warwick.ac.uk/lib-publications

Chaperonins in *Rhodobacter sphaeroides*
and *Pisum sativum*

Gregory Michael Francis Watson
B.Sc. (Hons) Rhodes University, South Africa.
M.Sc. University of Warwick, Coventry
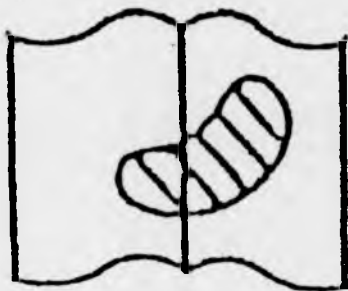

A thesis presented for the degree of
Doctor of Philosophy

Biol. Sci

University of Warwick
September 1992

VARIABLE PRINT QUALITY

NUMEROUS ORIGINALS
IN COLOUR

"Man selects only for his own good;
Nature only for that of the being which she tends."

Charles Darwin
The Origin of Species
(1859)

CONTENTS

Chapter 1

Introduction

Chapter 2

Materials and Methods

Chapter 3

Detection, purification and characterization of a *Rhodobacter sphaeroides* cpn60

Chapter 4

The stress response of *Rhodobacter sphaeroides*

Chapter 5

Attempts to identify and purify a putative cytosolic chaperonin from the leaves of *Pisum sativum*

Chapter 6

Discussion and Conclusions

List of Tables

List of Figures

Acknowledgements

I should like to thank my supervisor, Dr Nick Mann, for his guidance and encouragement during this work, and for displaying the patience of Job while I was writing up.

Special thanks go to Prof John Ellis, who became an unofficial second supervisor during the course of this work, for his critical input and the numerous delightfully stimulating conversations about the chaperones in particular and science in general.

Of all the people who always seem able to find time to give aid and advice when it is sorely needed I should especially like to thank Professor Roger Whittenbury for standing up for my rights when I wasn't sure I had them, Dr Helen Saibil and her group at Birkbeck for the superb electron microscope work and Dr Linda Barnett for the harvests of peas and support during the dark days of the TCP-1 story. Thanks also go to the three Bs. Bryan Dunbar for the N-terminal sequencing work, Brian Wells for superb tuition in immunocytochemical techniques and Bryan Sadler for good beer and conversation. Finally, I would like to thank Anni for her constant love, support and assistance.

Declaration

I hereby declare that the work described in this thesis was, unless otherwise stated, conducted by myself under the supervision of Dr N. H. Mann.

None of the information contained herein has been used in any previous application for a degree.

Gregory M. F. Watson

Dedication

To my parents, Frank and Sheila,

with love and admiration.

Abstract

The work presented in this thesis involves the isolation and characterization chaperonin 60 (cpn60) proteins from *Rhodobacter sphaeroides* and the cytosol of *Pisum sativum*.

Non-denaturing-PAGE analysis of *R. sphaeroides* cell-free extracts fractionated in sucrose density gradients revealed the presence of a M_r 670,000 protein that was considered to be a cpn60 candidate protein. This protein was purified by a combination of ion exchange and size exclusion chromatography. The M_r 670,000 multimer was shown to consist of a single polypeptide of M_r 58,000. Anti-sera raised against the *Escherichia coli* cpn60 (GroEL) and the pea plastid cpn60 did not cross-react with this protein. However, anti-sera raised against the *R. sphaeroides* protein was shown to cross-react with the *E. coli* GroEL protein. Subsequent N-terminal amino acid sequence analysis demonstrated that the *R. sphaeroides* polypeptide was identical to GroEL at 15 of the 19 residues determined and showed significant similarity to other known cpn60 sequences. Like other chaperonins the native protein tended to dissociate in the presence of MgATP, in this case into what appears to be a dimeric form. Examination of the *R. sphaeroides* protein in the electron microscope revealed 'top' views with seven-fold symmetry and rectangular 'side' views with four equally spaced, approximately equally dense, transverse striations. The protein appeared to be a cylinder of diameter 12 nm and height 9 nm. This structure is characteristic of the majority of the cpn60s examined so far. Rotational symmetry analysis revealed structural details extending beyond (20 Å)-1. The image of the chromosome-encoded *R. sphaeroides* chaperonin (Rs cpn60) differed from similarly generated images of plasmid-encoded GroEL. The possible significance of these differences is discussed.

The effects of various environmental stresses on protein synthesis in *R. sphaeroides* was investigated by pulse-labelling cells. Particular attention was paid to the expression of Rs cpn60. Overall the stress responses observed were similar to those reported for other prokaryotes. Of particular note are the quite different views of the stress responses presented by non-denaturing- and SDS-PAGE analyses. Attempts were made to determine the range of polypeptides that might be bound to Rs cpn60 before and after heat shock. These studies possibly indicate that there is a size constraint on polypeptides that interact with Rs cpn60 and that the flux of polypeptides through Rs cpn60 alters during heat shock.

The second part of this thesis concerns attempts to identify a cytosolic chaperonin in *P. sativum*. Until very recently no chaperonins had been identified in the cytosol of eukaryotes. Sequence analysis had raised the possibility that the TCP-1 protein from mice might be a cytosolic chaperonin (van der Vies, 1989). Monoclonal antibodies raised against the mouse TCP-1 protein cross-reacted with a M_r 57,000 polypeptide with a possible cytosolic location in *P. sativum* leaf extracts. Subsequent attempts to purify and determine the sub-cellular distribution of this polypeptide were unsuccessful. However, the sedimentation behaviour of the native protein indicated that the polypeptide was part of a multimeric complex of similar size to other chaperonins. The behaviour on ion exchange columns was also similar to other known chaperonins. The main obstacles to purification and localization of the native protein were: a) the monoclonal antibody used did not recognise the native protein and b) the multimeric complex appeared to dissociate during purification.

Chapter 1

Introduction

1.1. Historical perspective

Developing an understanding of the rules that govern the
way in which polypeptide chains fold up into the unique
three-dimensional structures of active proteins is an
endeavour that has occupied biological chemists for more
than half a century. This review will describe some of the
major advances in the understanding of protein folding and
outline the problems and observations that have led to the
emergence of the molecular chaperone concept.

The reversible denaturation of proteins was first
observed and discussed in the 1930s (see Ghélis and Yon,
1982). These early works clearly outlined the correlation
between the biological activity of a protein and the
native structure. The native structure was defined in
terms of activity and some physical properties such as
solubility, ability to crystallize and hydrodynamic
properties (see Ghélis and Yon, 1982). Also at this time
the reversibility of the denaturation-renaturation process
was demonstrated for a few proteins such as haemoglobin,
chymotrypsinogen and trypsinogen (see Anson, 1945). So
little was known about proteins at this time that the
period was principally marked by the success in protein
crystallization, an event that rebutted the old arguments
of the vitalists concerning the mysterious nature of

- 1 -

enzymes (see Ghélis and Yon, 1982).

The late 1950s and early 1960s saw the emergence of what Epstein *et al.* (1963) called the "thermodynamic hypothesis". Anfinsen (1973) defined this hypothesis as follows:

> "The three-dimensional structure of a native protein in its normal physiological milieu is the one in which the Gibbs free energy of the whole system is the lowest; that is that the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given natural environment."

The studies that gave rise to this hypothesis were mainly carried out on bovine pancreatic ribonuclease (Anfinsen, 1973). This protein has four disulphide bonds and the fully reduced protein thus has eight sulphhydryl groups which can then form a possible 105 isomeric disulphide bonded forms. Haber and Anfinsen (1962) demonstrated that when the fully reduced protein was allowed to reoxidize under denaturing conditions such as exist in a solution of 8 M urea, a mixture of products was obtained containing many or all of the possible 105 isomeric disulphide bonded forms. This mixture was essentially inactive. When the denaturant was removed and the unfolded protein exposed to a small amount of reducing reagent, the mixture was eventually converted into a

homogeneous solution indistinguishable from the native
protein. This process did, however, take several hours as
opposed to the couple of minutes required for synthesis of
the active enzyme *in vivo*. Those engaged in this research
went on to discover protein disulphide isomerase
(Goldberger *et al.*, 1963), and demonstrated that
ribonuclease correctly refolded in the presence of this
enzyme in less than 2 minutes. Renaturation of some other
enzymes that did not contain disulphide bridges had been
shown to take a few seconds or less (see Anfinsen, 1973).
In 1972 Anfinsen shared the Nobel Prize for Chemistry with
Moore and Stein for "... studies on ribonuclease, in
particular the relationship between amino acid sequence
and the biologically active conformation ..." (Anfinsen,
1973; Moore and Stein, 1973).

