour to pellet aggregated/insoluble protein. 0.5ml of the solution before and after centrifugation (and the pellet from 0.5ml of preparation) was precipitated with acetone and analysed by SDS-PAGE and Western blotting.

Lanes 1, 2 and 3 represent fraction 2 before, supernatant after and pellet after centrifugation respectively. For the pooled fractions 40-42, lane 4 represents the solution before centrifugation, lane 5 refers to 0.5ml of the protein supernatant after high speed centrifugation, and lane 6 refers to the corresponding pellet. Lane 7 represents 1 $\mu g$  of rRTA.

It was interesting to note the appearance of a slightly smaller fragment in fraction 44 which appears to be the same size as the recombinant A-chain control (lane 44 in Fig. 3.55). The fact that this fragment elutes in this position relative to the mature protein indicates that it has a greater positive charge density than the mature form. It is postulated that this could represent the A-chain polypeptide with all or some of the N-terminal 10 amino acids removed.

The concentration of D177 protein was estimated to be 10.3 $\mu$ g/ml in the fractions pooled. An equivalent amount is certainly present in those fractions not retained because of slight contamination. Thus it can be calculated that the yield of D177 was approximately 1.4mg per litre of culture. D177 was shown to have been purified in an active form, as is discussed in a later section (3.5.2.2).

Purification of the A29 mutant was also attempted. The substitution of an alanine for an arginine decreases the predicted pI to 6.53. Thus the protein will be almost neutral at pH 6.5 and therefore CM-Sapharose chromatography at pH 6.5 would be inefficient. However, to check that this was the case, a sample of A29 was applied to such a column. For this mutant, the entire crude sonicate was initially subjected to a 100,000 xg spin to prepare only soluble protein. The majority of the A29 appeared to be insoluble and only a small portion was recovered in the 'soluble' supernatant. When applied to a

CM-Sepharose column at pH 6.5, the soluble A29 mutant was recovered in the pregradient wash as expected. A second preparation was made from 1 litre of LB culture. Approximately 550mg of protein was recovered in the crude sonicate, of which 65% was shown to be present in the soluble fraction. Analysis of expression of the ricin A-chain A29 showed there to be an amount of soluble protein, although it was present as mature sized product with at least two degradation products (Fig. 3.34).

The CM-Sepharose column was re-equilibrated to pH 5.8 with 10mM phosphate buffer (pH 5.8). This pH was chosen since it was less than the predicted pI of the A29 but greater or equal to the majority of E. coli proteins so as to effect an efficient separation of the mutant protein. The crude preparation was dialysed in the same buffer and loaded onto the column. A linear salt gradient was started after the column had been washed with 230ml of buffer (elution profile in Fig. 3.57). Analysis of the Western blot indicated an extremely faint A-chain cross-reactive band in fraction 43 (Fig. 3.58), which was semi-purified. However, the majority of the A-chain polypeptides were recovered in the low salt wash (the pregradient fractions in Fig. 3.57).

With the previous observations of the differential behaviour of E. coli proteins with different strength ion-exchange matrices, it was interesting to examine the elution profile of A29 when applied to the S-Sepharose

Fig. 3.57 Elution profile determined from CM-Sephacose chromatography of E. coli extracts containing A29.

A sample of a crude E. coli preparation containing A29 was loaded onto a CM-Sephacose column (matrix volume 30ml) and washed with 230ml 10mM phosphate buffer (pH 5.8) (pregradient fractions). A linear gradient was initiated from 0mM to 300mM NaCl and fractions assayed for OD280nm (gradient fractions) and SDS-PAGE (Fig. 3.58).

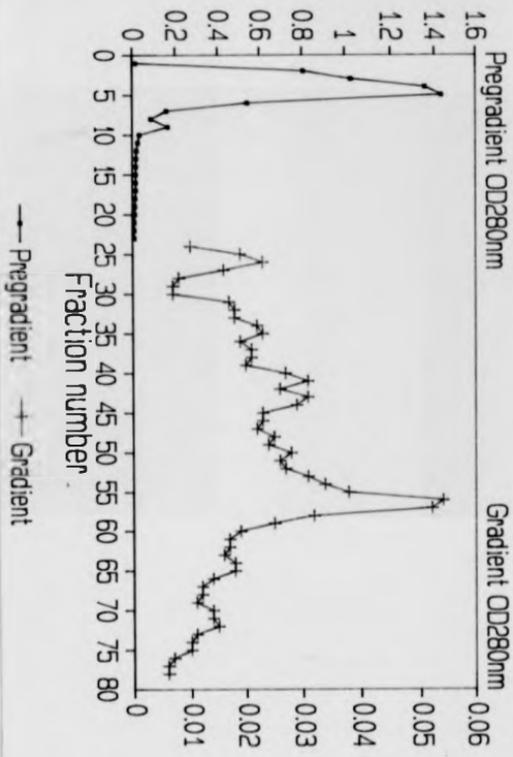
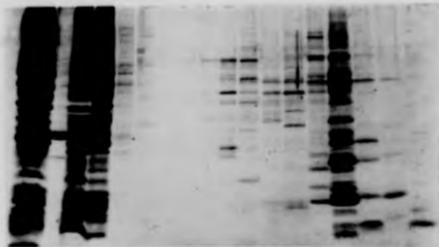


Fig. 3.58 Attempted purification of A29 from CM-Sepharose at pH 5.8.

A sample of A29 in a crude E. coli sonicate was loaded directly onto a CM-Sepharose column equilibrated with 10mM phosphate buffer (pH 5.8) and washed with 230ml of the same buffer to elute unbound proteins. A 600ml linear gradient was initiated and 10ml fractions collected (gradient fractions). 0.75ml of selected fractions were precipitated with acetone and analysed by SDS-PAGE (silver stain in A) and Western blotting (B).

Lane C refers to approximately 400µg of crude protein before purification, whilst lane B refers to 1µg of rRTA. The remaining lanes refer to the eluted fractions (see Fig. 3.57).

C R 3 5 9 31 37 43 50 56 64  
26 34 41 48 54 59 72



**A**

C R 3 5 9 31 37 43 50 56 64  
26 34 41 48 54 59 72



**B**

column at pH 5.8. In this case, the material to be purified was taken from fractions 8 to 12 from the CM-purification experiment. This sample was independent of the initial column wash (likely to contain the aggregated A29) and had already been semi-purified. An extremely low amount of A29 mutant of the correct size eluted soon after the salt gradient was initiated. No A29 was seen in the pregradient fractions. However the most striking rRTA species was an apparent degradation product corresponding to the lower rRTA band in Fig. 3.34 which, eluted late in the gradient. It was also possible to isolate this degradation product away from the mature protein by passing a crude A29 preparation through a DEAE-Sephacel column at pH 8.0 and identifying this fragment in the gradient at approximately 0.15M NaCl. Attempts to analyse the N-glycosidase activity of this fragment were unsuccessful and the amino acid sequence and thus its relationship with the A-chain sequence is unknown.

At this stage A29 has not been purified by ion-exchange chromatography. However A29 appears to be a candidate for immunoaffinity chromatography or Cibacron-blue chromatography (see discussion) and I feel that purification would be achieved by one of these methods.

The mutant K177 has not been purified because of lack of time. The predicted pI of this mutant is 8.12. It would be envisaged that the CM-Sepharose column would be appropriate for this task with a change in buffer pH to pH

column at pH 5.8. In this case, the material to be purified was taken from fractions 8 to 12 from the CM-purification experiment. This sample was independent of the initial column wash (likely to contain the aggregated A29) and had already been semi-purified. An extremely low amount of A29 mutant of the correct size eluted soon after the salt gradient was initiated. No A29 was seen in the pregradient fractions. However the most striking rRTA species was an apparent degradation product corresponding to the lower rRTA band in Fig. 3.34 which, eluted late in the gradient. It was also possible to isolate this degradation product away from the mature protein by passing a crude A29 preparation through a DEAE-Sephacel column at pH 8.0 and identifying this fragment in the gradient at approximately 0.15M NaCl. Attempts to analyse the N-glycosidase activity of this fragment were unsuccessful and the amino acid sequence and thus its relationship with the A-chain sequence is unknown.

At this stage A29 has not been purified by ion-exchange chromatography. However A29 appears to be a candidate for immunoaffinity chromatography or Cibacron-blue chromatography (see discussion) and I feel that purification would be achieved by one of these methods.

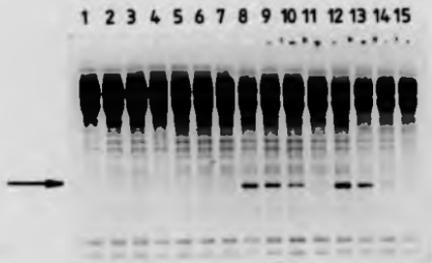
The mutant K177 has not been purified because of lack of time. The predicted pI of this mutant is 8.12. It would be envisaged that the CM-Sepharose column would be appropriate for this task with a change in buffer pH to pH

7.0, although yields are likely to be low because of the low proportion of soluble protein.

### 3.4.3 Storage of rRTAs

Recombinant ricin A-chain purified by CM-Sephadex and S-Sephadex chromatography was frozen at  $-70^{\circ}\text{C}$  for long-term storage. Pooled rRTA fractions which were shown to be pure were stored in the column elution buffer with the addition of sterile glycerol to 15%. This concentration of glycerol was chosen on the basis of previous information relating to RIP storage, in the cases of recombinant abrin A-chain (Kathy Wood, PhD thesis, 1991) and ricin A-chain (ICI).

In order to ascertain the temperature for storage, samples of rRTA (in 15% glycerol) were prepared immediately following purification and stored at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  for a period of 7 weeks. A defined amount of rRTA was tested for N-glycosidase activity using non-salt-washed yeast ribosomes (MSWT) as shown in Fig. 3.59.  $30\mu\text{g}$  of ribosomes ( $0.24\mu\text{M}$ ) was incubated with ricin A-chain ( $0.5\text{nM}$  to  $30\text{nM}$ ) for 30 minutes at  $30^{\circ}\text{C}$ .  $5\mu\text{g}$  of extracted RNA was used for the aniline assay. As can be seen in Fig. 3.59, samples stored at  $4^{\circ}\text{C}$  had negligible activity even at  $30\text{nM}$  ( $27.8\mu\text{g}$  rRTA) (lane 4), whereas samples stored at  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  appeared to retain activity (lanes 8-11 and 12-15 respectively).



**Fig. 3.59** N-glycosidase activity of recombinant ricin A-chain stored at three different temperatures.

Purified recombinant ricin A-chain (stored at  $-70^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  in 15% glycerol), at concentrations of 30, 10, 1 and 0.5 nM, was incubated with yeast ribosomes for 30 minutes at  $30^{\circ}\text{C}$ . The rRNA was extracted and treated with aniline as previously described and analysed by electrophoresis on a denaturing agarose/formamide gel.

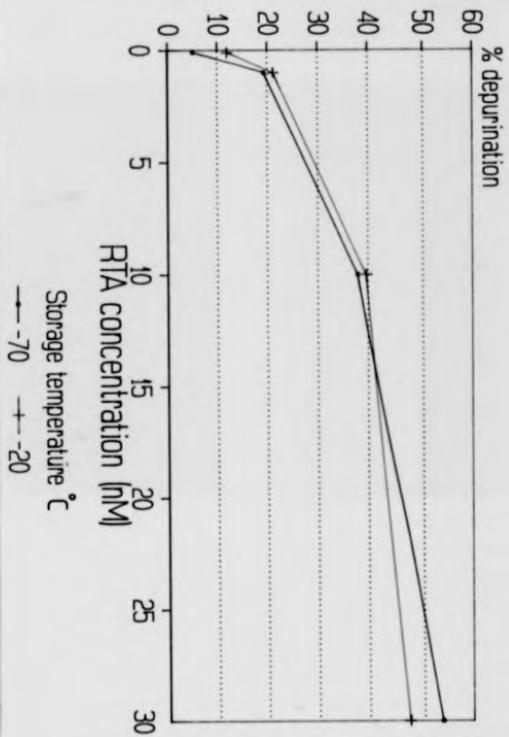
Lanes 4 to 7 refer to decreasing concentrations of A-chain for samples stored at  $4^{\circ}\text{C}$  whilst lanes 8 to 11 and 12 to 15 refer to  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  samples respectively. Lanes 1, 2 and 3 represent non-aniline treated samples of lanes 4, 8 and 12 respectively. The cleavage product is highlighted with an arrow.

The intensity of the aniline fragment was measured by using a Molecular Dynamics densitometer (as described in section 3.5.2) and related to the intensity of the 5.8S rRNA fragment to produce a measure of X RNA depurination. A plot of X depurination against rRTA concentration is shown in Fig. 3.60. It can be seen that after 30 minutes 30nM rRTA stored at  $-70^{\circ}\text{C}$  has depurinated 55% of the ribosomes, or 45% when stored at  $-20^{\circ}\text{C}$ . Wild-type recombinant ricin A-chain and purified recombinant mutants were thus stored at  $-70^{\circ}\text{C}$  after purification.

Although the integrity of the proteins was not analysed by SDS-PAGE, previous experiments using ricin A-chain preparations from a 40% ammonium sulphate precipitation had shown the rRTA to remain in the soluble fraction in an active form with no increase in degradation products, when stored in the absence of glycerol at  $4^{\circ}\text{C}$  for 2 weeks (data not shown). It would appear that the rRTA may be more stable in the presence of a protease-free crude E. coli preparation, than in dilute solution, although quantitative activity measurements to assay the N-glycosidase activity of the crude A-chain were not possible.

Fig. 3.60 Graphical representation of the effect of temperature on the storage of recombinant ricin A-chain.

See text for experimental details and refer to Fig. 3.59 for experimental data. The % depurination was calculated for each assay relative to the amount of 5.8S rRNA visualised on the agarose/formamide gel. The data were plotted as %depurination against rRTA concentration for samples stored at -20°C and -70°C. No data is shown for experiments where rRTA was stored at 4°C because only negligible catalytic activity was retained by these samples.



### 3.5 ACTIVITY AND KINETIC ANALYSIS OF RICIN A-CHAIN DERIVED FROM IN VIVO EXPRESSION.

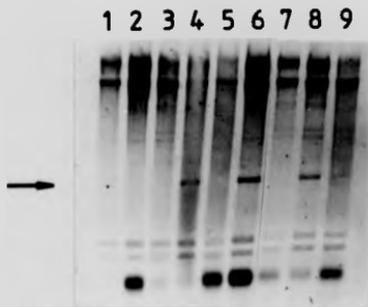
#### 3.5.1 N-glycosidase activity measurements

Of the five rRTA species expressed in vivo, two (wild-type rRTA and D177) were examined in detail and kinetic data recorded. The three remaining mutants (A177, K177 and A29) were analysed for N-glycosidase activity from crude preparations or from purified protein (A177). Analysis of crude protein preparations often had to be performed in the presence of the vanadyl ribonuclease complex (VRC) ribonuclease inhibitor to prevent RNA degradation during incubation. This was in addition to the use of Kirby buffer (Kirby, 1968) during the extraction of the rRNA which also has ribonuclease-inhibitory properties. Since it is difficult to accurately quantitate the rRTA in a crude E. coli preparation, these data represent a qualitative estimation of activity and can only be used to verify the presence of ricin A-chain within the limited assay volumes available.

Mutations of glutamate 177 were assayed for N-glycosidase activity using 30µg of yeast ribosomes incubated (60 minutes/30°C) with 9µl of a soluble supernatant taken from a sonicate of transformed E. coli. 9µl of a dialysed 40% ammonium sulphate precipitation from these sonicates was also tested. 3µg of RNA was cleaved

with aniline and the products visualised (Fig. 3.61). Under these conditions only A177 appeared active (lanes 6 and 8). Neither K177 nor D177 produced a cleavage product (lanes 5, 7 and 9). The fact that D177 appeared inactive is important since, as will be shown later, D177 was later purified and shown to retain a fraction of activity when assayed in detail. Thus the limitation of low sample volume affected the interpretation of the data shown in Fig. 3.61. One cannot therefore be absolutely sure that K177 is fully inactive from this experiment, however in light of the in vitro data presented in section 3.2.5.2 it would be a strong possibility.

During the purification of A177 from E. coli culture (3.4.2.6), samples were removed and assayed for N-glycosidase activity in the absence of additional ribonuclease inhibitors. The results are presented in Fig. 3.62. Salt-washed rabbit reticulocyte ribosomes (SWR) were incubated with A177 preparations for 30 minutes at 30°C before the RNA was extracted and 5µg treated with aniline. Fig. 3.62 indicates that a fraction (43) from the A177 purification experiment (Fig. 3.52) was clearly active (lane 10), as were dilutions of the crude sonicate (lanes 6 to 8 compared with lane 2). An aliquot of the insoluble pellet appeared to have no activity (lane 9) when compared to the control (lane 3), although the increased degradation of the RNA in this experiment makes the results from the pellet difficult to interpret.



**Fig. 3.61** N-glycosidase activity of in vivo expressed glutamate 177 mutants.

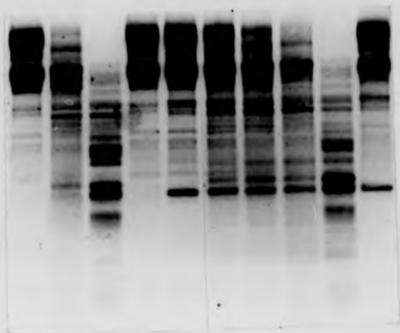
Aliquots of crude E. coli sonicate were assayed for N-glycosidase activity using yeast ribosomes as shown in lanes 4 to 6 (rRTA, K177 and A177 respectively). Samples of the supernatant from a 40% ammonium sulphate precipitation were also assayed (lanes 7 to 9 representing K177, A177 and D177 respectively). Lanes 1 to 3 represent the non-aniline treated controls for lanes 4, 6 and 8 respectively. The fragment released by aniline treatment of the isolated rRNA is highlighted with an arrow.

**Fig. 3.62** N-glycosidase activity of mutant A177 during purification by CM-Sepharose chromatography.

Samples of A177 were removed during purification and assayed for N-glycosidase activity on 40 $\mu$ g of salt washed rabbit reticulocyte ribosomes (as discussed in the text). Lanes 1 to 4 and 5 to 10 refer to non-aniline treated and aniline treated samples respectively.

Samples of crude E. coli sonicate (lanes 6, 7 and 8 representing 0.6 $\mu$ g, 5.8 $\mu$ g and 29 $\mu$ g protein respectively) demonstrated activity when compared to the non-aniline control (lane 2). The aniline cleavage fragment is arrowed. Lane 5 represents 21ng of purified RTA and a cleavage fragment is clearly visible (compared to lane 1). Lane 9 (compared to lane 3) indicates the results obtained from a sample of the pellet following centrifugation of the crude E. coli sonicate at 100,000 xg for 1 hour. Lane 10 refers to approx. 40ng protein from fraction 43 (see Fig. 3.52) and a cleavage fragment is visible compared to lane 4.

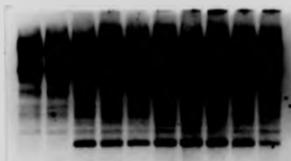
1 2 3 4 5 6 7 8 9 10



With the presence of active, soluble protein established using rabbit reticulocyte ribosomes, the ribosome-inactivating properties of purified A177 were assessed relative to the wild-type rRTA as shown in Fig. 3.63. Various combinations of rRTA concentration and incubation time, as described in the legend for Fig. 3.63, were used to demonstrate differences between the activity of A177 and wild-type rRTA. The most striking observation was shown in lanes 9 and 10 when 1ng of ricin A-chain was incubated for 5 minutes with 30µg SWR ribosomes. A177 is clearly less active than the wild-type rRTA. When the  $\Sigma$  depurination was assessed by scanning densitometry it was found that the A177 in lane 10 had approximately 50% of the  $\Sigma$  depurination activity of wild-type rRTA. Readings taken from the other lanes showed less variation between A177 and rRTA. Unfortunately time did not allow a full characterisation of the N-glycosidase activity of A177 by kinetic analysis.

Mutant A29 was shown to be active towards yeast ribosomes over a 20 minute incubation at 30°C, although the cleavage product was faint (Fig. 3.64; lane 5). A comparable sample from E. coli transformed with the pDS5/3 vector alone showed no cleavage product (lane 4). The results from the experiment using A29 are described in Fig. 3.64.

1 2 3 4 5 6 7 8 9 10



**Fig. 3.63** N-glycosidase activity of A177 relative to rRTA.

The N-glycosidase activity of CM-Sepharose purified A177 was compared directly with the activity of CM-S-Sepharose purified rRTA. Assays were performed with salt-washed reticulocyte ribosomes at 30°C. Reaction conditions were as shown:

|        |      |      |            |
|--------|------|------|------------|
| lane 3 | 10ng | rRTA | 2 minutes  |
| lane 4 | 10ng | A177 | 2 minutes  |
| lane 5 | 10ng | rRTA | 5 minutes  |
| lane 6 | 10ng | A177 | 5 minutes  |
| lane 7 | 10ng | rRTA | 30 minutes |
| lane 8 | 10ng | A177 | 30 minutes |
| lane 9 | 1ng  | rRTA | 5 minutes  |
| lane10 | 1ng  | A177 | 5 minutes  |

Lanes 1 and 2 are non-aniline treated control samples of lanes 7 and 8 respectively.

1 2 3 4 5

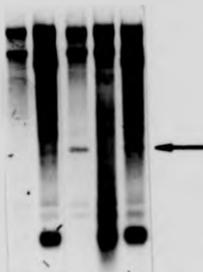


Fig. 3.64 N-glycosidase activity of mutant A29.

Samples from a crude E. coli sonicate containing A29 were assayed for N-glycosidase activity using the aniline assay. Crude samples were incubated with 30 $\mu$ g yeast ribosomes for 20 minutes at 30°C and the rRNA extracted.

Lanes 1 and 2 represent the non-aniline treated control rRNA for lanes 3 and 5 respectively. lane 3 represents 1.6 $\mu$ g of rRTA and lane 4 represents 9 $\mu$ l of a crude sonicated E. coli transformed with the vector pDS5/3. Lane 5 indicates 9 $\mu$ l of a crude sonicated E. coli transformed with pDSA29. The aniline cleavage fragment is arrowed.

### 3.5.2 Kinetic studies with wild-type rRTA and mutant D177

In order to investigate the roles of the mutated residue in the functioning of ricin A-chain, the kinetics of ribosome-inactivation were assessed. Wild-type ricin A-chain was initially used to measure the wild-type kinetics of depurination of ribosomes, to which results obtained for mutants would be compared. Yeast ribosomes were used for the kinetic measurements as prepared in section 2.3.3.1. The kinetics of inactivation of yeast ribosomes by ricin A-chain had not been published, although values were known for reticulocyte ribosomes (Endo & Tsurugi, 1988) and wheatgerm ribosomes (Osborn, 1991). Yeast ribosomes had been shown to be almost as sensitive to ricin A-chain action as those from reticulocytes when salt-washed to remove bound elongation factors and tRNA (Osborn, 1991). By using the method of preparation described in 2.3.3.2, ribosomes were in plentiful supply, when compared to using reticulocyte ribosomes.

The parameters to be measured were the  $K_m$  and the  $k_{cat}$  for the mono-substrate ricin A-chain catalysed reaction. The  $K_m$  (Michaelis-Menten constant) represents the substrate concentration required to achieve a half-maximal rate of reaction. A prediction of the Michaelis-Menten equation (see appendix) is that  $K_m$  becomes a measure of the binding affinity of the enzyme for the substrate, providing that the backward reaction  $K_2$  is

greater than the turnover rate  $K_3$ .

Experiments performed by Hedblom et al. (1976) have shown that the dissociation constant for the binding of 1 molecule of RTA to 1 rat liver ribosome is  $2\mu\text{M}$  which is in agreement with the published  $K_m$  for the reaction. Thus the  $K_m$  can be regarded as being a measure of the substrate binding and alterations in  $K_m$  by mutagenesis can be proposed to be due to a difference in the substrate binding of the mutant. The  $k_{cat}$  (turnover number) for the reaction is a measure of the rate of catalysis. It represents the product of relating the maximal velocity achieved by the enzyme saturated with substrate to the total concentration of enzyme needed to achieve this velocity (i.e.  $k_{cat} = V_{max}/[E]$ ). If a ricin A-chain mutant was shown to have an altered  $k_{cat}$ , then it could be proposed that the residue targeted for mutagenesis was involved in the catalytic mechanism.

The rate of catalysis can be measured at a given time point and Michaelis-Menten kinetics apply, providing certain conditions are met. At the time of assay the reaction rate must be linear. Michaelis-Menten kinetics are based on measuring the initial rate of reaction. The enzyme concentration is supposed to be negligible in comparison to the substrate concentration and the substrate concentration should not be limiting. Also, in order to make accurate measurements of the  $K_m$  and  $k_{cat}$ , the substrate concentration should be of the order of the

greater than the turnover rate  $K_3$ .

Experiments performed by Hedblom et al. (1976) have shown that the dissociation constant for the binding of 1 molecule of RTA to 1 rat liver ribosome is 2 $\mu$ M which is in agreement with the published  $K_m$  for the reaction. Thus the  $K_m$  can be regarded as being a measure of the substrate binding and alterations in  $K_m$  by mutagenesis can be proposed to be due to a difference in the substrate binding of the mutant. The  $k_{cat}$  (turnover number) for the reaction is a measure of the rate of catalysis. It represents the product of relating the maximal velocity achieved by the enzyme saturated with substrate to the total concentration of enzyme needed to achieve this velocity (i.e.  $k_{cat} = V_{max}/[E]$ ). If a ricin A-chain mutant was shown to have an altered  $k_{cat}$ , then it could be proposed that the residue targeted for mutagenesis was involved in the catalytic mechanism.

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predicted  $K_m$ . The design of the experiments discussed below attempted to meet these criteria.

Kinetic experiments with ricin A-chain have been performed by other workers using the inhibition of protein synthesis as a measure of activity (Ready *et al.*, 1991). However this indirect method is not ideal since it measures an effect of ribosome inactivation and not the actual catalytic process itself. Superior measurements of the rate of depurination have been performed by Endo & Tsurugi (1988) with reticulocyte ribosomes and Osborn (1991) with wheatgerm ribosomes. In the former case, values of  $2.6\mu\text{M}$  and 1777 ribosomes/minute were published for the  $K_m$  and  $k_{cat}$  respectively. In the latter case, a  $K_m$  of  $2.1\mu\text{M}$  and a  $k_{cat}$  of 1.1 ribosomes/minute were observed. These authors made use of the aniline assay to assess depurination of the rRNA during a controlled ribosome-inactivation experiment. The amount of depurination at any one time during the reaction can be measured by assessing the proportion of aniline fragment released after treatment of the depurinated rRNA with aniline. Endo & Tsurugi used radiolabelled ribosomes and measured the radioactive counts in the fragment.

In this study, and in that of Osborn, the amount of fragment was measured directly from ethidium bromide stained gels. It is possible to relate the amount of cleavage product to the amount of 5.8SrRNA visualised on the same gel. Since it has been shown that the 5.8SrRNA

(Rubin, 1973), then the amount of aniline fragment released can be related to the amount of 26SrRNA present before ricin A-chain action. The lengths of the aniline fragment (369b) and 5.8SrRNA (160b) are known (Noller, 1984) and thus a percentage depurination value can be calculated.

Relating the amount of the aniline fragment to the amount of the 5.8S fragment has the advantage that each lane of the RNA gel has an internal control to counteract differences in loading. Assumptions are made that in the conditions of the denaturing agarose gel the labelling of the RNA with ethidium bromide is directly proportional to the lengths of the RNA fragments.

The accuracy of the scanning method could be assessed by measuring the intensity of the 5SrRNA band in addition to the 5.8SrRNA and the aniline fragment. Since the 5S and the 5.8SrRNA are in equimolar proportions, and the lengths of each is known, it is possible to work out the ratio of their intensities and compare this to the predicted 5.8S : 5S ratio of 1.33.

The amount of each fragment was assessed by scanning Polaroid 665 negative photographs with a Molecular Dynamics computing densitometer using the volume integration function of ImageQuant software. Data was obtained which measured the absorbance for each band and related this to the background absorbance. It was important to maintain the background as low as possible

while still achieving efficient staining of the RNA fragments. This required careful staining/destaining procedures and RNA samples with low amounts of background degradation. Collection of good gels for accurate scanning proved to be time-consuming. A rectangle was drawn around the computer-enhanced band to determine an area within which to integrate the volumes. Additional measurements were performed of the background absorbance close to each band to be assessed, such that the absorbance of each band of interest could be related to the background in that immediate area. A value was generated for each RNA band by summing the total absorbances (A) within the rectangle and subtracting the background (B) (equation 1). This number was referred to as the 'amount' of RNA fragment.

$$(\text{Sum of A}) - B = \text{'amount'}$$

Equation 1.

The % depurination could then be calculated using equation 2 shown below.

$$\frac{\text{Amount of aniline fragment} \times 160 \times 100}{\text{Amount of 5.8SrRNA} \quad 369} = \% \text{ depurination}$$

Equation 2.

For the initial experiments to determine the conditions for depurination that would fulfil the criteria above, this  $\bar{X}$  depurination value was the figure used. Once the conditions had been set, the initial rate of reaction could be calculated as follows.

Let  $V_0$  = initial velocity.

Let  $x$  =  $\mu\text{g}$  ribosomes per reaction.

Let  $y$  =  $\bar{X}$  depurination calculated as in equation 2.

Let  $v$  = volume of the reaction.

Let  $t$  = time of incubation.

Thus in a single experiment:  $y\bar{X}$  depurination of  $x$   $\mu\text{g}$  ribosomes in a  $v$   $\mu\text{l}$  reaction volume over a period of  $t$  minutes was observed.

$\frac{xy}{100t}$   $\mu\text{g}$  of ribosomes were depurinated/minute/ $v$   $\mu\text{l}$   
100t

$\frac{xy}{100vt}$   $\mu\text{g}$  of ribosomes were depurinated/minute/ $\mu\text{l}$   
100vt

It is known that the molecular weight of yeast ribosomes is  $4.2 \times 10^6$  (Wool, 1979). Thus 1  $\mu\text{mole}$  is  $4.2 \times 10^6 \mu\text{g}$ . To calculate the initial rate of reaction in terms of  $\mu\text{M}$  ribosomes, the equation above was adjusted as shown overleaf.

$$\frac{xy}{(100vt)(4.2 \times 10^6)} \quad \mu\text{moles ribosomes/minute}/\mu\text{l}$$

and thus:

$$\frac{(xy)(10^6)}{(100vt)(4.2 \times 10^6)} \quad \mu\text{moles ribosomes/minute/litre}$$

$$i.e. V_o = \frac{xy}{420vt} \quad \mu\text{M ribosomes/minute} \quad \text{Equation 3.}$$

### 3.5.2.1 Kinetics of wild-type recombinant ricin A-chain

All experiments performed with wild-type recombinant ricin A-chain used the rRTA purified in as in section 3.4.2.5 and stored at  $-70^{\circ}\text{C}$  in 15% glycerol. Glycerol was shown not to affect the reaction at the concentrations used (data not shown). In order to ascertain the conditions necessary for rRTA depurination activity, a number of depurination experiments were performed.