The thermodynamic hypothesis was modified by Levinthal
(1968) who proposed a kinetic control of protein folding.
The idea of kinetic control of protein folding has been
defined by Wetlaufer and Ristow (1973), who wrote that:

"This would mean that in many, perhaps most,
proteins the native structure is not the
structure of the lowest Gibbs free energy
(the global minimum). Of course, it would be
a structure in a free energy minimum, the
lowest free energy of the kinetically
accessible structures."

Thermodynamic versus kinetic control of protein folding

greatly influenced several studies in the 1970s. However,
in most cases no decisive arguments favouring one
hypothesis above the other were reached. In more recent
times the view taken has been that these two hypotheses
are not mutually exclusive (see Ghélis and Yon, 1982). The
importance of kinetic control of folding pathways is now
well established (see Creighton, 1984).

Observation of the refolding of many proteins has led to
the formulation of the principle of self-assembly. The
fundamental premise of this principle is that all the
information required to specify the final structure and
function of a protein resides within the amino acid
sequence of that protein. The assembly process is held to
be spontaneous and does not require either the input of
energy, or any steric factors which are extrinsic to the
polypeptide itself.

In spite of the general acceptance of the principle of
self-assembly, exceptions to the rule are known. Examples
of such exceptions are relatively rare. Creighton (1984)
has expressed the view that a possible reason for this is
that proteins that did not refold *in vitro* were generally
assumed to have undergone some form of interfering
covalent modification, or loss of some cofactor required
for the folded state. Some credence for this point of view
is provided in a series of recent papers, appearing after
the formulation of the the molecular chaperone concept, in
which *in vitro* studies of the assembly of proteins

- 4 -

translocated into the mitochondrion has shown that
glutamate dehydrogenase (West and Price, 1988), citrate
synthase (West *et al.*, 1990) and aspartate
aminotransferase (West and Price, 1989) cannot be
reactivated after they have been extensively unfolded by
guanidinium chloride. Examples that predate the emergence
of the molecular chaperone concept include the assembly of
certain bacteriophage that require the presence of what
has been described as a scaffold protein. These include
the λ protein gp*Nu3* (see Friedman *et al.*, 1984) and the T4
protein gp31 (Laemmli *et al.*, 1970).

The general acceptance of the principle of self-assembly
has led researchers to largely ignore or discount the
potential involvement of other proteins in the *in vivo*
folding process (Creighton, 1984). However, the yield of
active enzyme in many *in vitro* refolding experiments is
low, concentration-dependant, and accompanied by the
formation of insoluble aggregates (Epstein *et al.*, 1963;
see Mitraki and King, 1989). In the *in vivo* situation,
protein folding takes place in a complex, dynamic milieu
containing high concentrations of other proteins. In such
an environment it is likely that a great number of
reactive surfaces would be exposed, and there is a high
probability that incorrect interactions would take place.

1.2. The molecular chaperone concept

Laskey *et al*. (1978) first coined the term molecular chaperone to describe the role of nucleoplasmin in nucleosome assembly. Nucleosomes could only be reconstituted from separated DNA and histones by prolonged dialysis from >1 M salt plus 5 M urea. At physiological ionic strengths the components of such a mixture simply precipitate out of solution. Laskey *et al*. (1978) isolated a heat-stable, pentameric, acidic protein from unfertilized eggs of the frog *Xenopus laevis*, and demonstrated this to be the active factor required for nucleosome assembly at physiological ionic strengths. This protein acts by binding histones and neutralizing their positive charges, which is thought to prevent non-specific ionic interactions between DNA and histones and allow only specific interactions to occur. Note that nucleoplasmin is not a component of the mature nucleosome. This work, clearly demonstrating that nucleoplasmin prevented the occurrence of improper DNA/protein interactions, does not challenge Anfinsen's (1973) thermodynamic hypothesis which only refers to protein folding. However, the work of Laskey *et al*. (1978) defined a situation in which the interaction between two molecules required an external factor to ensure that the interaction took place correctly. Given these observations, the possibility could not be excluded that intramolecular and particularly intermolecular protein-protein interactions could be

- 6 -

modulated in a similar way.

Two protein families that have been crucial to the
formulation of the molecular chaperone concept will be
briefly discussed here. These are the hsp70 and GroEL type
proteins. Pelham (1986, 1988) suggested that the heat-
shock proteins, particularly hsp70 and hsp90, might play a
role in the assembly and disassembly of proteins and
protein-containing structures. Drawing on his own
observations and those of other researchers (see 1.3.8.)
Pelham (1986) postulated that hsp70, besides having a role
in specific cellular processes, has a general affinity for
denatured or abnormal cellular proteins. Pelham's model
(1986, 1988) supposed that during heat shock, proteins
become partially denatured, exposing hydrophobic regions
which then tend to interact to form insoluble aggregates.
He suggested that hsp70 binds tightly to such exposed
hydrophobic regions, thus limiting such interactions and
promoting disaggregation. Hsp70 then uses the energy of
ATP hydrolysis to free itself from the substrate protein
and partially unfold this substrate. The released
substrate protein is then considered to be able to refold
or reassemble into its pre-heat-shock state. These and
subsequent observations (see 1.3.8.) suggested a situation
in which a protein factor may play a role in preventing
improper interactions between substrate proteins, and
indeed may have a role in ensuring that correct folding
takes place.

The final observations that led directly to the formulation of the molecular chaperone concept were made by Ellis (1987) and Hemmingsen *et al.* (1988). This work developed from the discovery of a pea chloroplast protein that bound non-covalently to newly synthesized large subunits of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) before their appearance in the holoenzyme (Barraclough and Ellis, 1980). The binding protein had a molecular weight of >700,000 kD (Hemmingsen and Ellis, 1986) and was composed of α and β subunits of M_r 61,000 and 60,000 respectively (Musgrove *et al.*, 1987). This protein appeared to play a role in the assembly of Rubisco in higher plants (Cannon *et al.*, 1986; Ellis and van der Vies, 1988). The amino acid sequences of the α subunits of the castor bean (*Ricinus communis*) and wheat (*Triticum aestivum*) binding proteins were shown to be very similar, approximately 50% identical, to that of the GroEL protein from *E. coli* (Hemmingsen *et al.*, 1988).

The *groE* locus is composed of two genes, *groEL* and *groES*, (Tilly *et al.*, 1981) which encode polypeptides with M_r s of 65,000 and 15,000 respectively (Tilly *et al.*, 1981; Georgopolous and Hohn, 1978). Significantly, the GroE proteins were already known to play a role in the assembly of several phage and to be essential for cell viability (see Hemmingsen *et al.*, 1988). Both of these proteins are found in the cell as multimeric structures. The purified GroEL protein is a homo-tetradecamer arranged in two

stacked rings of seven subunits each (Hendrix, 1979; Hohn
et al., 1979). The GroES protein is composed of 6-8
subunits arranged in a single ring (Chandrasekhar *et al.*,
1986). The structure and function of these proteins will
be extensively reviewed in a later section (1.4.).

   Hemmingsen *et al.* (1988) pointed out that GroEL
homologues had been identifed in a number of bacterial
species. Furthermore, an abundant protein purified from
the mitochondria of *Tetrahymena thermophila*, had been
shown to be related to the GroEL protein both
immunologically and structurally (McMullin and Hallberg,
1987; 1988). Antibodies to the *Tetrahymena* protein cross-
reacted with a protein of M_r 58-64,000 in extracts of
mitochondria from yeast, *Xenopus*, maize and human,
suggesting that it may occur in all mitochondria (McMullin
and Hallberg, 1988). Hemmingsen *et al.* (1988) argued that
they had "...described a ubiquitous, conserved, abundant
protein that is associated with the post-translational
assembly of at least two structurally distinct oligomeric
protein complexes. We conclude that the role of this
protein is to assist other polypeptides to maintain or to
assume conformations which permit their assembly into
oligomeric structures."

   In the year previous to this work Ellis (1987) had
proposed the term "molecular chaperone" to describe a
class of cellular proteins whose function is to ensure

that the folding of certain other polypeptides, and their assembly into oligomeric structures, occurs correctly. The GroEL type proteins appeared to meet the criteria suggested for molecular chaperones and Hemmingsen *et al.* (1988) proposed that the term chaperonin be used to describe this specific group of sequence-related proteins. Hemmingsen *et al.* (1988) recognized three classes within the molecular chaperone family of proteins. These were; 1) nucleoplasmin, 2) *hsp70*-immunoglobin heavy chain binding protein class, and 3) the chaperonins. The molecular chaperones, with the exception of nucleoplasmin, have also been referred to as polypeptide chain binding proteins (Rothman, 1989).

The current definition of a molecular chaperone as proposed by Ellis and Hemmingsen (1989) is as follows:

> "A family of cellular proteins which mediate the correct folding of other polypeptides, and in some cases their assembly into oligomeric structures, but are not components of the final functional structures."