Ricin A-chain is active towards ribosomes at low concentrations and initial experiments were designed to measure the concentration of rRTA required for adequate depurination. During these experiments it was shown that salt washed ribosomes from rabbit reticulocytes were extremely sensitive to ricin A-chain catalysed depurination. Depurination was observed when only 0.1nM rRTA was incubated with 0.4 $\mu\text{M}$  SWR for only 5 minutes

at 30°C. Experiments with salt washed yeast ribosomes (SWY), to determine the amount of rRTA to use for a linear rate of reaction, indicated that rRTA at a concentration of 0.5nM was suitable (data not shown).

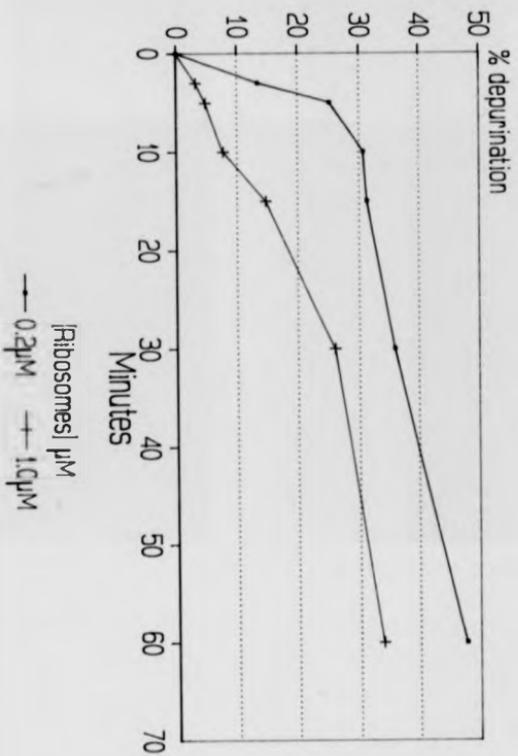
To determine the time of incubation at 30°C, two experiments were performed using ribosomes at 0.2µM and 1µM. A reaction mix was prepared consisting of SWY at the appropriate concentration, 2xENDO buffer and water. The reaction volume was determined by the number of time points that were assayed and the requirement for 30µg of ribosomes for each assay. The reaction mix was incubated at 30°C for 5 minutes to equilibrate the components to the correct temperature. rRTA was added and a sample immediately removed into an ice-cold mix of 100µl 2 x Kirby buffer and water to 100µl. Samples were removed at various time points to 60 minutes. 5µg of RNA was cleaved and the subsequent gel scanned and X depurination calculated. It was shown that for ribosomes at 0.2µM and 1µM the reaction was in an initial linear rate at 5 minutes (Fig. 3.65).

Using these data (0.5nM rRTA + 5 minutes at 30°C) experiments were performed with varying substrate concentrations from 0.1µM to 3.0µM. Reactions were incubated for exactly 5 minutes before stopping the catalysis by the addition of ice-cold Kirby buffer to 1x concentration.

Fig. 3.65 Estimation of the linear rate of reaction for recombinant ricin A-chain.

Purified rRTA (0.5nM) was assessed for N-glycosidase activity against salt-washed yeast ribosomes (0.2 $\mu$ M and 1 $\mu$ M) at various time points between 0 and 60 minutes. Experimental details are described in the text. The depurination of RNA at each time point was assessed by scanning densitometry and related to the amount of <sup>32</sup>P-5.8SrRNA to create a % depurination value.

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This experiment to determine the  $K_m$  and  $V_{max}$  was performed three times. For the first experiment (#1), two gels were prepared and each was scanned independently. The results were pooled and average initial velocity ( $V_o$ ) values calculated. Mean 5.8S : 5S ratio for these two scans was 1.326 (sd of 0.073 and 0.064). A Lineweaver-Burk plot was prepared for these data from the first experiment and this is shown in Fig. 3.66. Linear regression analysis of the data proposed a straight line with x-intercept ( $-1/K_m$ ) of -0.794 and y-intercept ( $1/V_{max}$ ) of 12.042. Calculated  $K_m$  and  $k_{cat}$  were 1.259 $\mu$ M and 166 ribosomes/min respectively. An Eadie-Hofstee plot was also prepared which proposed figures of  $K_m = 0.911\mu$ M and  $k_{cat} = 137$  ribosomes/min (Fig. 3.67).

For the second and third experiments (#2 and #3) a single gel was prepared but it was scanned twice in an attempt to reduce the error involved in the scanning procedure. The Lineweaver-Burk plot for these results are also shown in Fig. 3.66. The line of best fit for #2 appeared to intersect the x-axis at -0.621 (i.e.  $K_m = 1.610\mu$ M) and the y-axis at 9.408 (i.e.  $V_{max} = 0.106\mu$ M/min). Calculated  $k_{cat}$  was shown to be 213 ribosomes/minute. Similar procedures were followed for #3 with the x- and y-intercepts being shown to be -0.745 and 10.239 respectively, leading to  $K_m$ ,  $V_{max}$  and  $k_{cat}$  figures of 1.342 $\mu$ M, 0.097 $\mu$ M/min and 195 ribosomes/min respectively.

Fig. 3.66 Lineweaver-Burk plot of recombinant ricin A-chain N-glycosidase activity from experiment #1, #2 and #3.

0.5nM purified rRTA was incubated with salt-washed yeast ribosomes at concentrations between 0.1 and 3 $\mu$ M for 5 minutes at 30°C. The initial rate of reaction ( $V_0$ ) was calculated from the % depurination as described in equation 3. The x- and y-intercepts for experiments #1, #2 and #3 were shown to be:

|    | x-intercept | y-intercept |
|----|-------------|-------------|
| #1 | -0.794      | 12.042      |
| #2 | -0.621      | 9.408       |
| #3 | -0.745      | 10.239      |

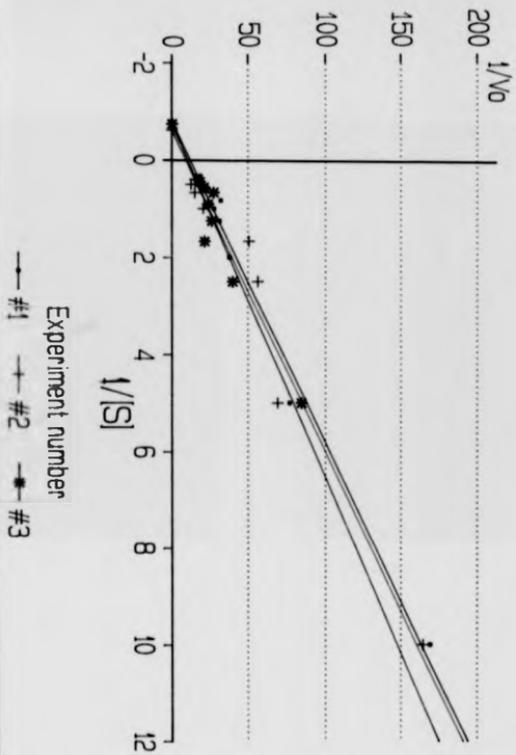
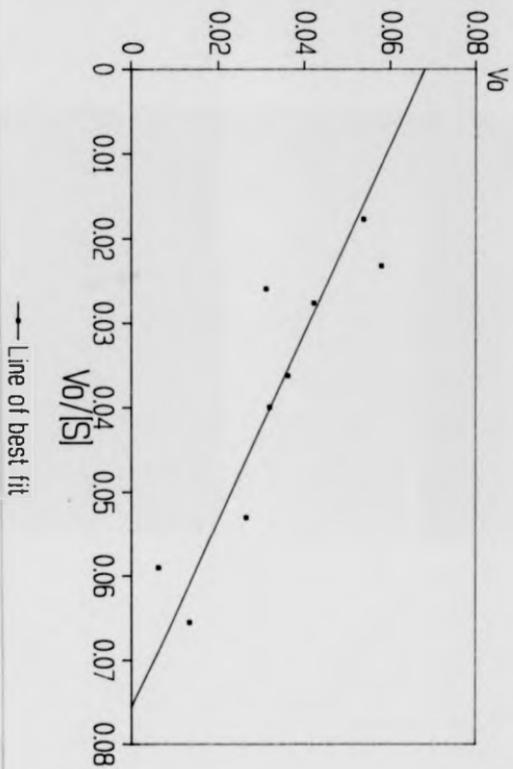


Fig. 3.67 Eadie-Hofstee plot of recombinant ricin A-chain N-glycosidase activity from experiment #1.

The initial velocity ( $V_0$ ) was calculated as previously described in equation 3. Linear regression analysis of the points proposed a y-intercept of 0.0686 and a slope of -0.911.



Mean initial velocity data generated in the three experiments were used to construct a mean Lineweaver-Burk plot ( $\phi_{\text{mean}}$ ), shown in Fig. 3.68. Linear regression analysis on these data proposed an x-intercept of -0.659 and a y-intercept of 9.953. The calculated  $K_m$  and  $k_{\text{cat}}$  are shown in Table 3 below. Analysis of the mean rETA results by Eadie-Hofstee plot proposed a  $K_m$  value of 1.02 $\mu\text{M}$  and a  $k_{\text{cat}}$  of 160.8 $\text{min}^{-1}$  (Fig. 3.69).

**Table 3** Calculated kinetic data for wild-type recombinant ricin A-chain.

| Experiment                  | $K_m$ ( $\mu\text{M}$ ) | $V_{\text{max}}$ ( $\mu\text{M}/\text{min}$ ) | $k_{\text{cat}}$ ( $\text{min}^{-1}$ ) |
|-----------------------------|-------------------------|---|--|
| $\phi_1$                    | 1.259                   | 0.0830  | 166                                    |
| $\phi_2$                    | 1.610                   | 0.1063  | 213                                    |
| $\phi_3$                    | 1.342                   | 0.0977  | 195                                    |
| Mean $\phi_1+\phi_2+\phi_3$ | 1.404                   | 0.0960  | 192                                    |
| $\phi_{\text{mean}}$        | 1.517                   | 0.1000  | 201                                    |

Fig. 3.68 Lineweaver-Burk plot of the mean recombinant ricin A-chain N-glycosidase activity from #1, #2 and #3.

Data were collected from the three rRTA activity measurements (#1, #2 and #3 in Fig. 3.66) and mean initial velocities recorded. With these data a mean Lineweaver-Burk plot was produced as shown. The x- and y-intercepts were shown to be -0.659 and 9.953 respectively.

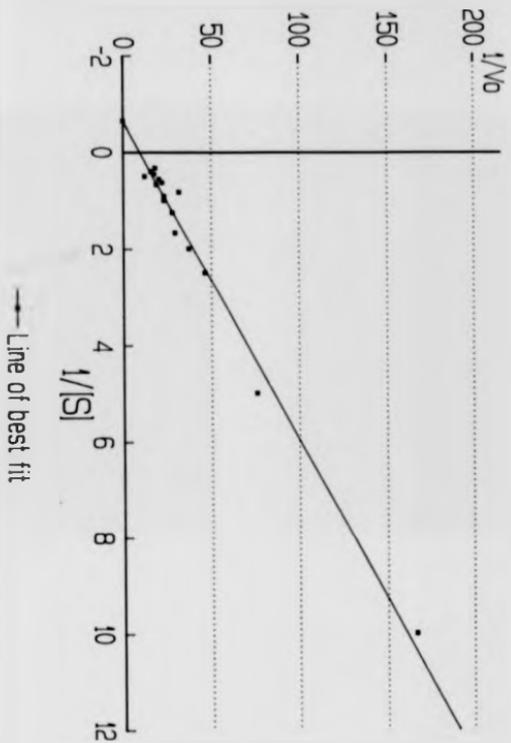


Fig. 3.69 Esdie-Hofstee plot of the mean recombinant ricin A-chain N-glycosidase activity.

Data were collected from the three rRTA activity experiments (#1, #2 and #3 in Fig. 3.66) and mean initial velocities recorded. From the y-intercept,  $V_{max}$  was calculated to be  $0.0804\mu\text{M}/\text{min}$ . From the slope of the line,  $K_m$  was calculated to be  $1.02\mu\text{M}$ .

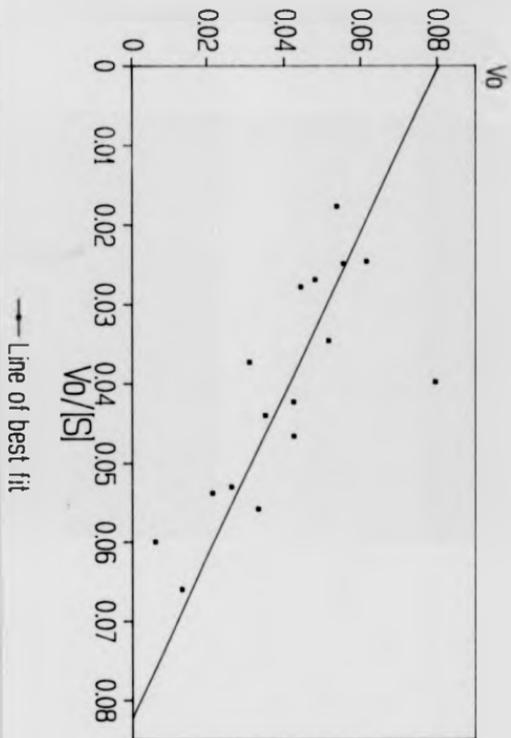


Fig. 3.70 Depurination of ribosomes by D177.

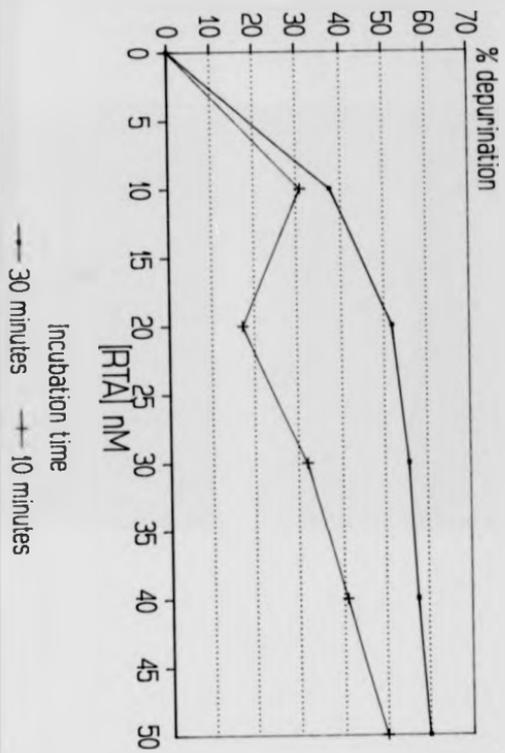
Various concentrations of ricin A-chain mutant D177 were incubated with 0.5 $\mu$ M SWY ribosomes for 10 or 30 minutes. Samples of rRNA were assessed and assayed for depurination using the aniline assay. Quantitation of this data was performed as described and the % depurination calculated.

Note that the point at 10 $\mu$ M D177 for 10 minutes was artificially high as indicated by an artificially low 5.8:5 SrRNA ratio of 0.93.

### 3.5.2.2 Kinetics of mutant D177

Of the two E177 mutants purified, Asp177 was perhaps the most interesting because of the unexplained greater decrease in activity relative to Ala177 that was observed in this work and by Schlossman *et al.* (1989). It was of interest to assess why the conservative mutation exhibited this property. D177 had previously been shown to be active in vitro but less active than wild-type ricin A-chain.

A preliminary assessment of D177 activity from a crude cell homogenate was unsuccessful in demonstrating N-glycosidase activity (Fig. 3.61). Preparation of pure protein (3.4.2.6) allowed the activity of D177 to be investigated more thoroughly. To ascertain the concentration of D177 that should be used for depurination and the time of incubation, an experiment was performed using 0.5 $\mu$ M SWY incubated at 30°C for 10 or 30 minutes with increasing concentrations of D177 (from 0 to 50nM). Analysis of the plot shown in Fig. 3.70 indicated that adequate depurination was achieved with 5.26nM D177 over a period of 30 minutes. The reaction rate was linear at this stage and, although one could use greater concentrations of D177 protein for a shorter incubation time (10 minutes in Fig. 3.70), this concentration was used to maintain the substrate : enzyme ratio as great as possible.



Using this D177 concentration in an incubation with varying concentrations of substrate for 30 minutes, the kinetic properties of D177 were examined. This experiment was performed twice (D#1, D#2) in the substrate range of 0.2 to 2.5 $\mu$ M. The calculated initial reaction rates were used for plots of each individual experiment as shown in Fig. 3.71. As described for the studies of wild-type rRTA, the mean  $V_o$  for the two experiments was calculated ( $D\phi_{\text{mean}}$ ) and a Lineweaver-Burk plot prepared for the average results as shown in Fig. 3.72. Linear regression analysis proposed the x-intercept to be -0.531 ( $K_m = 1.883\mu\text{M}$ ) and the y-intercept to be 45.34 ( $V_{\text{max}} = 0.0221\mu\text{M}/\text{min}$ ). The corresponding Eadie-Hofstee plot (Fig. 3.73) proposed a  $K_m = 1.778\mu\text{M}$  and  $V_{\text{max}} = 0.0216\mu\text{M}/\text{min}$ . A summary of the data generated for D177 is shown in Table 4 below.

Table 4 Calculated kinetic data for mutant D177.

| Experiment            | $K_m$ ( $\mu\text{M}$ ) | $V_{\text{max}}$ ( $\mu\text{M}/\text{min}$ ) | $k_{\text{cat}}$ ( $\text{min}^{-1}$ ) |
|-----------------------|-------------------------|---|--|
| D#1                   | 1.548                   | 0.0222  | 4.22                                   |
| D#2                   | 1.972                   | 0.0198  | 3.76                                   |
| Mean D#1+D#2          | 1.760                   | 0.0210  | 4.00                                   |
| $D\phi_{\text{mean}}$ | 1.883                   | 0.0221  | 4.19                                   |

Fig. 3.71 Lineaever-Burk plot of D177 N-glycosidase activity from experiments DØ1 and DØ2.

5.26nM purified D177 was incubated for 30 minutes at 30°C with salt-washed yeast ribosomes at concentrations ranging from 0.2 to 2.5µM. The initial rate of reaction ( $V_0$ ) was calculated from the X depurination as described in equation 3. The x-intercepts for DØ1 and DØ2 were shown to be -0.646 and -0.507 respectively, whilst the y-intercepts were identified as 45.133 and 50.58 respectively.

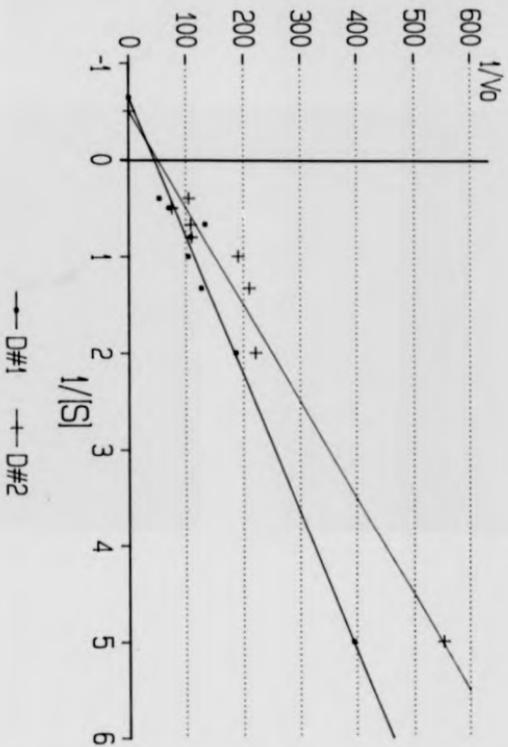


Fig. 3.72 Lineweaver-Burk plot of mean D177 N-glycosidase activity.

The N-glycosidase activity of mutant D177 was assessed using an enzyme concentration of 5.26nM with a substrate range of 0.2 to 2.5 $\mu$ M over a period of 30 minutes. Mean initial velocity rates were calculated from the X depurination derived from the two experiments.

Linear regression analysis proposed the x-intercept to be -0.531 and the y-intercept to be 45.34.

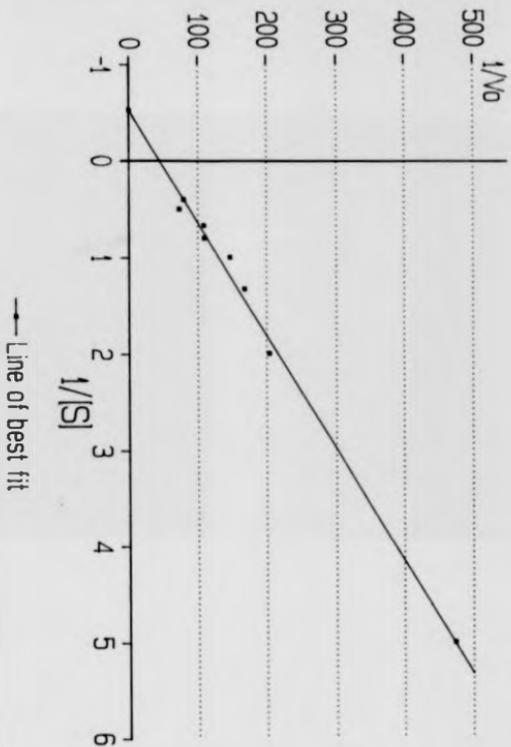
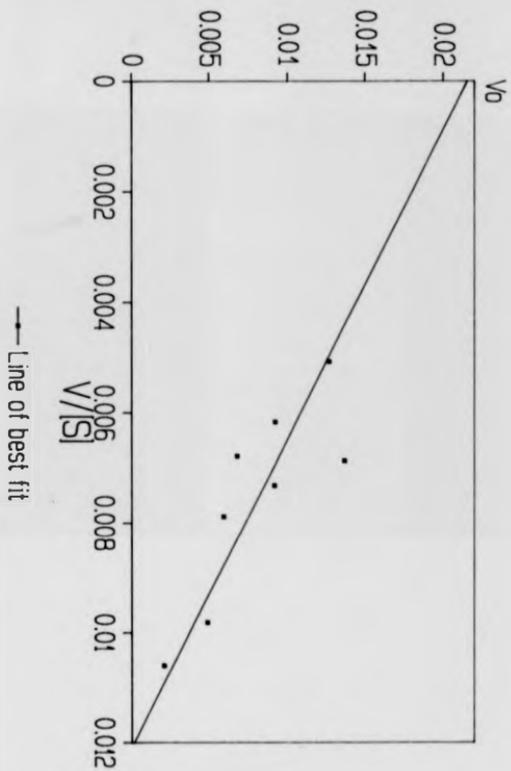


Fig. 3.73 Eadie-Hofstee plot of mean D177 N-glycosidase activity.

The mean initial velocity measurements for D177 were calculated from the percentage depuration values observed. An Eadie-Hofstee plot was prepared from these measurements. The proposed y-intercept was  $0.0216\mu\text{M}/\text{min}$  and the slope was  $-1.778\mu\text{M}$ .

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Comparison of the results for D177 (shown in Table 4) with those for rRTA (shown in Table 3), indicates that D177 has a 1.25 fold increased  $K_m$  value and a 48 fold increased  $k_{cat}$  leading to an overall 60 fold decrease in activity. This difference is discussed at length in the following chapter.

The cofactor requirement for the catalytic activity of RIPs has been the subject of much debate. In the case of PAP (Ready et al., 1983) it was shown that there was a requirement for ATP and a factor present in the postribosomal fraction for efficient ribosome-inactivation. The salt concentration during the depurination was also indicated to be important, with the greatest difference in activity observed as a change in the  $K_m$  value. A requirement for ATP and tRNA has been suggested during experiments with tritin (Roberts & Stewart, 1979). Additional evidence for this phenomenon was indicated by experiments using gelonin (Sperti et al., 1991), in which the presence of ATP and a factor from the postribosomal supernatant were shown to increase the  $k_{cat}$  and decrease the  $K_m$  for the inactivation of Artemia salina ribosomes. These workers also measured the ribosome-inactivation by ricin A-chain and found that it was similar, in the absence of cofactors, to that of gelonin in the presence of these factors ( $K_m = 2.02\mu M$ ,  $k_{cat} = 317 \text{ min}^{-1}$ ). Recently published data by Ready et al. (1991) suggest a cofactor dependency for ricin A-chain when

assayed for protein synthesis inhibition of Artemis salina ribosomes. In the absence of cofactors the  $K_m$  was shown to be  $1.3\mu M$  and the  $k_{cat}$  was  $300\text{min}^{-1}$ . In the presence of ATP and a complex supernatant fraction the  $K_m$  decreases to  $0.4\mu M$  and the  $k_{cat}$  increases to  $1000\text{min}^{-1}$ .

The data presented in this study suggested a  $K_m$  of  $1.52\mu M$  and a  $k_{cat}$  of  $201\text{min}^{-1}$  for rRTA. These data would appear to be in agreement with that presented by Sperti et al. and Ready et al. in the absence of cofactors. However, observations during the preparation of kinetic data for this study have not indicated a requirement for ATP nor an effect of salt. Additional work by Andrea Massiah has also not shown this ATP requirement (unpublished observations). This phenomenon will be discussed in section 4.

### 3.5.3 Summary of in vivo data

The data indicated in Fig. 3.74 represents a summary of the major results obtained within sections 3.3, 3.4 and in this section. Of the 10 ricin A-chain constructs used in this study, expression in E. coli was achieved in 6 cases. N-glycosidase activity was assessed and the purification of selected rRTA species was attempted. Purified protein was obtained from wild-type rRTA, A177 and D177 using an ion-exchange chromatography procedure. The N-glycosidase activity of wild-type rRTA and the

mutant D177 was examined using yeast ribosomes and kinetic parameters recorded. It would appear that D177 is approximately 60 fold less active than wild-type rRTA with the majority of the effect being observed with the  $K_m$  of the mutant.

Fig. 3.74 Summary of in vivo experiments.

| A-chain | Expression     | Crude    | Purified | $K_m(\mu M)$ | kat(/min) |
|---------|----------------|----------|----------|--------------|-----------|
|         | <u>in vivo</u> | Activity |          |              |           |
| rRTA    | Yes            | Yes      | Yes      | 1.52         | 201       |
| K177    | Yes            | No       | No       | ND           | ND        |
| A177    | Yes            | Yes      | Yes      | ND           | ND        |
| D177    | Yes            | No       | Yes      | 1.88         | 4.2       |
| A29     | Yes            | Yes      | No       | ND           | ND        |
| SEAR    | No             | ND       | ND       | ND           | ND        |
| Q180    | No             | ND       | ND       | ND           | ND        |
| A180    | No             | ND       | ND       | ND           | ND        |
| M180    | No             | ND       | ND       | ND           | ND        |
| p+      | Yes            | ND       | ND       | ND           | ND        |

ND - Not determined

*Chapter Four*

**DISCUSSION**

#### 4.1 General discussion

At the onset of this project the knowledge relating to the mode and site of action of ricin A-chain and other RIPs was limited. It was known that ricin A-chain catalytically removed a single adenine residue from eukaryotic ribosomal RNA by an unknown mechanism of hydrolysis (Endo & Tsurugi, 1987), and that the ribosomal substrate took the form of a defined stem-loop structure (Endo & Tsurugi, 1988). Ricin had been crystallised and a representation of the structure was available (Montfort et al., 1987). Some chemical modification studies had been performed but their results were of limited value because the modifications were relatively non-specific and thus the proteins were difficult to characterise.

This project was initiated to use a more specific approach to investigate the relationship between the structure of ricin A-chain and its enzymatic function. Several key amino acids were identified as being potentially important, predominantly on the basis of conservation of the residue between different RIPs. These were then altered by a specific mutagenesis approach and the mutant products characterised with in vitro and in vivo experiments. This more defined approach has also been utilised by several other groups and has led to an explosion of information relating to the individual residues within ricin A-chain.

Occasionally, mutants have been fully characterised and kinetic parameters determined, such as D177 presented in this study. This information is particularly useful since it gives more detailed data which can be used to propose a likely role for the residue. More often, residues have been assessed with more simple activity measurements such as inhibition of protein synthesis, inactivation of a translation system or with a more direct assay of the extent of depurination of the rRNA. Activity measurements of this kind have led to a number of residues within ricin A-chain being assigned putative roles. The focus of the research has been predominantly towards the catalytic mechanism and the associated rRNA/ribosome binding function of this protein. Other aspects of ricin A-chain activity such as translocation into the cytosol have not been examined by this approach. The suggested roles of the residues identified by such an approach will be discussed later when I present a putative mechanism of action proposed by Ready et al. (1991), with which my data concurs.

Whilst experiments have been designed to investigate the roles of amino acids within ricin A-chain, other aspects of working with ricin A-chain have come to light as the project progressed. In this study, the expression of recombinant ricin A-chain in vitro and in Escherichia coli, and purification of soluble, active recombinant ricin A-chain from a heterologous expression system have

been investigated.

Mutated constructs were initially expressed in vitro to examine their ribosome-inactivation properties and to ascertain any alterations in activity as a result of mutagenesis. The reduced sensitivity of wheatgerm ribosomes to ricin A-chain was exploited when in vitro transcripts were translated in a wheatgerm lysate such that mutant polypeptides (active and inactive) could be visualised and their molecular sizes estimated. The extreme toxicity of ricin A-chain towards rabbit reticulocyte ribosomes made it important to have a system in which the in vitro produced polypeptide could be visualised to confirm the fidelity of the reading frame after the earlier cloning procedures. It is as yet unknown why RIPs have such different activities toward ribosomes from different species. In the case of wheatgerm, they have been shown to be 1000 fold less susceptible to ricin A-chain than reticulocyte ribosomes (Osborn, 1991). The major kinetic difference is seen with the  $K_{cat}$  of the reaction since the  $K_m$  is relatively unchanged, suggesting that the binding of ricin to the substrate (rRNA within a ribosome) is similar. The difference in  $k_{cat}$  may probably be due to slightly different interactions between the rRNA target sequence and the enzyme active site. The fact that the mutants produced in this study were translated by the wheatgerm ribosomes to equivalent levels to wild-type A-chain, indicated that the mutant residues did not affect

these complex enzyme-substrate interactions to increase the Kcat and therefore increase the activity of the A-chain towards wheatgerm ribosomes.

Translation of recombinant wild-type and mutant ricin A-chain species in rabbit reticulocyte lysate to assess in vitro activity has previously been reported by May et al. (1989). The two experiments that were performed to assess the in vitro activity (double translation and aniline assay) provide the most reliable information when both are used to assess the activity of mutants. The aniline assay is particularly suitable for assessing active toxins since it is both extremely sensitive and gives a clear indication of depurination. A non-toxic protein however will produce a negative result which is less satisfactory. By using the double translation experiment, non-toxic proteins can be clearly visualised by the presence of the toxin polypeptide and the reporter protein after SDS-PAGE, demonstrating that the toxin was translated in the lysate but was inactive towards the protein synthesis system. Conversely, a toxic protein produces no visible product in the double translation experiment but is visualised by a cleavage product in the aniline experiment.

In the double translation experiment it was difficult to assess the differences in activity quantitatively because the A-chain species was present throughout the assay, continually inactivating the ribosomes as the reporter protein was translated. To more accurately

estimate the N-glycosidase activity, one could inactivate the rRTA action (by the addition of anti-rRTA antibodies) after rRTA translation, and assess the residual protein synthesis capability of the translation system. However, to apply this method to generate quantitative data is difficult because of the uncontrollable inactivation of ribosomes during translation. A feature of this system is that active proteins will inactivate the translation system quickly and their own synthesis will be decreased. A mutant with lower activity will be translated to a greater degree over the same time period and therefore there will be more enzyme molecules in the translation mix to effect depurination. Thus the actual quantity of RIP produced in the translation system may be different.