1.3. Classes and functions of molecular chaperones

The molecular chaperone concept has rapidly garnered support within the scientific community. With a definition to hand there has been a veritable explosion in the number of classes of protein now regarded as molecular chaperones. In this review no attempt has been made to

Table 1.1. **Proteins regarded as molecular chaperones**

Name	Proposed roles
1. Nucleoplasmins	Nucleosome assembly
	Transcription?
2. Chaperonins	Protein folding
	Oligomer assembly
	Protein trasport
	DNA replication
	mRNA turnover
	Stress protection
3. BiP-hsp70	Protein transport
	Oligomer assembly
	Oligomer disassembly
4. Signal recognition particle	Protein transport
5. Pro-sequence of subtilisin	Subtilisin folding
6. Ubiquitinated ribosomal proteins	Ribosomal assembly in eukaryotes
7. Trigger factor	Protein transport
8. SecB protein	Protein transport
9. PapD protein	Pilus assembly

Table after Ellis (1990).

describe all of the classes that could be described as
molecular chaperones. Instead those classes identified by
Ellis (1990; see Table 1.1.) will be described in some
detail with particular emphasis on the chaperonins. It is
hoped that this review will illustrate the remarkable
diversity, both structural and functional, of the
molecular chaperones and outline some of the problems in
correctly identifying members of this group.

Before discussing these classes in detail, it is
necessary to discuss the proposed nature of chaperone
action. The current view of protein folding is that there
are protein folding pathways in which there may be well-
defined intermediate stages where protein folding may
pause before continuing the journey to the final folded
state (see Dobson, 1992). The possible steps in such a
pathway are shown in Figure 1.1. The first steps in which
a protein in a disordered, unfolded state collapses into a
more compact state occur very rapidly while the subsequent
appearance of highly ordered tertiary structure occurs
somewhat more slowly (see Dobson, 1992).

It is thought that molecular chaperones interact with
intermediates on the protein folding pathway and guide the
folding protein towards its correctly folded state. Figure
1.2. shows a graphical representation of these concepts.
In case (A) the formation of the correct structure is
favoured both kinetically and thermodynamically and
molecular chaperones may not be required. In case (B) the

| Protein in disordered, unfolded state | Collapse into more compact state with fluctuating native-like or non-native-like secondary structure | Formation of persistent native-like secondary structure in absence of highly ordered tertiary structure | Formation of native state with well-defined secondary and tertiary structure and closely packed, solvent-excluded core |

After Dobson (1992)

The vertical axis (F) represents the free energy of protein folding from the unfolded state (U) within the cellular environment, while the horizontal axis represents the two possible results of folding – either an inactive misfolded structure (I) or a functional, correctly folded structure (C). In case (a) the formation of the correct structure is favoured both kinetically and thermodynamically; in this case chaperones may not be required. In case (b) the formation of the incorrect structure is favoured kinetically; the addition of a chaperone, indicated by the dotted lines, could either block the misfolding pathway or lower the activation energy of the correct folding pathway. This method of representation was suggested by R. B. Freedman.

After Ellis (1990).

formation of an incorrect structure is favoured
kinetically: the effect of addition of a molecular
chaperone, shown by the dotted lines, could either block
the misfolding pathway or lower the activation energy of
the correct folding pathway. In cases in which the
molecular chaperone is involved in protein transport, the
chaperone would "freeze" the substrate protein in a
transport-competent form by binding to a folding
intermediate.

1.3.1. Nucleoplasmins

As mentioned earlier (section 1.2.) the first protein to
be described as a molecular chaperone was nucleoplasmin
(Laskey *et al.*, 1978). This protein was purified from
Xenopus egg extracts, which assemble purified DNA into
nucleosomes using either the endogenous histone pool or an
exogenous source of purified histones (Laskey *et al.*,
1978). Nucleosome core particles consist of 140 base pairs
of DNA bound to a histone octamer. This structure is the
basic repeating unit of chromatin in the eukaryotic
nucleus.

Nucleoplasmin itself is an acidic, heat-stable protein
(Laskey *et al.*, 1978). Nucleoplasmin is the most abundant
protein in the *X. laevis* oocyte nucleus, constituting
approximately 10% of the nuclear protein (Mills *et al.*,
1980). The native protein has been shown to be pentameric,
being composed of five subunits of M_r 29-30 kD. In the

electron microscope the protein appears as a disc of 75 Å
diameter (Earnshaw *et al.*, 1980). The subunits have two
distinct domains, a protease-sensitive tail, carboxy-
terminal region and a relatively protease-resistant core
region that remains in solution as a pentamer after
proteolysis. The core monomer, after protease treatment,
has an M_r of approx. 23,000 (Dingwall *et al.*, 1982).
Cloning and sequencing of the cDNA for nucleoplasmin has
revealed a number of clusters of acidic amino acids within
the polypeptide. In particular, an almost uninterrupted
tract from residue 121 to 148 contains three aspartic acid
and eighteen glutamic acid residues. On the amino-terminal
side of this tract the core region is fairly acidic while
the tail region has only seven acidic residues out of 51
(Dingwall *et al.*, 1987; Burglin *et al.*, 1987).

A model for histone binding has been proposed in which
the polyacidic regions of the pentamer produce a large
negatively charged region, which functions as a single
binding site that neutralizes the positive charge of
histones before transfer to DNA (Dingwall *et al.*, 1987).
Estimates of the stoichiometry of the histone complexes in
egg extracts indicate that one nucleoplasmin pentamer
binds to one or two histone molecules (Kleinschmidt *et
al.*, 1985; Dilworth *et al.*, 1987). Moreau *et al.* (1986)
have demonstrated that nucleoplasmin also interacts with
ribonucleoprotein particles. These observations suggest a
potential role for nucleoplasmin in ribonucleoprotein

- 16 -

assembly.

Nucleoplasmin has been shown to be highly phosphorylated. Nucleoplasmin isolated from *Xenopus* eggs has about twenty phosphates per monomer, while that isolated from oocytes has less than ten phosphates per polypeptide chain (Cotten *et al.*, 1986). These phosphate groups are located predominantly in the core, histone-binding region, of the protein (Sealy *et al.*, 1986). Since the function of nucleoplasmin in the oocyte is principally the storage of the large histone pool (Woodland and Adamson, 1977), while in the egg it mediates the assembly of nucleosomes during rapid DNA replication, one might expect that the more highly phosphorylated form would be more efficient at assembling nucleosomes. Sealy *et al.* (1986) purified nucleoplasmin from oocytes and eggs and showed that this is the case. However, egg and oocyte extracts were equally efficient in nucleosome assembly (see Dingwall and Laskey, 1990).

Another feature of nucleoplasmin is a nuclear location sequence. Dingwall *et al.* (1982) demonstrated that the tail region of nucleoplasmin is necessary and sufficient for migration into the nucleus and rapid accumulation there. The nuclear location sequence has been shown to consist of two interdependent domains containing basic amino acids (Dingwall *et al.*, 1989). Studies with nucleoplasmin have been instrumental in demonstrating that protein uptake occurs via the the nuclear pore complex,

and that uptake is by a two-step process; the first step
being an energy-independent binding to the nuclear
envelope and the second an energy dependent translocation
step (see Dingwall and Laskey, 1990).

Finally it should be noted that nucleoplasmin is a
representative of a family of proteins. The cloning of
cDNA from oocyte mRNA resulted in the isolation of two
very closely related sequences (Dingwall *et al.*, 1987;
Bürglin *et al.*, 1987). Another nucleolar protein, XLNO-38,
has also been cloned and sequenced and shown to be
strikingly similar to nucleoplasmin (Schidt-Zachmann *et
al.*, 1987). This protein may carry out nucleolus specific
chaperoning functions (see Dingwall and Laskey, 1990).

1.3.2. Signal recognition particle

In mammalian cells, most proteins translocated across or
inserted into the ER membrane need the signal recognition
particle (SRP) to do so. The SRP is an 11S cytoplasmic
particle composed of six distinct polypeptides and a 7S
RNA. The signal sequence of a nascent polypeptide is
cotranslationally bound by SRP54, the 54 kD subunit of the
particle. The complex of SRP, nascent chain and ribosome
then interacts with the SRP receptor (docking protein) at
the ER membrane to be followed by the actual protein
transfer (Rapoport, 1990; 1991). Recently a particle
resembling the SRP has been found in *E. coli* (Ribes *et
al.*, 1990; Poritz *et al.*, 1990). A 4.5S RNA and a 48 kD

- 18 -

protein, P48, were shown to be part of a 10S
ribonucleoprotein. The 7S eukaryotic RNA can bind P48 and
is even able to take the place of the 4.5S RNA of *E. coli*
in vivo, whereas the 4.5S RNA binds SRP54 and can replace
the 7S RNA in an enzymatic assay. Other eubacteria and
archaebacteria have been shown to contain at least the SRP
RNA (Struck *et al.*, 1988).