Translation systems have been used to generate kinetic data (Ready et al., 1991) using purified protein (i.e. of a known quantity) to inactivate the system before neutralisation by the addition of anti-A-chain antibodies. The residual capacity for translation was then measured. In this way the processes of ribosome-inactivation and polypeptide translation are distinct events and kinetic measurements are valid.

The aniline assay is considered to be the most accurate of the methods available for assessing RIP activity. This is because it measures the actual effect of ricin A-chain (i.e. depurination) and not the secondary effect of protein synthesis inhibition. Technically it is

more difficult to perform due to the sensitivity of the ribosomes and the rRNA to degradation by non-specific RNases. Hence the kinetic measurements presented in this study, although more representative of the actual activity, required a more labour-intensive approach to prepare data of sufficient quality.

It is necessary to be aware of the assay system used to generate activity measurements so that comparisons of the results between groups can be made. A significant criticism of using inhibition of protein synthesis as a measure of activity is that this method should theoretically overestimate the activity of a RIP. This would occur because inactivating one ribosome in a polysome would effectively inactivate more than one ribosome per depurination reaction and protein synthesis from that polysome would cease. This has been determined experimentally by Fodstad & Olanes (1977) who suggest that the unaffected ribosomes in an arrested polysome are still capable of translation if released from the polysome. It was interesting therefore to compare results published by Olanes et al. (1975) in which the Kcat was estimated to be 1500 ribosomes per minute using an assay based on protein synthesis inactivation, or 1777 ribosomes per minute using the aniline assay reported by Endo & Tsurugi, (1988). The difference between these results is surprisingly small.

The in vitro results generated using the double translation and aniline methods indicate the importance of

certain residues within the ricin A-chain for the catalytic action. From the mutagenesis of glutamate 177 it would appear that some activity is retained by altering glutamate to alanine or aspartate. In the case of D177, the amount of residual activity is less than that retained by A177. This was a curious result since one would suspect that the conservative change of Glu to Asp would have less effect than the relatively large change of Glu to Ala. The results of Hovde et al. (1988) had suggested that E177 may be a crucial residue for the activity of the enzyme, probably being centrally involved in the catalytic mechanism. The results from this in vitro study suggested that the role of Glu177 could be substituted to a certain extent by alanine. However, the Glu residue must have some function because its replacement with a lysine (K177) caused the total loss of enzymatic activity.

Therefore one had the scenario of a residue that had been shown to be required for in vitro activity and whose function could apparently be replaced by an alanine residue but not to the same extent by an aspartate. The chemistry of the side-chain of aspartate is obviously more akin to that of the glutamate than the methyl group of an alanine residue. Similar differences were observed by Schlossman et al. (1989) using purified E177A (Glu177 to Ala) and E177D (Glu177 to Asp) in a protein synthesis inhibition assay. E177A was shown to be approx. 20 fold less active than wild-type recombinant ricin A-chain

whereas E177D had 80 fold reduced activity. A possible reason for this observation was later presented by Frankel et al. (1990) during an investigation into the role of Glu208.

It was hypothesized, on the basis of examination of the ricin A-chain structure, that Glu208 could substitute for Glu177 by rotation around the alpha-carbon atom to bring the carboxylate side-chain into the active-site area. The role of Glu177 would thus be performed by Glu208 in this model. Mutagenesis of Glu208 to Asp retained 100% of the activity, with Asp208 presumably fulfilling the structural role proposed for Glu208. However if a double mutant (E177A + E208D) was created, effectively no activity remained (Frankel et al., 1990). Thus it was proposed that the Asp208 side-chain could no longer compensate for the loss of the carboxylate side-chain of Glu177 because the shorter Asp could not fill the stereochemical requirements of Glu177. E208 is a conservative but not invariant residue in the RIP group (although it is conserved in the 5 RIPs detailed in Fig. 3.1), therefore it is difficult to understand why this residue evolved to be present in ricin A-chain in a position where it could perform such a 'backup' role. Of course, it may be a fortuitous secondary role of a residue whose primary role is structural- this area of the A-chain (helix G) is involved in the formation of a hydrophobic pocket that holds the C-terminus of the B-chain.

In the K177 mutant, the positive charge of the lysine side-chain probably prevents rotation of E208 into the active site area, thus there is no residue to substitute for Glu177. It is likely that the lysine residue will form interactions with negatively charged species in the immediate locality, and also repel nearby basic residues (such as Arg180) causing strain on the molecule. This may result in poor protein folding and consequently the poor in vivo expression of soluble K177.

Therefore contrary to initial theories, ricin A-chain does not have an absolute requirement for glutamate at position 177 for ricin A-chain activity. Although it is invariant amongst RIPs, it is not the crucial residue it had earlier been proposed to be.

Attention focussed to Arg180 and, as shown in this study, mutagenesis of Arg180 has a dramatic effect on the activity of the rRTA. When altered to glutamine, alanine or methionine, no N-glycosidase activity remained, suggesting that this arginine was important to the structure and/or function of the rRTA. Arg180 is also invariant amongst the RIPs and it has been shown to be located at the base of the putative active-site cleft.

Frankel et al. (1990) also published data relating to the R180 mutants I prepared at Duke University, indicating that the mutants (Q, A and M180) were insoluble in his in vivo expression system and were unsuitable for purification. No attempt was made to perform in vitro

assays or refolding protocols to try and generate soluble protein that could be fully assessed. Since I have shown these mutants to be partially soluble in vitro and yet no N-glycosidase activity or protein synthesis inactivation could be demonstrated, it is likely that the lack of activity observed is a direct result of removal of a key catalytic residue. The in vitro results presented in section 3.2.6 suggested that this inactivation of N-glycosidase activity may, in part, be due to a reduced affinity of binding to the ribosome, either as a direct result of altering Arg180 or as an indirect result of disturbing the structure by mutagenesis at position 180. If arginine 180 is indeed a crucial residue for activity, it would be assumed that the chemistry of the side-chain is an important factor. Arginine is the most basic of the amino acids and possesses a positive charge at pH 7, as does Lys.

Additional mutagenesis at Arg180 has been performed by Frankel et al. (1990) with the creation of R180K and R180H. Activity measurements have revealed that R180K was almost fully active (a 4 fold decrease was observed) which rules out a role for the guanidine moiety as a necessary nucleophile in catalysis. However the lysine mutant would be able to fulfil other possible roles of the arginine side-chain i.e. coordinate with an active-site water or formation of an ion pair with Glu177. Unsurprisingly, R180H was inactive towards ribosomes, the imidazole ring

structure probably being inadequate for performing the two functions above. Thus it would appear that the residue at position 180 required a positive charge, although the stereochemistry of this charge distribution is not critical for activity.

When the mutations SEAAR and A29 were assessed for activity in vitro, it was found that the SEAAR deletion was inactive using both methods whereas A29 appeared to retain its activity. The presence of the correct mutants had been confirmed by sequencing and restriction enzyme digests. The result for SEAAR was not surprising. Although this mutation had been created previously (May et al., 1989), it was difficult to justify the earlier result of activity in light of an accumulating amount of data suggesting the importance of residues within this region. Even if one does not consider the structural effects of a pentapeptide deletion at the centre of the protein, the conservation and mutagenesis data strongly suggested that this region must be important in the functioning of the molecule.

It was important to clarify the actual behaviour of this SEAAR deletion since this region contained six of the site-specific mutants I had created. Structural information from Katsin et al. (1991) has shown the amino acid sequence SEAAR to form the C-terminal end of helix E of the A-chain. This region is fundamental in distorting the helix to form a characteristic bend. Both Glu177 and

Arg180 are able to interact in the solvent due to this bend in the helix and interdigitate with Ala178 and Phe181. In doing so, the latter two residues are orientated towards the hydrophobic core and are proposed to be strong determinants of the folding dynamics and stability of the active-site cleft.

Even though no N-glycosidase activity was observed, it was perhaps surprising to find a portion of SEAAR in the 'soluble' phase of a reticulocyte translation system. It is difficult to explain this apparent solubility. It is possible that within a reticulocyte lysate there are factors or ionic conditions that aid the stabilisation of such a molecule, even though the structural modifications are likely to be great. Alternatively, it may demonstrate an inherent weakness of the solubility criterion that small aggregated protein complexes may not be pelleted by centrifugation at 100,000 xg.

The in vitro results for Ala29 also have structural significance. It has been reported (Katzin et al., 1991) that Arg29 may play a role in stabilising the bend in Helix E by formation of a hydrogen bond to the O of A178, although the authors suggested that the bond distances were long and they were unclear as to why other polar residues could not substitute for arginine. As described earlier, arginine 29 is invariant and yet there is no obvious crucial role that specifically requires an arginine. This residue was chosen for study with the

hypothesis that it was involved in ribosome recognition / binding. However, when tested non-quantitatively in a rabbit reticulocyte lysate, A29 would appear to be almost as active as the wild-type protein. This would suggest that the removal of the arginine side-chain has little effect on the functioning of ricin A-chain. It may be that the hydrogen bonding to helix E does not play a major role in stabilising the structure. Alternatively, the role of Arg29 could be substituted for by another amino acid, in a similar way to the E177A-E208 phenomenon.

In a recent publication, Funatsu et al. (1991) has suggested Arg29 to be an essential residue for catalytic activity, on the basis of its total conservation throughout 11 RIFs. However, the results presented in this study demonstrate that R29 is not essential for the N-glycosidase activity. The possibility remains that it is involved in anti-viral effects, if indeed the two activities are performed by separate functional domains of the protein.

A more likely role for Arg29 could be to bind the rRNA during catalysis. During examination of the ricin A-chain structure at Duke University, I observed that R29 was located distant from the putative active site cleft, amongst many positively charged residues (mainly arginines). I postulated that this region may be involved in rRNA binding. It has also been postulated by Katsin et al. (1991) that Arg29 may play a role in binding RNA,

maybe in a ribosomal recognition site away from the catalytic site.

A hypothesis of a second site involved in ribosome recognition and binding has been given greater credibility by examining the observed  $K_m$  for ricin A-chain on a variety of rRNA substrates. The  $K_m$  for various substrates is similar, as shown in Table 5 below.

Table 5 Apparent  $K_m$  of ricin A-chain when tested on a variety of substrates.

| $K_m(\mu M)$ | Substrate  | Reference                   |
|--------------|--|-----------------------------|
| 2.6          | Rat liver 80S ribosomes  | Endo & Tsurugi, 1988        |
| 5.8          | Rat liver naked 28SrRNA  | Endo & Tsurugi, 1988        |
| 3.3          | <u>E. coli</u> naked 23SrRNA                                     | Endo & Tsurugi, 1988        |
| 0.4          | <u>A. salina</u> ribosomes (+factors) Ready <u>et al.</u> (1991) |                             |
| 1.3          | <u>A. salina</u> ribosomes (-factors) Ready <u>et al.</u> (1991) |                             |
| 0.1          | Reticulocyte ribosomes   | Olsnes <u>et al.</u> (1975) |
| 2.1          | Wheatgerm ribosomes  | Osborn, (1991)              |
| 1.5          | Yeast 26SrRNA (salt-washed)                                      | This work (3.5.2.1)         |

Therefore it would appear that the RNA binding event (as estimated by the  $K_m$ ) is a relatively consistent process that may be separate from the catalytic function. It may be the case that this binding event involves

residues distant from the active site (such as Arg29) for preliminary recognition, and residues within the active-site (e.g. Arg180) for specific RNA binding and enzyme orientation. It would appear that the mutation of Glu177 to Asp had a negligible effect on the  $K_m$  of the reaction. This suggests that a glutamate at position 177 is not an absolute requirement for substrate binding - either D177 fulfils an equivalent binding role, or E177 is not involved in substrate binding at all.

The final 'mutant' to be studied in vitro was the E138D 'pseudo-positive' construct prepared as a control for the R180 mutants. As explained in the Results Chapter, the mutation of Glu138 to Asp was present in the original template used for mutagenesis of R180. This construct was active in vitro to the same extent as wild-type. E138 is located between the alpha-helices C & D away from the active-site. It is not conserved and in three out of the five RIP sequences presented in Fig 3.1 the position is filled with proline residues. However, as reported by Gould et al. (1991), wild-type rETA produced from this clone (possessing the E138D mutation) is always less active than native (plant purified) ricin A-chain (3-fold) and it could be speculated that this mutation could be one of the factors affecting the activity.

The in vitro data obtained for the mutants are an indication of their relative activities. The next stage was to express the constructs in E. coli and attempt to

purify, and further characterise, the mutant A-chains. Initial experiments to purify wild-type ricin A-chain using the method outlined in O'Hare et al. (1987) were only partially successful. The conditions of expression and purification were investigated using the method of O'Hare as a model.

It is not the job of this discussion to review recombinant ricin A-chain expression protocols and purification procedures at length, highlighting the problems and making extensive comparisons between different techniques. However, since a large proportion of this project was devoted to the expression of recombinant ricin A-chain in Escherichia coli and the subsequent purification of protein for kinetic studies, it is appropriate to spend a little time explaining the observations that were made, commenting on results generated by other workers, and critically assessing work that has been performed in this area.

The first hurdle when starting to express a protein of interest is the choice of expression host. In the case of ricin A-chain species, or Type 1 RIPs, the choice was immediately limited by the fact that the protein was toxic to eukaryotic ribosomes. This excluded expression of recombinant ricin A-chain in oocytes or in yeast where the host ribosomes would be sensitive to the toxin. However, as will be shown later, expression of a recombinant ricin A-chain mutant has been successfully performed in Xenopus

oocytes in this laboratory, exploiting the reduced activity of this mutant. Yield is low however, and the procedure is labour intensive, making it unsuitable for large-scale expression. Fortunately, ricin A-chain is inactive towards E. coli ribosomes (Endo & Tsurugi, 1988), enabling expression to be carried out in the cytoplasm or periplasm of the cell. For some RIPs that are active towards E. coli (e.g. MAP, PAP, Dianthin (Hartley et al., 1991)) expression procedures are difficult and often result in extremely poor yields.

Assuming the RIP is not active towards the host ribosomes, further problems arise because of physical characteristics of the RIP, e.g. the pI. Most E. coli proteins have an acidic pI, however most RIPs possess a basic pI (in the case of ricin A-chain the pI is approximately 7.5). This may increase the rate at which these proteins are recognised as being foreign by the bacteria, causing degradation or aggregation of the protein.

One approach to avoid the degradation of expressed proteins is to express them in an insoluble form in the inclusion bodies of the cell. A case in point would be the expression of Pseudomonas exotoxin constructs by the group of Pastan. These proteins are routinely expressed as insoluble aggregates, which are later solubilised with guanidine and refolded to produce active, cytotoxic molecules (Chaudhary et al., 1990).

However, the most common route to isolating RIPs from E. coli is to express the protein in a system designed to express a proportion of soluble protein and accept that there will be losses due to aggregation. At least six methods of expressing ricin A-chain in E. coli have been reported. The method most similar to that described by O'Hare et al. (1987) is that of Shire et al. (1990) which utilises the same promoter ( $P_{N25}$ ) fused to the lac operator. Expression of this synthetic gene is achieved at 30°C in L-B medium. Purification of the recombinant ricin A-chain was performed using an anti-rRTA affinity column prepared with monoclonal antibodies. Elution was achieved with 0.1M glycine (pH 3.0) and a single polypeptide was eluted representing a yield of approximately 1.5mg/l culture. Reconstitution of this A-chain with biochemically purified ricin B-chain was performed and the cytotoxicity compared to other reconstituted forms of ricin. It was apparent that ricin reconstituted from the recombinant sample was of lower cytotoxicity than ricin reconstituted using native or deglycosylated A-chain (approximately 10 fold). There are numerous reasons why this could be the case. However, given the results presented in this study (3.4.2.2) which casts doubt over the stability of rRTA on low pH elution, it is possible that this purification strategy has had a deleterious effect on the activity of the rRTA protein.

Piatek et al. (1988) examined a number of promoter

systems and also the effect of temperature. Maximal expression was achieved with the ricin A-chain cDNA in a cistron-like arrangement with the alkaline phosphatase (*phoA*) signal peptide. Expression was directed by the *phoA* promoter and the ribosome binding site contained within the cloned *phoA* fragment. rRTA was expressed intracellularly at 6-8% of the total cell protein in a predominantly soluble form at 37°C. Lowering the temperature to 30°C increased expression of soluble protein to 10-11% total cell protein. Increased expression of soluble protein was also observed in this study during expression of rRTA at 30°C.

Using a different vector system based on the expression of insoluble aggregated rRTA (up to 6% total cell protein) under the control of the phage lambda P<sub>L</sub> promoter, Piatak and colleagues examined the effect of temperature on expression of soluble protein. They observed an increase in soluble protein when expressed at 37°C instead of 42°C. Insoluble rRTA could be produced in vivo by switching a culture expressing at 37°C to 42°C, however this could not be repeated in vitro. Factors relating to the intracellular environment and not a characteristic of the A-chain would therefore appear to be important.

The recently described, but increasingly reported concept of the protein chaperone may have a role to play in the stabilisation of the protein under different

expression conditions (Gething & Sambrook, 1992). It could be postulated that a chaperone-like molecule is present in E. coli which can aid the production of correctly folded proteins.

This concept of protein folding in vivo and in vitro is one of the central dogmas in expression studies. Protein folding is a poorly understood concept and prediction of the folding tendencies of a protein are difficult. In a recent report by Wilkinson & Harrison (1991), factors affecting inclusion body formation were assessed by a statistical analysis of the solubility of proteins expressed under similar conditions. The goal of this approach was to use the data to predict, from a primary sequence, the solubility of proteins in E. coli. Expression of recombinant ricin A-chain at 37°C revealed insoluble protein, as previously noted, and this was predicted using the Wilkinson model. Shein & Noteburn (1988) have linked inclusion body formation to temperature sensitive denaturation and noted that lowering the temperature to 30°C reduced the frequency of inclusion bodies.

Wilkinson noted that in vivo solubility is different, in at least one respect, to in vitro solubility, being related more to the solubility of the folding intermediates than to the solubility of the mature protein. In a bacterial cell the amount of folding intermediates will depend on the rate of folding which, in

turn, is dependant on the number of 'turn' residues. Ricin A-chain has a high percentage of such residues (Asp, Asn, Pro, Gly, Ser) which may indicate a likely slow-folding regime, involving many folding intermediates. High concentrations of folding intermediates may lead to poor in vivo solubility.

Expression in E. coli of rETA mutants in this study was partially successful. All the mutations at Glu177 were produced in a soluble form (although the recovery of K177 was extremely low). This would indicate that the Glu177 mutants were correctly folded into a thermodynamically stable conformation, and revealing that Glu177 is not an essential structural residue. N-glycosidase activity was retained in the A177 mutant and to a lesser degree in the D177 mutant. The rETA mutant K177 (shown to be inactive when produced in E. coli) has been successfully expressed in Xenopus laevis oocytes by Richard Argent in our laboratory. Soluble protein was recovered, confirming that at least a small proportion of the protein folded correctly.

Soluble product, that was shown to retain N-glycosidase activity, was also recovered for the A29 mutant. However, the proportion of degradation products with this mutant was greater than for wild-type rETA on every expression experiment. This could be due to a perturbed structure leading to increased accessibility to proteases, or possibly the altered amino acid sequence

creates a more optimal recognition sequence for proteolysis by bacterial enzymes.

Expression of the pseudo-positive construct (E138D) was also achieved as expected. However, expression of the mutants at the Arg180 locus and the SEAAR deletion was unsuccessful. This suggests that in E. coli these mutations are not tolerated during the folding process and the products are degraded. The polypeptides cannot be simply packaged into insoluble protein bodies since analysis of the total cell protein led to no rRTA-cross reactive bands on Western blots. Thus the proteins must be efficiently degraded. Since these mutants have been shown to be inactive in vitro, it would be of interest to attempt to express them in an alternative expression system (e.g. yeast) to ascertain if soluble, inactive protein could be recovered.

One approach to stabilise proteins during expression in a heterologous system is to create a protein fusion. One such fusion has been produced for recombinant ricin A-chain by Schlossman et al. (1989) with  $\beta$ -galactosidase (previously shown to stabilise foreign proteins in the E. coli environment (Germino & Bastia, 1984)). Schlossman expressed rRTA, linked to the N-terminus of  $\beta$ -galactosidase via a 60 amino acid collagen linker, under the control of the lambda P<sub>R</sub> promoter. Cells were grown at 30°C, induction was initiated at 42°C for 15 minutes, and the protein expressed at 37°C. The fusion protein was

expressed at 5% of the total cell protein and after purification the overall yield of rRTA was 2.4mg/litre.

This was the expression system used by Frankel *et al.* (1990) when performing expression studies with rRTA mutants comparable to those studied here. There are two main problems with this approach, the first of which is the high temperature expression conditions. Although the  $\beta$ -gal fusion has been shown to stabilise certain proteins, there is no evidence to suggest that it is particularly suitable for ricin A-chain. In fact approximately 20% of the fusion protein is lost when the disrupted cell suspension is centrifuged to remove cellular debris, suggesting a degree of aggregated material. Solubility prediction by the method of Wilkinson & Harrison (1991) suggests that  $\beta$ -galactosidase is a satisfactory, but not ideal, partner for fusion protein production.

Unlike other fusion protein techniques where a cleavable linker with a specific cleavage point is introduced between the subunits, the collagen linker is an inefficient and unreliable linker. Preparations of collagenase vary, leading to inefficient cleavage of the collagen, giving rise to ricin A-chain species with heterogeneous C-termini. This rRTA is obviously unsuitable for kinetic studies since it is impossible to determine the molecular weight of the protein accurately. It was also reported that overdigestion can sometimes occur, leading to non-specific cleavage of the rRTA by proteases

present in the collagenase and a purification procedure complicated by many A-chain degradation products.

Using this procedure Frankel et al. (1990) expressed the following ricin A-chain mutants for activity studies: E177A (0.8mg/l), R180K (0.8mg/l), R180H (1.3mg/l) and E177A+E208D (0.2mg/l). R180H and E177A+E208D were interesting in that there was no detectable activity remaining after mutagenesis and yet pure soluble protein had been isolated. The single mutation (E208D) control for the E177A+E208D mutant was strangely difficult to express and a yield of only 1-10 $\mu$ g/l was achieved. The yield of A177 presented in this thesis of 0.4 mg/l compares favourably with the data shown above. The mutation of Glu177 to Asp was also purified using the fusion protein method (Schlossman et al., 1989) with a yield of 1mg/l. As reported in this work, D177 was prepared at 1.4mg/l, the final product having a known amino acid sequence and being produced with a simple, potentially less damaging approach.

A weakness of the in vivo ricin A-chain construct presented here is the presence of a 10 amino acid N-terminal extension. This sequence (MGSSRVEDNN) was a result of the cloning strategy used for the construction of pDS5/3RA (O'Hara et al., 1987). It is understandable that this extension may be an unsatisfactory feature of recombinant ricin A-chain for use in immunotoxin construction, since it is likely to increase the chances

of the protein being recognised by the host and thus increase degradation and decrease plasma retention time. However it was shown to have virtually identical protein synthesis inhibitory properties, to reassociate with ricin B-chain, and to have a cytotoxic potency indistinguishable from that of native ricin (O'Hare et al. 1987). Since the focus of this work was not directed towards the construction of mutant immunotoxins, this in vivo produced A-chain was satisfactory for the requirements of this study.

Large yields of protein have been reported by Ready et al. (1991) expressing rRTA under the control of the lac promoter at 30°C. These workers also found expression at 37°C to be unsatisfactory. Up to 15-30mg/l was reported after purification by phenyl-Sepharose and then CM-Sepharose, however expression of mutants was much more difficult. Certain mutants (E177Q) were expressed at extremely low levels making purification impossible. In addition Q-Sepharose and Cibacron blue dyes were used for individual mutants (Y80F, Y123F & N209S) whose characteristics made them unsuitable for purification by the standard methods used for wild-type recombinant ricin A-chain.

A multitude of different separation techniques have been used for the purification of rRTA and other RIPs. Predominantly the separation processes have been based on ion-exchange, as was the protocol developed in this study.

Cation-exchange had been used by Stirpe et al. (1981) during the purification of dianthin from carnation leaves. Application of cation exchange in this study in the form of a CM-Sepharose column at pH 6.5 has proved to be successful in separating rRTA from the majority of E. coli proteins. Addition of the S-Sepharose step further refined this procedure. CM-Sepharose has also been recently used in the purification of MAP 30 (Lee-Huang et al., 1990).

An alternative method, and one particularly suited to mutant proteins with significantly altered pIs, is the use of triazine dye chromatography. It was reported by Appukuttan & Bachhavat (1979) that ricin A-chain (but not ricin) could interact with the blue dye Cibacron blue F<sub>3</sub>GA. This binding was further investigated by Watanabe & Funatsu (1987) who estimated 1.4 molecules of dye bound per molecule of A-chain. Stabilisation of the dye on a matrix such as Sepharose CL-4B or Affi-gel has led to the development of dye-ligand columns that can bind RTA.

It was thought that interaction took place through the RIP active site region leading to a useful purification step to isolate 'active' RTA. However this may not be the case since a recent publication by Munoz et al. (1990) demonstrated that although 80% of ricin A-chain is retained by an Affi-gel Blue matrix, no PAP-I was retained. Triazine dyes have been reported to interact with several types of nucleotide-dependant enzymes and thus this column is best suited for use as a final

purification step. In the case of ricin A-chain, the dye is proposed to mimic the ribonucleotides that interact with the putative active site residues. PAP and RTA have similar  $K_m$  values for ribosomes although the  $K_{cat}$  of PAP is approximately 5 fold lower. In addition, PAP is active towards E. coli ribosomal RNA whereas RTA is not (Hartley et al., 1991). Could it be that the active site region of PAP is perturbed relative to ricin A-chain such that PAP no longer interacts with Cibacron blue? The X-ray crystal structure of PAP has recently been determined (J. Robertus-personal communication) and comparison with that of recombinant ricin A-chain should give exciting information as to the structure of the active sites.

For the purposes of this study, the dye-binding approach would appear to be particularly suited to the purification of A29 from a semi-purified, CM-Sephacrose (pH 5.8) pregradient wash. This experiment would be interesting to perform since, if the hypothesis of a second RNA binding domain is correct, and if R29 is involved in this binding, then the interaction with the Cibacron Blue dye should be the same but the N-glycosidase activity may be different. It would also be interesting to assess the binding and activity of the major degradation product from A29 expression. It has previously been shown by Wood et al. (1991) that a major contaminating degradation product (approx. 27.5kD) copurified with recombinant abrin A-chain on a Blue-Sephacrose column,

leading to the suggestion that its binding function was intact. It was also found to be active towards rabbit reticulocyte ribosomes although it was not further characterised. The breakdown product for A29 is smaller but unfortunately the N-glycosidase activity has not been successfully tested and no data has been determined as to the sequence of the fragment.

The use of ion-exchange chromatography in this study was successful in separating recombinant ricin A-chain (expressed at 1% of the total cell protein) from the background E. coli proteins. It was often difficult to differentiate ricin A-chain from the background E. coli proteins when stained with silver or coomassie blue. However visualisation (to 10ng) in a Western blot was possible using sheep anti-rRTA IgG purified during this study. Since it was unnecessary to have an absolutely pure preparation of IgG, these antibodies were not affinity purified on a ricin A-chain column. The antibodies were shown not to react with E. coli proteins but were shown to bind recombinant ricin A-chain, or ricin, specifically.

These IgGs were also used in the construction of an anti-ricin A-chain column. A column of this type had potential in purifying recombinant ricin A-chain from a crude E. coli sonicate in one step. Such columns have been used previously for ricin A-chain (Shire et al., 1990), for Pseudomonas aeruginosa Exotoxin A (Douglas & Collier, 1990) and diphtheria-toxin conjugates (Williams et al.

1990) with varying elution conditions. It was found in this study that although elution of recombinant ricin A-chain was achieved with a change in pH to pH 2.15, such a pH had a detrimental effect on the activity of the toxin. This made this approach unsuitable for the preparation of rRTA for kinetic studies. It was preferential to maintain the A-chain in relatively neutral conditions to have a minimal effect on the protein structure. This made the CM-Sephacrose column at pH 6.5 particularly suitable.

Initially DEAE-ion-exchange chromatography was attempted, however the rRTA passed through the column in the pre-gradient low salt wash. The behaviour of recombinant ricin A-chain under these conditions is not as expected since at a pH greater than 7.5, ricin A-chain should have an overall negative charge. Revising the literature showed that this effect had been also observed by Olsnes & Pihl (1973) with a DEAE-cellulose column using 100mM Tris (pH 8.5) as buffer. The interaction between a protein and an ion-exchange matrix is complex. The actual distribution of charged groups on the external surface of the protein probably plays a major role in this interaction. The pI is a measure of the overall property of the protein, however clustering of charged residues may create areas of localised pI differences which may affect the interaction with an ion-exchange matrix. However, this column was shown to act as a purification step since the *E. coli* proteins bound strongly to the column.

The use of a S-Sepharose column after a CM-Sepharose column was designed to exploit the differential interaction of some proteins when presented with a weak (CM) or strong (S) ion-exchanger. However, as a single purification step using crude sonicate, it was not as successful as the CM-column, demonstrating the altered interaction.

Recombinant ricin A-chain produced in E. coli by the method outlined in this study produces ricin A-chain with a 10 amino acid N-terminal extension. Ricin A-chain has been examined for its tolerance to deletions and addition of amino acids to its structure in order to ascertain the minimum structure necessary for N-glycosidase activity. This relatively crude approach has yielded conflicting results. At the N-terminus it would appear that the addition of 19 amino acids does not affect the activity (Sundan et al., 1989) and neither does the deletion of 4 (Funatsu et al., 1989) or 9 residues (May et al., 1989). According to May and coworkers, the deletion of 12 amino acids removed the activity of rRTA, however Sundan et al. (1989) demonstrated the deletion of the first 28 residues without affecting the inhibition of protein synthesis (although they could not demonstrate N-glycosidase activity). Bradley et al. (1989) created a deletion of residues 19-40 and showed the production of an insoluble and inactive protein. It has been reported (Katrin et al.. 1991) that the first 6 residues of rRTA interact loosely

with the remainder of the protein, the first 4 residues are thought to be in a state of disorder. However a deletion of residues 18-30 would remove the helix A from the structure. There are 2 invariant and 3 conservative residues within this region and they are thought to interact with the  $\beta$ -sheet structure of domain 1. This  $\beta$ -sheet possesses several of the proposed RTA active-site residues and presumably the conformation is important for activity.

Various deletions have been performed at the C-terminus and again the results have been conflicting. Funatsu et al. (1989) deleted the C-terminal 27 amino acids and reported 1.8% activity retained, suggesting a role in rRTA N-glycosidase action for these residues. Sundan et al. (1989) deleted the last 70 and the last 45 and found these constructs also to be incapable of inactivating a protein synthesis system. However Sundan also reported the deletion of residues 156 to 267 and the retention of protein synthesis inhibition in an in vitro system. However again no N-glycosidase activity could be observed with this construct.