The main question to be asked about SRP is whether its
functions are consistent with it being a molecular
chaperone. SRP appears to function exclusively during
preprotein synthesis. The established view (see Saier *et
al.*, 1989) is that SRP binds loosely to ribosomes: this
becomes a tight-binding complex following synthesis and
exposure on the ribosomal surface of the signal sequence
of the partially translated signal protein. Signal
recognition then gives rise to translational elongation
arrest followed by binding to the docking protein. The
signal sequence then becomes bound to the signal sequence
receptor and the SRP-ribosome complex dissociates (see
Saier *et al.*, 1989). In the absence of ribosomes no
specific binding of SRP to signal sequences has been
demonstrated (see Bernstein *et al.*, 1989). Thus it would
appear that recognition must depend to a large extent on
on the proper juxtaposition of the signal sequence binding
site of SRP54, and the nascent chain on the ribosome.

In view of these observations it is somewhat surprising
that free SRP has been shown to promote post-translational

translocation activity in yeast (Sanz and Meyer, 1988) and bacterial (Crooke *et al.*, 1988b) *in vitro* systems. However, Bernstein *et al.* (1989) suggest that since SRP is known to be hydrophobic, these experiments could be explained in terms of non-specific hydrophobic interactions retarding folding of the substrate protein, and are not necessarily related to actual functions *in vivo*. Overall the weight of evidence suggests that SRP acts primarily by modifying the rate of protein translation. There is no compelling evidence that SRP plays a role in protein folding *per se* and thus it should not be considered to be a molecular chaperone. However, this case does highlight the problem of improper interactions between proteins *in vitro* being interpreted as physiologically significant.

1.3.3. Pro-sequence of subtilisin

Subtilisin E, an alkaline serine protease consisting of a single polypeptide chain of 275 amino acids, is produced from a pre-pro-protein and is devoid of disulphide linkages. The 21 amino acid pre-sequence functions as a signal sequence for protein secretion. Pro-subtilisin denatured in 6M guanidine-HCl can be processed to the active enzyme with concomitant cleavage of the pro-sequence, when dialysed against a renaturation buffer (Ikemura and Inouye, 1988). On the other hand, deletion of the 77 amino acid pro-sequence yields mature but inactive

subtilisin (Ikemura *et al.*, 1987). An active site mutant of pro-subtilisin, which is not processed to active enzyme because intramolecular processing is prevented, was constructed (Ikemura *et al.*, 1987). When this was denatured and mixed with denatured mature enzyme and dialysed against renaturation buffer, as much as 20% of the activity of the mature enzyme could be recovered and the amount of activity recovered was proportional to the concentration of the mutant pro-subtilisin (Zhu *et al.*, 1989). These workers went on to demonstrate that the mutant pro-subtilisin will catalyse the correct folding, albeit somewhat less efficiently, of subtilisins from other sources (Zhu *et al.*, 1989). It has since been shown that a synthetic pro-sequence will also catalyse folding (Ohta *et al.*, 1991). Thus, the pro-sequence fulfils the role of a molecular chaperone and has the unusual advantage of being covalently linked to its substrate.

The evidence that pro-sequences can catalyse protein folding has been complemented by studies on other enzymes. For example Silen *et al.* (1989) have demonstrated that the 166 amino acid pro-sequence of α-lytic protease, an extracellular serine protease of *Lysobacter enzymogenes 495*, is required for the formation of the active protease. Independent expression of the pro- and protease domains has demonstrated that, like subtilisin, covalent linkage is not required for production of active protease (Silen and Agard, 1989).

Baker *et al.* (1992) have shown that omission of the α-lytic protease pro-sequence from *in vitro* folding reactions resulted in the formation of an inactive, but folding-competent state of the protease. Structural characterization of this state showed it to have properties intermediate between the native and denatured states. In particular this state contained nearly as much secondary structure as the native protein but little or no organized tertiary structure. These characteristics, as well as an expanded hydrodynamic radius, are hallmarks of the 'molten globule' state. Both the intermediate and the native states were stable under identical conditions.

The intermediate state may be the first example of a 'molten globule'-like conformation that is stable under the same conditions as the native state. Addition of the pro-region to the intermediate state resulted in its rapid conversion to the active native state. These observations strongly suggest that the folding of the α-lytic protease must be under kinetic and not thermodynamic control. Addition of the pro-region increased the rate of folding by over seven orders of magnitude and the free energy barrier for conversion of the intermediate to the native state in the absence of the pro-region was calculated to be in excess of 27 kcal mol^{-1}. Baker *et al.* (1992) argue that: "By lowering the height of a limiting energy barrier, the pro-region provides a means of access to new regions of conformational space." The most significant

aspect of these findings is that the energy barriers
separating minima in polypeptide chain conformational
space can exceed 27 kcal mol^{-1}. The presence of such
barriers on folding free energy surfaces of proteins
suggests that large regions of conformational space would
be kinetically inaccessible. This raises the possibility
that in such cases the native conformation might be at a
local and not a global free energy minimum.

1.3.4. Ubiquitin

Ubiquitin is a highly conserved, eukaryotic, 76-amino
acid polypeptide. Monomeric ubiquitin is produced from
hybrid proteins and can be conjugated to the ε-amino
nitrogen of lysyl residues in proteins (Hershko, 1988).
The covalent attachment of ubiquitin to various acceptor
proteins in eukaryotic cells participates in, and
regulates, a number of cellular processes. These include
selective protein degradation, DNA repair, progression
through the cell cycle and a variety of stress responses
(see Rechsteiner, 1987 for review).

A number of hybrid proteins are the initial source of
ubiquitin. Linear ubiquitin adducts are formed as the
translational products of natural gene fusions. For
example, in *S. cerevisiae* ubiquitin is generated
exclusively by proteolytic processing of precursors in
which ubiquitin is joined either to itself, as in the
polyubiquitin protein UBI4, or to unrelated tail

sequences, as in the hybrid proteins UBI1, UBI2 and UBI3.
In normally growing yeast cells most ubiquitin is
generated from the UBI1-UBI3 hybrid proteins, while
polyubiquitin, UBI4, becomes essential as the main
supplier of ubiquitin when cells are stressed (Finley *et
al.*, 1987).

The tail sequences of ubiquitin precursors have been
shown to be highly conserved between yeasts and mammals
(Ozkaynak *et al.*, 1987), suggesting that they function
similarly in all eukaryotes. In *S. cerevisiae* the tail
sequences of UBI1 and UBI2 have been shown to be identical
52-residue polypeptides, while UBI3 has an unrelated 76-
residue tail (Ozkaynak *et al.*, 1987). Finley *et al.* (1989)
were able to demonstrate that *ubi3* deletion mutants were
defective in the assembly of small, 40S, ribosomal
subunits and that *ubi1* and *ubi2* deletion mutants were
defective in large, 60S, ribosomal subunit assembly. All
of these mutants displayed a slow growth phenotype. The
UBI3 and UBI1 tails were then shown to be components of
the 40S and 60S ribosomal subunits respectively (Finley *et
al.*, 1989). Further experiments demonstrated that while
the ubiquitin-coding portion of the *ubi3* gene was not
necessary for ribosome biogenesis, its presence improved
the efficiency of incorporation of the tail sequence into
the nascent 40S ribosomal subunit (Finley *et al.*, 1989).
Finley *et al.* (1989) suggest that before assembly into
ribosomes the UBI1-UBI3 tails are likely to be short-lived

proteins and that the transient association with ubiquitin
could facilitate the transport of tails to, or assembly
within, the nascent ribosome, or prevent degradation of
nascent tails.

Fusions of ubiquitin to the N-termini of heterologous
proteins that are normally poorly expressed in *E. coli*,
have resulted in greatly enhanced yields of such proteins
(Butt *et al.*, 1989). This, and the observation that
expression of human steroid receptors in *E. coli* gives
rise to insoluble aggregates while ubiquitin fusions give
rise to soluble, biologically active proteins (Butt *et
al.*, 1989), lend support to the view that ubiquitin can
act as a molecular chaperone.