A project has been initiated by Morris et al. to produce consecutive deletions of the RTA protein and assess N-glycosidase activity. Reports from a recent conference have suggested a surprising number of mutants that were both soluble and active (221 residues altered out of 263) (Frankel et al., 1991). However this work has

yet to be published and it is awaited with interest.

The pentapeptide deletion DVTNAY (residues 75-80) produced by May et al. (1989) and the deletion SEAAR (residues 176-180) presented in this study, have resulted in inactive proteins. In order to rationalise the SEAAR result with the Sundan deletion (156-267), one would have to assume that the SEAAR deletion caused a structural change in the C-terminal region which either interacted with the N-terminal region forcing it into a strained conformation, or the altered folding of the C-terminus masked a site on the N-terminus involved in the mechanism of protein synthesis inhibition. Sundan presents no theories as to why protein synthesis inhibition, but no N-glycosidase activity, was observed. Presumably the possibility of a two site model would be favoured by the Sundan results, maybe with the protein competing for the RNA and effectively 'slowing down' translation by interaction alone.

In addition to the large-scale deletions discussed previously, mutations at many loci within the ricin A-chain have been performed. All the A-chain mutations and their activity compared to wild-type (if measured), are listed in Fig. 4.1. Also included are eight random A-chain mutants generated by Frankel et al. (1989) which allowed yeast, bearing the rRTA plasmid, to survive.

**Fig. 4.1** List of ricin A-chain mutants whose protein synthesis inhibition activity or N-glycosidase activity has been measured.

Amino acids are designated with the single letter code. Mutagenesis reactions are described by the common notation of native amino acid-residue number (according to Lamb et al. (1985))-mutant residue. Deletions are indicated by 'del.'.

| Mutation       | Reference                    | Activity             |
|----------------|------------------------------|----------------------|
| G212W          | Frankel <u>et al.</u> (1989) | Inactive in yeast    |
| G212E          | "                            | Inactive in yeast    |
| G212 del.      | "                            | Inactive in yeast    |
| S215P          | "                            | Inactive in yeast    |
| W211R          | "                            | Inactive in yeast    |
| E177D          | "                            | Inactive in yeast    |
| E177K          | "                            | Inactive in yeast    |
| I252R          | "                            | Inactive in yeast    |
| R48A           | May <u>et al.</u> (1989)     | Active               |
| R56A           | "                            | Active               |
| R56 del.       | "                            | Active               |
| D75-Y80 del.   | "                            | Inactive             |
| F2S            | Bradley <u>et al.</u> (1989) | 100% active          |
| Q19A           | "                            | 100% active          |
| H40Q+E41A+I42L | "                            | 100% active          |
| I1-H40 del.    | "                            | insoluble & inactive |
| I1-V28 del.    | Sundan <u>et al.</u> (1989)  | Active               |
| T156-F267 del. | "                            | Active               |
| I1-A27 del.    | Funatsu <u>et al.</u> (1989) | 1.8% active          |

(continued overleaf)

| Mutation    | Reference                       | Activity             |
|-------------|---------------------------------|----------------------|
| E177D       | Schlossman <u>et al.</u> (1989) | 80 fold less active  |
| E177A       | "                               | 18 fold less active  |
| E208D       | Frankel <u>et al.</u> (1990)    | 100% active          |
| E208D+E177A | "                               | Inactive             |
| R180Q       | "                               | Insoluble & inactive |
| R180A       | "                               | Insoluble & inactive |
| R180M       | "                               | Insoluble & inactive |
| R180K       | Frankel <u>et al.</u> (1990)    | 4 fold less active   |
| R180H       | "                               | <0.1% active         |
| W211F       | Bradley <u>et al.</u> (1990)    | 9 fold less active   |
| E177Q       | Ready <u>et al.</u> (1991)      | 180 fold less active |
| N209S       | "                               | 6 fold less active   |
| Y80F        | "                               | 15 fold less active  |
| Y123F       | "                               | 7 fold less active   |
| S203N       | Gould <u>et al.</u> (1991)      | 100 fold less active |

**This study has indicated:**

|                |                     |
|----------------|---------------------|
| E177K          | Inactive            |
| E177A          | Active              |
| E177D          | 60 fold less active |
| R180Q          | Inactive            |
| R180A          | Inactive            |
| R180M          | Inactive            |
| R29A           | Active              |
| S176-R180 del. | Inactive            |
| E138D          | Active              |

Analysis of these mutants, and analogy with other N-glycosidases, has led Ready et al. (1991) to propose a putative mechanism of action of ricin A-chain. It was known that the cleavage of the N-glycosidic bond is typically carried out by acid hydrolysis (Michelson, 1963). A model of an acid-catalysed reaction suggests that the adenine base is protonated, inducing electron flow from the ribose ring oxygen into the ring to form an oxycarbonium intermediate with partial bonding between the ribose and the adenine. The reaction is completed by nucleophilic attack by water on the C1 carbon of the ribose ring. This model has been confirmed by Mentch et al. (1987) during analysis of the mechanism of action of AMP nucleosidase from Azotobacter vinelandii. In addition, it was shown that a transition state analogue for the AMP nucleosidase reaction (formycin monophosphate (FMP)) binds to the active site of rRTA.

Using this data as a working model for RIP N-glycosidase activity, Robertus and colleagues initiated the examination of a series of rRTA mutants to examine the roles of some of the conserved residues in ricin A-chain, as shown in Fig. 4.1. From these studies, it was shown that Glu177 and Arg180 are crucial residues for the catalytic activity of rRTA, although it is apparent that both these residues can be substituted with certain alternative amino acids and activity maintained. Other residues were implicated as being important in the

functioning of ricin A-chain. Asn209 was proposed to be involved in substrate binding, since mutation to serine causes an increase in the  $K_m$  (to  $8\mu M$ ) with no effect on the  $k_{cat}$ . Amide residues are frequently used by proteins for specific recognition of nucleic acid bases and this would appear to be a case in point. The two invariant tyrosines (80 and 123) have a role to play in the function of ricin A-chain. However, removal of the hydroxyl group in both cases had only a small effect on the activity of the mutant protein.

When one examines the structural position of these invariant residues (Y80, Y123, N209 & W211), it is apparent that they form a cluster around the active site area. It is thought therefore, that they interact with the substrate and contribute additively to the tight binding of RTA to the ribosome. This binding is most likely in conjunction with other groups around the active site. The total energy of such interactions is suggested to put the substrate adenosine (4324 in rat liver, see Fig. 1.4) into a strained syn conformation. From X-ray analysis, FMP is known to exist in the syn conformation. Arg180 may aid the formation of this structure by ion-pairing to the phosphate backbone, hence the need for a positive charge at this position. This would explain the lack of N-glycosidase activity of the Arg180 mutants presented in this study. It may also explain the apparently lower binding of the mutants to rabbit reticulocyte ribosomes.

The role of Glu177 is probably to stabilise the positive charge that develops on the ribose ring as a result of partial bond breakage of the N-C bond. It has already been explained how it is thought that the Glu177 mutation, A177, can accomplish this task. Presumably the stereochemistry of the charge distribution of the mutant D177 is less suitable for interacting with the developing oxycarbonium ion than substitution of Glu177 by Glu208. In the case of Shiga-like toxin, in which the equivalent residue to Glu177 (Glu167) was mutated to aspartate and a 1000 fold reduction in activity was observed (Hovde et al., 1988), the equivalent residue to Glu208 is a threonine which could not fulfil the role of Glu208. Hence the large reduction in activity for the E167D mutant.

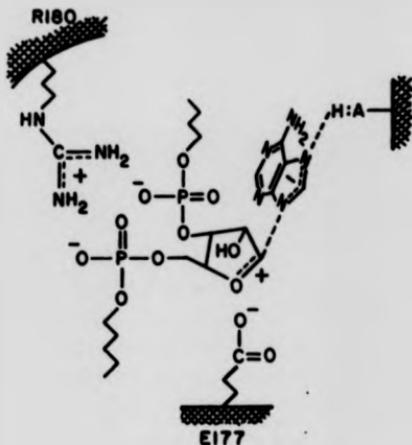
The N-C bond, which has been destabilised by the combination of interactions described above, would then be susceptible to nucleophilic attack by water at the C1 of the ribose moiety. It is suggested that this water molecule is water #323 which usually occupies a position in the active site, coordinated between Arg180 and Glu177. The leaving group (adenine) may be protonated by partial hydrogen bonding at more than one of the nitrogen atoms, which, in an additive system, would stabilise the adenine making the reaction energetically favourable.

A schematic representation of this proposed mechanism is shown in Fig. 4.2. The origin of the hydrogen bond for protonation of the adenine is unspecified.

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A schematic representation of this proposed mechanism is shown in Fig. 4.2. The origin of the hydrogen bond for protonation of the adenine is unspecified.



**Fig. 4.2** Proposed mechanism of action of ricin A-chain.

This mechanism of action has been proposed by Ready et al. (1991). It is suggested that residues Arg160 and Glu177 act to stabilise the putative transition state oxycarbonium ion, making the reaction more energetically favourable. The substrate adenine is shown in the syn conformation with partial breakage of the N-glycoside bond. Protonation of the adenine is performed by an unspecified residue. Additional interactions with other conserved residues (W211, Y123, Y80) are likely to occur with the ribose and purine rings of the substrate. A water molecule displaces the N-C bond by nucleophilic attack on the C1 of the ribose moiety.

#### 4.2 Overall conclusions

It has been demonstrated in this study that specific mutagenesis of potentially important amino acids can be used as a powerful tool to examine the relationship between the structure of a protein and its function. Analysis of the mutations by in vitro and in vivo techniques has led to information relating to the stability and activity of mutated protein.

Mutagenesis of the putative active site residues Glu177 and Arg180 has resulted in various degrees of inactivation of the protein. Arg180 would appear to have a crucial role in catalysis since its conversion to Gln, Ala or Met results in an protein with no remaining N-glycosidase activity, as assessed by in vitro activity analysis. Mutagenesis studies by other workers have shown that the role of Arg180 can be filled by a lysine residue, indicating that a positive charge at this location is crucial. It is thought that this residue interacts with the RNA substrate during catalysis. Substitution of Arg180 by other amino acids has led to difficulties in expression of the mutant in E. coli.

Mutagenesis of Glu177 to Lys, Ala and Asp has indicated that a negative charge at position 177 is important for catalysis, although it can be substituted to some degree by a second glutamate residue at position 208 if there is no steric interference (as in the cases of

K177 and D177). Glu177 is thought to interact with the developing oxycarbonium ion during catalysis and thus make the reaction more energetically favourable. The presence of a positive charge at position 177 is structurally unfavourable and leads to low levels of soluble protein expression in E. coli.

Deletion of a pentapeptide (SEAAR), shown to be located at the active site of ricin A-chain, results in an inactive mutant protein that possesses no detectable N-glycosidase activity and potentially decreased binding affinity for the substrate.

Mutagenesis of Arg29 to Ala resulted in a protein with similar N-glycosidase activity to wild-type recombinant ricin A-chain. The location, and conservation, of Arg29 made it a potentially important residue for substrate recognition and/or binding. The results of in vitro experiments would suggest that the catalytic rate was unchanged, suggesting that the residue is not crucial for N-glycosidase activity. At present it is uncertain what the role of this residue may be.

The explosion of information over the past few years, relating to the properties of ribosome-inactivating proteins, is a direct result of an appreciation of the importance of this class of proteins. It has been established that a study of RIFs may lead to important

advances in many areas, including therapeutics and anti-viral studies. It is my hope that the results presented in this thesis, in combination with the work carried out by many other groups, may assist the determination of the mechanism of action of potentially one of the most important protein families that is documented.

# **APPENDIX**

APPENDIX A: The cDNA sequence and deduced protein sequence of preproricin.

The nucleotide sequence for the ricin precursor cDNA according to Lamb et al. (1985) was found to lack a single methionine codon at its 5' end when compared to the sequence of a genomic clone isolated by Halling et al. (1985).

The amended cDNA sequence described here encodes a single polypeptide precursor form of ricin (termed preproricin). Nucleotide and amino acid numbering throughout this thesis refers to the sequence presented here, unless otherwise indicated. The deduced primary amino acid sequence is shown below the DNA sequence. Translation of the precursor is initiated at methionine (position -35) and extends through the N-terminal region (35 amino acid residues), a 267 amino acid residue A-chain, a 12 amino acid residue linker peptide and a 262 amino acid residue B-chain region.

The N-terminal region and the linker peptide are removed during post-translational processing of the precursor and these regions are highlighted with a dashed line between the DNA and polypeptide sequence.

-100

ARG AAA CCB DGB DGA <sup>30</sup> AAT ACT ATT BTA BTA TGB <sup>30</sup> TAT TCA BTA BTA BCB <sup>30</sup> CCB CCB CCB CCB CCB  
 Met Cys Arg Gly Ser Thr Thr Ile Val Ile Phe Met Tyr Ala Val Ala Thr Thr Leu Cys  
 -35

ITT DGB TCC ACE TCA <sup>10</sup> DGB TGB TCT TTC ACA TTA GAT GAT AAC <sup>10</sup> AAT TTC CCC AAT CAA CAA  
 Phe Gly Ser Thr Ser Gly Trp Ser Phe Thr Leu Glu Asp Asn Ser Ile Phe Pro Leu Cys

50

TAC CCA ATT ATA AAC <sup>10</sup> TTY ACC ACA CCG GAT GCT AAT BTA CAA AAC <sup>10</sup> TAC ACA AAC TTT ATC  
 Tyr Pro Ile Ile Asn Phe Thr Thr Ala Gly Ala Thr Val Ile Ser Tyr Thr Asn Phe Ile

100

AGA GCT GTT CCG GAT <sup>10</sup> CBT TTA ACA ACT DGA GCT GAT BTA BBA CAT <sup>10</sup> BAT ATA CCA ATA TTB  
 Arg Ala Val Arg Gly Val Thr Thr Thr Gly Ala Asp Val Arg His Asp Ile Pro Val Leu

150

CCA AAC ABA GTT GAT <sup>10</sup> TTB CCT ATA AAC CAA CCG TTT ATT TTA GTT <sup>10</sup> GAA CTC TCA AAT CAT  
 Pro Asn Arg Val Gly Leu Phe Ile Asn Glu Arg Phe Ile Leu Val Glu Leu Ser Asn His

200

GCA GAG CTT TCT GTT <sup>10</sup> ACA TTA GCC CTA GAT GTC ACC AAT ACA TAT <sup>10</sup> BTA BTA GGC TAC CBT  
 Ala Gly Leu Ser Val Thr Leu Ala Leu Asp Val Thr Asn Ala Tyr Val Val Gly Tyr Arg

250

GCT GAA AAT AAC GCA <sup>10</sup> TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT <sup>10</sup> GCA GAG GCA ATC ACT  
 Ala Gly Asn Ser Ala Tyr Phe Phe His Pro Asn Asn Ile Glu Asp Ala Gly Ala Ile Thr

300

CAT CTT TTC ACT GAT <sup>10</sup> GTT CAA AAT CBA TAT ACA TGC GCC TTT GAT <sup>10</sup> GAT AAT TAT GAT ABA  
 His Leu Phe Thr Asp Val Glu Asn Arg Tyr Thr Phe Ala Phe Gly Gly Asn Tyr Asp Arg

350

CTT GAA CAA CTT GAT <sup>10</sup> GAT AAT CTA GAA GAA AAT ATC GAG TTB GAA <sup>10</sup> AAT GAT CCA CTA GAA  
 Leu Gly Ile Leu Ala Gly Asn Leu Arg Glu Asn Ile Glu Leu Asn Gly Thr Leu Glu

400

GAG GCT ATC TCA GCA <sup>10</sup> CTT TAT TAT TAC AAT ACT GAT GGC ACT <sup>10</sup> CBT CTT CCA ACT CTA GCT  
 Glu Ala Ile Ser Ala Leu Tyr Tyr Ser Thr Gly Tyr Thr Glu Leu Pro Thr Leu His

450

CBT TCC TTT ATA ATG <sup>10</sup> TBC ATC CAA ATG ATT TCA GAA GCA GCA <sup>10</sup> AAT TTC CAA TAT ATT GAA  
 Arg Ser Phe Ile Ile Cys Ile Glu Met Ile Ser Glu Ala Ala Phe Asp Pro Ser Val Ile

500

GBA GBA ATG CBC ACB <sup>10</sup> ABA ATT ABA TAC AAC CBB ABA TCT GCA <sup>10</sup> CCA GAT CCT ABC ATA ATT  
 Gly Glu Met Arg Thr Arg Ile Arg Tyr Asn Arg Arg Ser Ala Phe Asp Pro Ser Val Ile

550

ACA CTT GAG AAT AAT <sup>10</sup> TGG GGG ABA CTT TCC ACT GCA ATT CAA GAA <sup>10</sup> TCT AAC CAA GBA GCC  
 Thr Leu Asn Asn Ser Trp Gly Arg Leu Ser Thr Ala Ile Glu Phe Ser Asn Glu Tyr Ile Ala

600

TTT GCT AAT CCA ATT <sup>10</sup> CAA CTB CAA ABA CBT AAT GAT TCC AAA <sup>10</sup> TTC AAT BTA TAC GAT BTA  
 Phe Ala Ser Pro Phe Ile Glu Leu Glu Arg Arg Asn Gly Ser Lys Phe Ser Val Tyr Asp Val

650

AAT AFA TTA ATC <sup>10</sup> CCT ACT ATA GCT CTA BTA BTA TAT ABA TAC GCA <sup>10</sup> CCT CCA CCA CCB TCA  
 Ser Ile Leu Ile Phe Ile Ile Ala Leu Met Val Tyr Arg Cys Ala Phe Pro Pro Ser Ser

700

750

800

800  
 CAG TTT TCI ITG STT ATA SSB CCB STS STV CCB SAI ITT SAI OCT GAT STT TST ATG DAT  
 31n Phe Ser Leu 290 11g Arg Pro Val Val Pro Ser Phe Asn 280  
 900  
 CCT GAG CCC ATA GTG CBT ATC GTA GGT CBA AAT GGT CTA TBT GTT GAT STT AGG GAT GGA  
 Phe Glu Phe Ile Val Arg Ile Val Gly Arg Ser Gly Leu Cys Val Arg Val Arg His Gly  
 290  
 990  
 AGR TTC CAC AAC GAA AAC SCA ATA CAG TTB TBB CCA TBC AAG TCT AAT ACA BAI ACA AAT  
 Arg Phe His Asn Gly Ser Ala Ile Glu Leu Trp Pro Cys Lys Ser Asn Thr Asp Ala Asn  
 310  
 1000  
 CAG CTC TGG ACT TTA AAA AGA GAC AAT ACT ATT CBA TCT AAT GAA AAG TBT TTA ACT ACT  
 Glu Leu Trp Thr Tyr Lys Arg Asp Ser Thr Ile Arg Ser Asn Gly Lys Cys Leu Thr Thr  
 320  
 1050  
 TAC GAA TAC AGT CCG GAA BTC TAT GTG ATG ATC TAT GAT TBC AAT ACT GCT SCA ACT GAT  
 Tyr Glu Tyr Ser Phe Gly Val Tyr Val Met Ile Tyr Asp Cys Ser Asn Thr Ala Ala Thr Asp  
 350  
 1100  
 GCC ACC CAC TGG CAA ATA TGG GAT AAT GAA ACC ATC ATA AAT CCC AAA TCT AGT CTA GTT  
 Ala Thr Arg Trp Glu Ile Trp Asp Ser Gly Thr Ile Ile Asn Phe Arg Ser Ser Leu Val  
 370  
 1200  
 TTA SCA CCB ACA TCA GAA AAC AGT GGT ACC ACA CTT ACA STG CAA ACC AAC ATT TAT ACC  
 Leu Ala Ala Thr Ser Gly Asn Ser Gly Thr Thr Leu Thr Val Glu Thr Asn Ile Tyr Ala  
 390  
 1250  
 GTT AAT CAA GBT TGG CTT CCT ACT AAT AAT ACA CAA CTT TTT GTG ACA ACC ATT GTT GGG  
 Val Ser Glu Gly Tyr Leu Pro Thr Asn Asn Thr Glu Pro Phe Val Thr Thr Ile Val Gly  
 420  
 1300  
 CTA TAT GBT CTB TCC TTB CAA SCA AAT AAT GAA CAA STA TBB ATG GAG SAG TBT AOC AAT  
 Leu Tyr Gly Leu Tyr Leu Glu Ala Asn Ser Gly Glu Val Trp Ile Glu Asp Cys Ser Ser  
 440  
 1350  
 GAA AAG GCT GAA CAA CAB TGG GCT TTT TAT SCA GAT GBT TCA ATA GBT CCT CAG CAA AAC  
 Glu Lys Ala Glu Gly Glu Trp Ala Leu Tyr Ala Arg Gly Ser Ile Arg Pro Glu Glu Asn  
 450  
 1400  
 CBA GAT AAT TBC CTT ACA AGT GAT TCT AAT ATA CBA SCA ACA GTT BTC AAG ATC CTC TCT  
 Leu Asp Ser Cys Leu Thr Ser Asp Ser Asn Ile Arg Glu Thr Val Val Lys Ile Leu Ser  
 470  
 1500  
 TST GAC CCT SCA TCC TCT GGC CAA CBA TGG ATB TTC AAG AAT GAT GAA ACC ATT TTA AAT  
 Cys Gly Pro Ala Ser Ser Gly Glu Arg Trp Met Phe Lys Asn Asp Gly Thr Ile Leu Asn  
 490  
 1550  
 TTB TAT AGT GGG TTB GTA TTA GAT GTG AGR SCA TCB BAI CCB AOC CTT AAA CAA ATC ATT  
 Leu Tyr Ser Gly Leu Val Leu Asp Val Arg Ala Ser Asp Pro Ser Leu Lys Glu Ile Ile  
 510  
 1600  
 CTT TAC CCT CTC CAT GBT SAG CCA AAC CAA ATA TBB TTA CCA TTA TTT TBA TAB  
 Leu Tyr Pro Leu His Gly Asp Pro Asn Glu Ile Trp Leu Pro Leu Lys Phe End End  
 530

APPENDIX B: The DNA sequence and deduced protein sequence of the mutagenesis template RTA20.

As described in section 3.1.2.1, the ricin A-chain DNA template for mutagenesis was provided by Dr. A. Frankel (then at Duke University, N.Carolina). It was constructed by cloning a 893bp BamHI fragment from the pAKG plasmid (Halling et al., 1985) containing a ricin genomic clone, into pUC119 (Vieira & Messing, 1987). Subsequent mutagenesis described in Schlossman et al. (1989) was used to create a full length ricin A-chain DNA sequence (810bp) with a BamHI site at the 5' and 3' ends.

In addition to the mutagenesis reactions detailed in Schlossman et al. (1989) this clone was also found to possess a single nucleotide difference to the sequence described in Appendix A. The alteration was a change of G to T at position 414 and was not reported in the original publication by Halling et al. (1985).

Nucleotide numbering is as described by Lamb et al. (1985). The underlined regions indicate the BamHI restriction endonuclease site.

GAT CCC AAC ATA TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT 50  
 Asp Pro Asn Ile Phe Pro Lvs Glu Tyr Pro Ile Ile 30 Phe Thr Thr Ala Gly Ala Thr

DTG CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CCG GGT CBT TTA ACA ACT GGA GCT GAT 100  
 Val Glu Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg Gly Arg Leu Thr Thr Gly Ala Asp

DTG AGA CAT GAT ATA CCA DTG TTG CCA AAC AGA GTT GGT TTS CCT ATA AAC CAA CCG TTT 150  
 Val Arg His Asp Ile Pro Val Leu Pro Asn Arg Val Gly Leu Pro Ile Asn Glu Arg Phe

ATT ITA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT GTT ACA TTA GCC CTG GAT GTC AAC 200  
 Ile Leu Val Glu Leu Ser Asn His Ala Glu Leu Ser Val Thr Leu Ala Leu Asp Val Thr

AAT GCA TAT GTG GTC GGC TAC CBT GCT GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT 250  
 Asn Ala Tyr Val Val Gly Tyr Arg Ala Gly Asn Ser Ala Tyr Phe Phe His Pro Asp Asn

CAG GAA GAT GCA GAA GCA ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CBA TAT ACA TTC 300  
 Glu Glu Asp Ala Glu Ala Ile Thr His Leu Phe Thr Val Glu Asn Arg Tyr Thr Phe

GCC TTT GGT GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GBT AAT CTS AGA GAA AAT ATC 400  
 Ala Phe Gly Glu Asn Tyr Asp Arg Leu Glu Glu Leu Ala Gly Asn Leu Arg Glu Asn Ile

JAT ITG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCS CTT TAT TAT TAC AGT ACT GBT 450  
 Asp Leu Gly Asn Gly Pro Leu Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr Ser Thr Gly

GGC ACT GCA CTT CCA ACT CTG GCT CCT TCC TTT ATA ATT TGC ACT CAA ATB ATT TCA GAA 500  
 Gly Thr Glu Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys Ile Glu Met Ile Ser Glu

JCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATB CBC ACC AGA ATT AGS TAC AAC CCG ABA 550  
 Ala Ala Arg Phe Glu Tyr Ile Glu Gly Glu Met Arg Thr Arg Ile Arg Tyr Asn Arg Arg

TCT CCA CCA GAT CCT ABC GTA ATT ACA CTT GAG AAT AGT TGB GGG AGA CTT TCC ACT GCA 600  
 Ser Ala Pro Asp Pro Ser Val Ile Thr Leu Glu Asn Ser Trp Gly Arg Leu Ser Thr Ala

ATT CAA GAG TCT AAC CAA GGA GCC TTT GCT AGT CCA ATT CAA CTS CAA AGA CBT AAT GBT 700  
 Ile Glu Gly Ser Asn Glu Gly Ala Phe Ala Ser Pro Ile Glu Leu Glu Arg Arg Asn Gly

TCC AAA TTC AGT STB TAC GAT STB AGT ATA ITA ATT CCT ATC ATA GCT CTC ATB STB TAT 750  
 Ser Lvs Phe Ser Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala Leu Met Val Tyr

AGA TGC GCA CCT CCA CCA TCG TCA CAG TTA GAT CCG 800  
 Arg Cys Ala Pro Pro Pro Ser Ser Glu Leu Asp Pro

**APPENDIX C: Ricin A-chain DNA sequencing primers.**

The following sequencing primers were designed to complement the ricin cDNA (Lamb et al., 1985) and were supplied by ICI. The concentration was adjusted to 10ng/ $\mu$ l for sequencing reactions.

CL582 5' 7 CCCAAACAATACCCAAT 23 3'

CL583 5' 250 ACCGTGCTGGAAATAGC 267 3'

CL584 5' 390 TGGTAATCTGAGAGAAA 406 3'

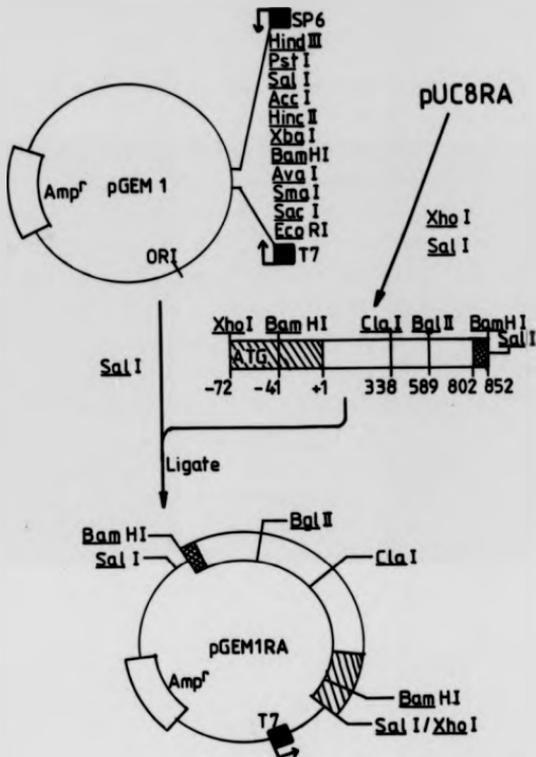
CL293 3' 301 CGTCTTCGTTIAGTGA 315 5'

CL294 3' 600 TCTAGGATCGCATTAA 615 5'

APPENDIX D: Construction of the recombinant ricin A-chain in vitro expression vector.

In order to express recombinant ricin A-chain in vitro, an expression vector was constructed in our laboratory based on pGEM1 (Promega). A DNA fragment from the preproricin cDNA (Lamb et al., 1985) was mutated to introduce a translation stop codon immediately after the codon for Phe267 of mature ricin A-chain (O'Hare et al., 1987). The DNA fragment containing the entire ricin A-chain coding sequence was excised from an earlier clone (pUC8RA) as a XhoI-SalI fragment and ligated into the SalI site of pGEM1 to give pGEM1RA.

Transcription of HindIII linearised constructs was performed using the T7 RNA polymerase promoter.



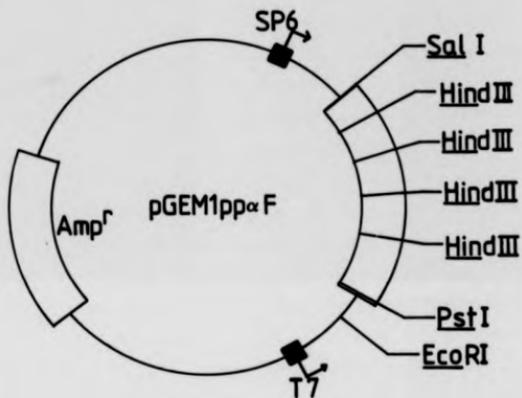
**APPENDIX E: pGEM1ppof**

Yeast  $\alpha$ -factor is a yeast mating pheromone produced from a precursor (preproalpha factor) which contains an 83 residue N-terminal sequence followed by 4 copies of the pheromone sequence, each separated by a spacer peptide (Kurjan & Herskowitz, 1982).

Preproalpha factor was used as a control polypeptide during in vitro N-glycosidase activity experiments. Previous work in our laboratory isolated the coding region for preproalpha factor in a HinfI/SalI fragment. The HinfI site was removed and the fragment cloned into the SmaI and SalI sites of pDS6. An EcoRI/SalI fragment from pDS6 was later cloned into pGEM2 to produce pGEM2 $\alpha$ 36.

The original in vitro construct in pGEM2 was suitable for transcription with SP6 polymerase. During this study the preproalpha factor coding region was cloned into pGEM1 as described in section 3.2.2.2. Linearised DNA for T7 RNA polymerase transcription was prepared by cleaving pGEM1ppof with SalI.

Note that the facing representation of the pGEM1ppof plasmid is not drawn to scale.



**APPENDIX F: Michaelis-Menten kinetics.**

The relationship between the initial velocity of an enzyme catalysed reaction and the substrate concentration is described by the Michaelis-Menten equation shown below:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

where  $V$  = rate of reaction.

$V_{\max}$  = maximum rate of reaction.

$[S]$  = substrate concentration.

$K_m$  = Michaelis-Menten constant

The enzymatic conversion of substrate to product is often written in the form:



Under the steady-state assumption, it is assumed that the concentration of the enzyme-substrate complex  $[E.S]$  will be small and constant. Thus a requirement of this model is that the enzyme concentration should be much lower than that of the substrate. Initial rates of reaction are measured before the substrate concentration becomes limiting.

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