1.3.5. Trigger factor

Trigger factor is a peripheral ribosomal protein of M_r
63,000. Approximately one trigger factor molecule is found
bound to each 50S subunit of 70S ribosomes (Lill *et al.*,
1988). A series of *in vitro* experiments with purified
trigger factor indicated it was a molecular chaperone,
since it could interact with denatured proOmpA and
maintain it in a translocation competent form (Crooke *et
al.*, 1988a; 1988b; Lill *et al.*, 1988; Kusukawa *et al.*,
1989; Lecker *et al.*, 1989). However, *in vivo* studies in
which the trigger factor chromosomal gene was placed under
the control of the arabinose promoter, showed that neither
an abundance nor a deficiency of trigger factor had any

affect on the synthesis or export of proOmpA (Guthrie and Wickner, 1990). The same result was also obtained in a strain bearing a *secB* null allele (see next section; Guthrie and Wickner, 1990). These results reinforce the importance of genetic studies to establish *in vivo* protein functions. Unexpectedly, trigger factor depletion or abundance caused filamentation a phenotype also observed in GroEL and DnaK depleted cells (Guthrie and Wickner, 1990). Overproduction of trigger factor and FtsZ, an essential cell division protein, returned cells to normal cell division (Guthrie and Wickner, 1990). These observations led Guthrie and Wickner (1990) to speculate that trigger factor might be needed as a chaperone for proteins involved in cell division. Currently there is no evidence that this is the case and thus trigger factor cannot be classified as a molecular chaperone.

These observation are somewhat similar to those made with SRP (see Section 1.3.2.). In particular it should be noted that the dilution of a protein from denaturant into another protein solution probably represents a set of conditions in which improper interactions between proteins are most likely to take place. Without supporting data from *in vivo* studies the significance of such interactions should be treated with some scepticism.

1.3.6. SecB protein

The *secB* gene was first identified in mutants defective in protein transport (Kumamoto and Beckwith, 1983). SecB is a cytosolic, oligomeric *E. coli* protein. The native protein appears to be a homotetramer of molecular weight 64 kD (Watanabe and Blobel, 1989) and is composed of subunits of M_r 16,600 (Kumamoto and Nault, 1989).

Both *in vivo* and *in vitro* experiments have implicated SecB in the transport of a subset of *E. coli* envelope proteins. These include periplasmic proteins such as MalE (maltose binding protein) and PhoA (alkaline phosphatase), and several outer membrane proteins such as LamB, OmpA, OmpF and PhoE (Kusukawa *et al.*, 1989; Kumamoto, 1989; Lecker *et al.*, 1989). *In vivo* (Randall and Hardy, 1986) and *in vitro* (Eilers and Schatz, 1986) experiments have strongly suggested that the passage of a protein through the membrane occurs in a folded state that is distinct from native. Randall and Hardy (1986) have shown that an unfolded conformation of pre-MalE is a prerequisite for assembly, and that both the leader sequence and interactions with other proteins govern presecretory protein conformation.

The interaction of SecB with MalE has formed the basis for most studies of the role of SecB in protein export (see Kumamoto, 1991). *In vivo* experiments have demonstrated: a) the transient association of SecB with MalE (Kumamoto, 1989), b) that in the absence of SecB pre-

- 27 -

MalE folds into an export incompetent, protease resistant, form (Kumamoto and Gannon, 1988) and c) that various slow folding MalE mutants are less dependent on SecB for export (Collier *et al.*, 1988). Collier *et al.* (1988) demonstrated that a number of MalE export-incompetent deletion mutants interfered with normal MalE export, and that overexpression of SecB eliminated this interference.

A sequence of 35 amino acids located in the mature part of the MalE protein was common to all interfering mutants, this region would thus appear to interact with SecB (Collier *et al.*, 1988). *In vitro* synthesis in the presence of SecB demonstrated that several of these mutants formed more stable complexes with SecB than did the normal protein (Weiss and Bassford, 1990). In the same study, a MalE mutant protein lacking its signal sequence was shown not to form any detectable complex with SecB. This mutant folds into a protease-resistant conformation at a far faster rate than normal pre-MalE (Weiss *et al.*, 1989). A second mutation that slows the folding rate of the leaderless protein gave rise to a species that formed a detectable complex with SecB (Weiss and Bassford, 1990). Purified SecB has been shown to retard the folding of normal pre-MalE (Collier *et al.*, 1988; Weiss *et al.*, 1988; Liu *et al.*, 1989). The above observations indicated that alterations in the folding kinetics of a particular protein profoundly influences its interaction with SecB.

In a series of competition experiments, Hardy and

- 28 -

Randall (1991) were able to demonstrate that as the
quantity of denatured competitor was increased, the
ability of SecB to block the folding of MalE was
decreased. SecB had high affinity for all the nonnative
polypeptides tested. However, some binding sites were
clearly preferred, since the relative affinity of SecB for
the nonnative polypeptides tested varied 50-fold. Hardy
and Randall (1991) proposed that the selectivity in
binding of ligands by SecB *in vivo* is not only a function
of affinity, but in addition is modulated by a kinetic
partitioning between the pathways of folding and
association. Thus, for rapidly folding polypeptides the
partitioning would favour folding, while polypeptides that
fold slowly would tend to associate with SecB. The
interaction of SecB with a range of MalE folding mutants
shows just this kind of partitioning (see above). The
presence of a leader sequence, which has been shown to
retard folding of precursor polypeptides (Park *et al.*,
1988; Laminet and Pluckthun, 1989), would favour
association with SecB.

Finally, SecB not only modulates the rate of protein
folding; it also enhances delivery of precursors to the
membrane (Kumamoto and Gannon, 1988), probably through a
specific interaction with SecA (Hartl *et al.*, 1990). Thus
the SecB molecular chaperone functions by a) retarding
protein folding and b) targeting precursor proteins to the
export machinery.

1.3.7. PapD protein

The *pap* operon is composed of 11 genes required for the synthesis and assembly of pap, or P, pili, which mediate attachment of nephritic *E. coli* strains to digalactoside residues on the surface of cells lining the urinary tract (Norgren *et al.*, 1984; Hultgren *et al.*, 1989). These pili are assembled in the periplasmic space from components that have been translocated across the inner bacterial membrane. Each pilus is composed of approximately 1000 repeating subunits of PapA, which form the shaft, and a tip composed of at least three proteins, PapE, PapF and PapG. PapC and PapD are not incorporated into the pilus, but are required for its formation. PapC appears to form a channel in the outer bacterial membrane through which the growing pilus emerges (Norgren *et al.*, 1987), while several observations indicate that PapD is a molecular chaperone.

Indirect evidence suggests that PapD interacts with PapA, PapF, PapH and PapJ (Hultgren *et al.*, 1989; Lindberg *et al.*, 1989), while stable PapD-PapE (Lindberg *et al.*, 1989) and PapD-PapG (Hultgren *et al.*, 1989) complexes have been isolated from the periplasm. Insertional mutations within *papD* abolish pili formation even though pilin antigen was present in cell extracts (Norgren *et al.*, 1984). A study of the association of PapD with PapG has been particularly rewarding. Hultgren *et al.* (1989) have mapped the digalactoside binding properties of PapG (Lund

et al., 1987) to the N-terminal half of the protein, while
the PapD binding domain involves the C-terminal 13 amino
acids. PapD has been found to protect PapG from
proteolytic cleavage, enhance processing of the PapG
signal peptide and be required for incorporation of PapG
into the pilus (Hultgren *et al.*, 1989).

The crystal structure of PapD has been determined
(Holmgren and Brändén, 1989). PapD is built from two
globular domains, each consisting of an antiparallel
structure formed from packed β-sheets. A wide crevice
between between these domains contains an exposed
hydrophobic patch flanked by basic and acidic residues.
Holmgren and Brändén (1989) suggest that this region is
involved in binding the pili subunits.

All genetically well characterized pilus systems, with
the exception of type IV pili, in Gram-negative
prokaryotes contain a gene that is analogous to *papD* (see
Hultgren *et al.*, 1991).

1.3.8. BiP-hsp70

The BiP-hsp70 class is one of the best understood and
most important of the molecular chaperone classes.
Attention was first drawn to hsp70 (heat shock protein 70
kD) when an organism, in this case *Drosophila*, was
subjected to heat-shock and the subsequent pattern of
protein synthesis analysed (Ashburner and Bonner, 1979).
Since this initial identification hsp70 homologues have

been found in all cells so far examined, including yeast
(Ingolia *et al.*, 1982), humans (Mues *et al.*, 1986) and *E.
coli* (Bardwell and Craig, 1984). Hybridization studies
have indicated the presence of a homologue in the
archaebacterium *Methanosarcina barkeri* (Bardwell and
Craig, 1984). Eukaryotes typically contain several hsp70
sequences (Lindquist and Craig, 1988). In *E. coli*, and
possibly other prokaryotes, there is only a single hsp70-
related protein, the product of the *dnaK* gene (Bardwell
and Craig, 1984).

The hsp70 proteins are highly conserved, showing 60-78%
identity among eukaryotic homologues, and 40-60% identity
between DnaK and the eukaryotic proteins (see Lindquist,
1986). The remarkable conservation of these proteins
indicates that they must be involved in vital cellular
processes. Although hsp70 was first identified as a heat
shock protein, it is abundant in the cell under normal
growth conditions. In *E.coli* cultures grown at 37ºC, DnaK
accounts for 1.4% of cellular protein and is the seventh
most abundant protein in the cell (Herendeen *et al.*,
1979). Transcripts of one of the *Drosophila* hsp70
homologues have been shown to be very abundant in cells at
all stages of development (Craig *et al.*, 1983).

Elucidating the roles of the hsp70s in cells has been a
long and fascinating process. Pelham's proposal (1986)
that hsp70 interacts with damaged proteins, partially
unfolds them, and enables the released protein to refold,

- 32 -

had its basis in a number of observations.

Upon heat shock of mammalian cells many nuclear proteins become insoluble and hsp70s migrate to the nucleus, subsequently concentrating in the nucleolus where they apparently bind to partially assembled ribosomes (Welch and Suhan, 1986). Pelham (1984) had already shown that the presence of a plasmid that over-produces hsp70 accelerated the recovery of nucleoli from heat shock. Lewis and Pelham (1985) went on to demonstrate that the tight association of hsp70 to heat shocked nucleoli could be reversed, *in vitro*, by the addition of ATP but not by non-hydrolyzable analogues. Pelham (1986) argued that the energy of ATP hydrolysis caused a conformational change in hsp70 which effects both the release and a conformational change in the substrate protein. This change was thought to weaken the interaction of the substrate with other proteins and allow it to refold or reassemble into its pre-heat shock state. A postulate of this model is that hsp70 has a general affinity for denatured or abnormal proteins. Evidence that overloading of the protein degradation system by such proteins induced hsp synthesis (Munro and Pelham, 1985; Ananthan *et al.*, 1986), supported this hypothesis.

Independent support for the idea that hsp70 uses ATP to disrupt hydrophobic aggregates was forthcoming when it was found that the uncoating ATPase required to release clathrin from coated vesicles was an hsp70 homologue.

hsc70 (heat shock cognate 70) (Chappell et al., 1986;
Ungewickell, 1985). Coated vesicles, intermediates in the
pathway of receptor-mediated endocytosis, are covered in a
cage-like structure formed by the association of clathrin
trimers. hsc70 can bind to clathrin cages and, using the
energy of ATP hydrolysis, disassemble them by disruption
of the clathrin-clathrin interactions (see Rothman, 1989).

Another important hsp70 homologue, grp78, is located in
the lumen of the endoplasmic reticulum. This is one of a
group of glucose-regulated proteins originally detected in
chicken fibroblasts and extensively studied in mammalian
cells (see Pelham, 1986). These proteins are not normally
heat inducible, but are overproduced when fibroblasts are
starved of glucose. Glucose deprivation inhibits N-linked
glycosylation of nascent polypeptides in the endoplasmic
reticulum. Inhibitors of N-glycosylation cause swelling of
the ER and the accumulation of at least some
underglycosylated proteins as insoluble aggregates (see
Pelham, 1986). Presumably these aggregates are substrates
for the hsp70 homologue.

The abundance of grp78 in secretory cells suggests that
it may play a role in the normal assembly of secreted
proteins. The discovery that grp78 is in fact
immunoglobulin heavy chain binding protein, BiP, confirms
this view (Munro and Pelham, 1986). BiP transiently
associates with newly synthesized Ig heavy chains,
apparently binding to the hydrophobic region of the heavy

chain that is subsequently covered by light chain. BiP is released from the heavy chain by ATP hydrolysis (Munro and Pelham, 1986). Thus, it seems likely that the role of BiP is to prevent or reverse the formation of heavy chain aggregates and thus aid the process of immunoglobulin assembly.

Biochemical characterization of the hsp70s has shown that they all bind ATP with high affinity and possess a weak ATPase activity (see Lindquist and Craig, 1988). These activities have been shown to be associated with the N-terminal two-thirds of the proteins, a region that is more highly conserved than the carboxy-terminal regions of the hsp70s (Chappell et al., 1987). This suggests that the less highly conserved carboxy-terminal region may have evolved to interact with particular sets of proteins. Initial studies of the peptide binding properties of BiP and hsc70 revealed that they could interact with a number of peptides, and that peptide binding elicited hydrolysis of ATP with subsequent release of bound peptide (Flynn et al., 1989). Synthetic peptides, ranging in length from 8 to 30 residues, stimulated the ATPase activity of BiP with varying efficiency, and hsc70 had a peptide-dependent ATPase similar to that of Bip (Flynn et al., 1989). More recent studies have shown that the peptide binding site of BiP is filled by a stretch of only 7 residues (Flynn et al., 1991). The peptide binding site prefers to bind aliphatic side chains, typically found in the interior of

folded proteins, but will tolerate binding of polar and
charged residues. Flynn *et al.* (1991) calculated that a
potential binding site will occur every 16 residues in a
typical globular protein with 20% aliphatic content. Such
stretches would be sterically accessible when the
polypeptide chain is in an extended form, such as occurs
during translation and translocation.

Hsp70 is known to facilate the translocation of proteins
into *S. cerevisiae* mitochondria, endoplasmic reticulum
(Deshaies *et al.*, 1988) and microsomes (Chirico *et al.*,
1988). Recently a study of the import of several precursor
proteins into yeast mitochondria, demonstrated that all
precursors bound transiently to the mitochondrial hsp70
(Manning-Krieg *et al.*, 1991). Beckmann *et al.* (1990) have
reported that newly synthesized proteins associate,
cotranslationally, with two cytosolic forms of hsp70 found
in HeLa cells. The results of Flynn *et al.* (1991) have
considerably deepened the understanding of the mode of
action of hsp70 in these processes.

DnaK, the *E. coli* hsp70 homologue, has also been
implicated in a number of cellular processes including DNA
synthesis, RNA synthesis, cell division, proteolysis,
phosphorylation of other proteins, autoregulation of the
heat shock response (see Georgopolous *et al.*, 1990; Gross
et al., 1990) and export of certain fusion proteins
(Phillips and Silhavy, 1990). Perhaps the best
characterized role of DnaK is in λ repication. DnaK,

folded proteins, but will tolerate binding of polar and charged residues. Flynn *et al.* (1991) calculated that a potential binding site will occur every 16 residues in a typical globular protein with 20% aliphatic content. Such stretches would be sterically accessible when the polypeptide chain is in an extended form, such as occurs during translation and translocation.

Hsp70 is known to facilate the translocation of proteins into *S. cerevisiae* mitochondria, endoplasmic reticulum (Deshaies *et al.*, 1988) and microsomes (Chirico *et al.*, 1988). Recently a study of the import of several precursor proteins into yeast mitochondria, demonstrated that all precursors bound transiently to the mitochondrial hsp70 (Manning-Krieg *et al.*, 1991). Beckmann *et al.* (1990) have reported that newly synthesized proteins associate, cotranslationally, with two cytosolic forms of hsp70 found in HeLa cells. The results of Flynn *et al.* (1991) have considerably deepened the understanding of the mode of action of hsp70 in these processes.

DnaK, the *E. coli* hsp70 homologue, has also been implicated in a number of cellular processes including DNA synthesis, RNA synthesis, cell division, proteolysis, phosphorylation of other proteins, autoregulation of the heat shock response (see Georgopolous *et al.*, 1990; Gross *et al.*, 1990) and export of certain fusion proteins (Phillips and Silhavy, 1990). Perhaps the best characterized role of DnaK is in λ replication. DnaK,

together with with DnaJ and GrpE, functions in the multi-
protein pathway that results in the activation of the λ
origin of replication (see Echols, 1990). DnaK and DnaJ,
also a heat shock protein, have been shown to cause the
ATP-dependent release of the λ P-protein from the
preribosomal complex, allowing the DnaB helicase to unwind
DNA near *oriλ*, and thus leading to λ DNA replication
(Dodson *et al*., 1989; Liberek *et al*., 1988). Again an
hsp70 homologue is implicated in the disruption of
protein-protein interactions.

   In discussing the role of the hsp70s it has often been
stated that they are capable of repairing damaged
proteins. Direct *in vitro* evidence that DnaK can interact
with denatured proteins and promote their renaturation has
recently been presented by Skowyra *et al*. (1990) and
Gaitanaris *et al*. (1990) who have demonstrated that DnaK
can use the energy of ATP hydrolysis to reactivate
denatured RNA polymerase and λ repressor protein
respectively.

1.3.9. **The stress response**

   The biological heat shock response is a universal
phenomenon and its components are among the most highly
conserved genetic elements presently known, involving
recognizable homology across the boundaries of the
prokaryotic, eukaryotic and archaebacterial kingdoms
(Neidhardt *et al*., 1984). Indeed, when *Saccharomyces*

cerevisiae and *E. coli* are subjected to similar heat
treatments, the patterns of synthesis of individual
cellular proteins as visualized by two-dimensional gel
electrophoresis are almost indistinguishable (Miller *et
al.*, 1979; 1982).

This "heat shock" response is in fact induced by a wide
range of environmental stresses and is often, more
correctly, referred to as the stress response (Ananthan *et
al.*, 1986). Briefly the stress response involves a
transient increase in the expression of a small number of
genes with the consequent production of about 20 proteins
that are commonly referred to as the heat-shock proteins
(hsps) or the stress proteins. These proteins are present
under normal growth conditions, but in lesser amounts. In
both eukaryotes and prokaryotes the first signs of the
heat-shock response can be detected within one minute of
the temperature shift and the response reaches a peak
after 20-60 minutes depending on the organism and the type
of stress (Neidhardt *et al.*, 1984). Concomitant with the
induction of synthesis of the stress proteins, the
synthesis of most other proteins is repressed. Thus, heat
shock or stress leads to a major perturbation of normal
gene expression. The genetics of regulation of the heat
shock response have been reviewed several times (Neidhardt
et al., 1984; Gottesman, 1984; Lindquist, 1986; Lindquist
and Craig, 1988).

The diversity of the heat shock proteins is obvious. In

E. coli there are about 20 known heat shock proteins (Kamath-Loeb and Gross, 1991). These vary in size from 10-94 kD. In abundance they vary from >1% of the total cell protein at normal growth temperatures while others can only be detected after induction by heat shock. Representatives are also amongst the most acidic and most basic proteins found in *E. coli* (Neidhardt *et al.*, 1984). However, until recently very little indeed was known about the function of these proteins. While it had been widely assumed that these proteins somehow protected cells from the effects of stress, an understanding of the mechanisms by which such a function is achieved has only recently begun to emerge.

The hsp70 family of proteins appear to play a pivotal role in the regulation of the cellular response to environmental stress. The broad features of this regulation, summarized in Figure 1.3., are very similar in prokaryotes and eukaryotes. Here the prokaryotic response will be briefly described. In *E. coli*, heat shock promotors are recognised by a RNA polymerase holoenzyme containing a 32 kD sigma factor, σ^{32}, the product of the *htpR* gene. Following temperature upshift, a transient increase in the concentration of σ^{32} leads to a transient increase in hsp synthesis. Mutations in *dnaK* also lead to increased hsp expression, suggesting that the DnaK protein acts as a negative regulator of hsp synthesis (Tilly *et al.*, 1983; Straus *et al.*, 1990). Mutations in *dnaJ* and

Figure 1.3.   **A speculative model for the role of hsp70 in controlling expression of heat shock proteins**
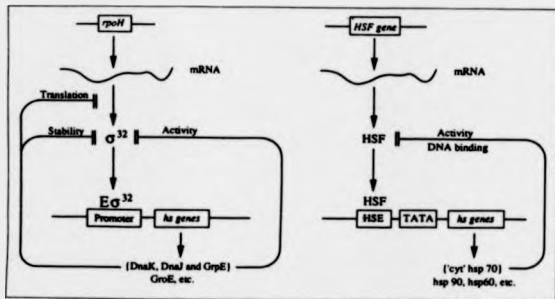
The model shows how the cytoplasmic hsp70 (DnaK), along with DnaJ and GrpE (and eukaryotic homologues), could act to control hsp expression in both prokaryotes and eukaryotes. Upon temperature upshifts, depletion of the free pool of hsps relieves their negative regulatory effects. Increased synthesis and stability of σ^{32} and increased activity of heat shock factor (HSF) permit increased transcription of the heat shock genes. HSE = heat shock element.

After Craig and Gross (1991).

grpE have the same phenotype as those in *dnaK*, suggesting
that all three gene products function together as negative
regulators of hsp expression (Straus *et al.*, 1990). A
eukaryotic homologue of DnaJ is already known to exist
(Sadler *et al.*, 1989), while hybridization studies have
revealed that a GrpE homologue is also probably present
(see Craig and Gross, 1991), indicating that the same sort
of regulatory triad may be functional in eukaryotic cells.
DnaK, DnaJ and GrpE are involved in every process known to
regulate σ^{32} (Craig and Gross, 1991) and have been shown
to negatively regulate σ^{32} in three ways: they are
required for translational regulation of σ^{32} synthesis at
high temperature, for inactivation of σ^{32} after shift to
low temperature and they facilitate degradation of σ^{32} at
all temperatures (Straus *et al.*, 1990; Tilly *et al.*,
1989).

Craig and Gross (1991) have suggested that hsp70 acts as
a cellular thermometer. In this model an increase in
temperature generates an increase in substrate, denatured
proteins, which in turn temporarily depletes the free pool
of hsp70, thus allowing σ^{32} to activate the transcription
of heat shock genes. This response would be self-limiting
because the ensuing overproduction of hsps would restore
the free pool of hsp70 and re-establish appropriate
regulation of σ^{32}. Conversely, a temperature downshift
would be the functional equivalent of overexpressing
hsp70, further repressing the action of σ^{32} and causing a

- 41 -

transient decrease in hsp synthesis. While this model fits
in with what is known of the regulation of the heat shock
response, the mechanism by which the putative hsp70
"thermometer" transduces the signal to increase the
concentration of σ^{32} is unknown. Craig and Gross (1991)
speculate that DnaK may be bound directly to σ^{32} allowing
the latter to be rapidly degraded by an as yet
unidentified proteolytic system. No doubt the true
mechanism of heat shock regulation will be elucidated in
the near future.

1.4. The chaperonins

The first chaperonins to be studied in any detail were
those isolated from *E. coli*. These were initially
identified in studies of host cell mutations that
interfered with the assembly of certain bacteriophage
(Takano and Kakefuda, 1972; Georgopoulos *et al.*, 1972).
These host genes were referred to as *mop* (morphogenesis of
phage) (Takano and Kakefuda, 1972) or *groE* (Georgopoulos
et al., 1972). The *groE* locus, as it is now called, was
shown to consist of two closely linked genes, *groEL* and
groES, encoding polypeptides of M_r 65,000 and 15,000
respectively (Tilly *et al.*, 1981). Since the coining of
the term chaperonin to describe the class of molecular
chaperone that includes the GroE proteins (Hemmingsen *et
al.*, 1988), it has become fashionable to refer to GroEL
and its homologues as cpn60, chaperonin 60 kD, while GroES

and its homologues are referred to as cpn10, chaperonin 10
kD (Goloubinoff *et al.*, 1989a). In the discussion of
individual experiments this terminology can be confusing.
For this reason I have tended to use the more traditional
notation, eg GroEL, when discussing the features of
specific chaperonins. Table 1.2. lists some properties of
the chaperonins.

Table 1.2. **Some properties of the chaperonins**

Origin	Alternative names	Subunit M$_r$	Oligomer
Bacteria	GroEL	57,259 (*E. coli*)	14-mer
	65 kD antigen	56,686 (*M. leprae*)	
	GroES	10,368 (*E. coli*)	6-8-mer
Mitochondria	hsp60	60,830 (yeast)	14-mer
	mitonin		
	HuCha60	57,939 (human)	
Plastid	Rubisco subunit binding protein		14-mer
	α subunit	57,393 (wheat)	
	β subunit	56,453 (rape)	

After Ellis, 1990.

1.4.1. Chaperonin structure

A large number of cpn60 sequences have now been
determined. However, to date relatively few have been
examined in the electron microscope and no X-ray
crystallographic analyses have been published.

Electron microscopic analyses have revealed that all the
native cpn60 proteins so far studied, are composed of
fourteen subunits organised in two rings of seven subunits

each, stacked one on top of the other. The seven-fold
rotational symmetry of these proteins is a rarity in
biological systems. Other proteins exhibiting this kind of
symmetry include the pyridine nucleotide transhydrogenase
of *Pseudomonas aeruginosa* (Louie *et al.*, 1972; Wermuth and
Kaplan, 1976) and multicatalytic proteases or prosomes
(Dahlmann *et al.*, 1989). Carazo *et al.* (1991) have
published evidence that the cpn60 proteins from both *E.
coli* and *Bacillus subtilis* show predominantly six-fold
symmetry when cultures are grown below 37°C, and
predominant seven-fold symmetry after a heat shock. Until
these observations are independently confirmed they need
to be treated with some scepticism, since no other studies
on the cpn60s have demonstrated the presence of a native
structure with sixfold symmetry.

In contrast to cpn60 very few cpn10 proteins have been
isolated and only one, from *E. coli*, has been studied in
the electron microscope (Chandrasekar *et al.*, 1986). These
studies indicated that the native cpn10 structure is
composed of 6-8 subunits arranged in a single ring. Given
the well known seven-fold symmetry of the cpn60s, there
has been a tendency to assume that the cpn10 will also
have seven-fold symmetry (see Gatenby and Ellis, 1990).
While it seems likely that this will turn out to be the
case, a definitive proof is still awaited.

There is ample evidence, both genetic and biochemical,
that the GroEL and GroEB proteins of *E. coli* are

functionally interactive (see below). GroEL and GroES cosediment in glycerol gradients in the presence of MgATP (Chandrasekhar *et al.* 1986). Recently Saibil *et al.* (1991) produced electron micrographic evidence showing that cpn10 binds to one face of the cpn60 protein, giving a domed appearance to one side of the molecule. Saibil (personal communication) has pointed out that the dimensions of the cpn10/cpn60 are somewhat larger than would be expected from the sum of the components. She has suggested that interaction of the two proteins causes some form of relaxation of the cpn60 structure.

1.4.2. Chaperonin function

The GroEL protein has been shown to possess a weak ATPase activity (Hendrix, 1979; Viitanen *et al.*, 1990; Gray and Fersht, 1991; Terlesky and Tabita, 1991) which is partially inhibited in the presence of GroES (Chandrasekar *et al.*, 1986; Viitanen *et al.*, 1990). Estimations of both the ATPase activity of GroEL and the degree of inhibition by GroES vary greatly between different groups. ATPase k_{cat} values of 4.4 s^{-1} at 25°C (Gray and Fersht, 1991), 0.8 s^{-1} at 37°C (Hendrix, 1979), 0.75 s^{-1} at 37°C (Terlesky and Tabita, 1991) and 0.1 s^{-1} at 22°C (Viitanen *et al.*, 1990) have been reported. Inhibition of this activity by GroES has been reported to be >95% (Viitanen *et al.*, 1990), 61% (Gray and Fersht, 1991) and 60% (Chandrasekhar *et al.*, 1986). Different conditions may to

some extent explain the variation in these results. However, inhibition by GroES may have important mechanistic implications and the differences reported above need to be resolved.

Despite its weak ATPase activity the GroEL protein binds ATP avidly, indicating that it might possess more than one binding site (see Georgopoulos and Ang, 1990). Gray and Fersht (1991) have calculated that there are at least three ATP binding sites on the GroEL tetradecamer. For reasons of symmetry, Gray and Fersht (1991) assume that the actual number of sites is either 7 or 14. The weak ATPase activity of GroEL is also possessed by all the cpn60s so far examined and GroES has been shown to interact with cpn60s from a variety of sources (see Goloubinoff *et al.*, 1989a; Terlesky and Tabita, 1991).

Since Hemmingsen *et al.* (1988) first proposed the existence of a chaperonin class of molecular chaperones, these proteins have been implicated in a wide variety of cellular functions, most of which appear to involve protein folding. The current view is that GroES couples K^+-dependent hydrolysis of ATP to the release of folded target protein from GroEL (Viitanen *et al.*, 1990).

1.4.2.1. Role in Rubisco assembly

Rubisco is the single most abundant protein in the biosphere (Ellis, 1979). This enzyme catalyses the initial reaction of the Calvin cycle, the photosynthetic fixation

of carbon dioxide, and is thus responsible for bringing
into organic combination virtually all the carbon atoms
found in living organisms. In addition to this activity
Rubisco also catalyses an oxygenase reaction, the first
step in photorespiration (see Miziorko and Lorimer, 1983).
Rubisco is a poor catalyst, having both a low affinity for
carbon dioxide and a small turnover number (see Gatenby
and Ellis, 1990). The major importance of this enzyme
allied with its severe catalytic limitations would appear
to make it an ideal target for study and improvement by
genetic manipulation.

The Rubisco isolated from plant chloroplasts and most
prokaryotic organisms that contain a Calvin cycle, is
composed of eight large subunits (M_r 50,000-55,000) and
eight small subunits (M_r 12,000-18,000) (see Gatenby and
Ellis, 1990). A simpler form of Rubisco composed
exclusively of large subunits is found in some
prokaryotes. The most extensively studied enzyme of this
kind is the homodimeric protein isolated from the purple,
non-sulphur, photosynthetic bacterium, *Rhodospirillum
rubrum*. The three-dimensional structure of this enzyme has
been determined at 2.9 Å by X-ray crystallography
(Schneider *et al.*, 1986). This work demonstrated that each
of the two active sites on the dimer have residues
contributed by both subunits. Thus, the monomeric protein
is held to be enzymatically inactive and the appearance of
enzymatic activity can be related to the successful

folding and assembly of the large subunits. This property
has been exploited to study the role of chaperonins in
Rubisco assembly (see below).

The first Rubisco gene to be successfully expressed and
assembled in *E. coli* was of the simple homodimeric type
from *R. rubrum* (Somerville and Somerville, 1984). Since
then, the more complex hexadecameric form from several
prokaryotes has also been expressed and given rise to
active enzyme in *E. coli* (see Gatenby and Ellis, 1990).
However, all attempts to express enzymatically active
Rubisco from crop plants in *E. coli* have met with failure
(Gatenby and Ellis, 1990). The discovery of the subunit-
binding protein (Barraclough and Ellis, 1980) provided the
first clues that another protein may be required as an
intermediate in the assembly process. This view was
supported by the observation that the assembly of *in vitro*
synthesized large subunits into the Rubisco holoenzyme,
was inhibited by the addition of antiserum raised against
the binding protein (Cannon *et al.*, 1986). The discovery
that the *E. coli* GroEL protein was a homologue of the
subunit binding protein (Hemmingsen *et al.*, 1988)
stimulated speculation on whether the correct assembly of
prokaryotic Rubisco in *E. coli* required the host
chaperonin.

A series of elegant experiments have been conducted to
test the validity of this hypothesis. Firstly, Goloubinoff
et al. (1989b) studied the expression and activity of

- 48 -

prokaryotic Rubisco from *Anacystis nidulans* (L$_8$S$_8$) and *R.*
rubrum in *E. coli*, in the presence and absence of a
multicopy plasmid containing the *GroE* operon. Increased
levels of the GroE proteins were shown to cause an
increase in Rubisco activity, even though expression of
Rubisco was unaltered. Induction of the heat shock
response was also shown to increase Rubisco activity.
Since the L$_8$ core of *A. nidulans* Rubisco is known to be
composed of four *R. rubrum*-like dimers (Schneider *et al.*,
1986; Chapman *et al.*, 1988), it was inferred that, at the
very least, the GroE proteins act at the stage of dimer
formation (Goloubinoff *et al.*, 1989b). Figure 1.4. shows
putative assembly pathway of the Rubisco hexadecamer as
suggested by Goloubinoff *et al.* (1989b).

In vitro studies on the chaperonin-dependent assembly of
R. rubrum Rubisco have lent further support to this model
Goloubinoff *et al.*, 1989a). *R. rubrum* Rubisco was
denatured with either 8 M urea, 6 M guanidine-HCl or by
treatment with acid (pH 3). Attempts to spontaneously
reconstitute activity under various conditions met with
failure. However, when the denatured enzyme was diluted
into a solution containing the GroE proteins and Mg-ATP,
Rubisco activity was recovered. Maximum reconstitution,
observed when GroES and GroEL were present in equimolar
concentrations, was approximately 80% for the guanidine-
HCl denatured material, while only about 40% of the
activity of the acid denatured material was recovered. The

- 49 -

Figure 1.4. **Proposed assembly pathway of Rubisco hexadecamer**
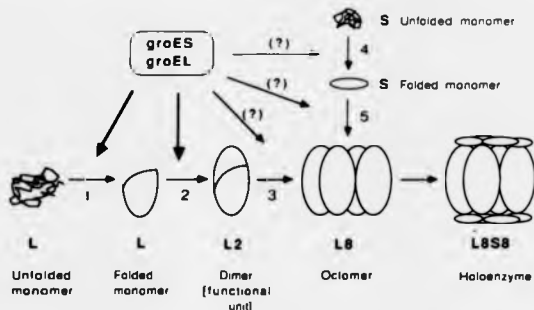
This model recognises that the L_2 dimer is the basic structural motif. Thus a minimum of five steps must be invoked to convert nascent, unfolded large and small subunits into the hexadecameric L_8S_8 holoenzyme: (1) folding of large subunit monomer; (2) formation of the L_2 dimer; (3) tetramerisation of L_2 dimers to give the L_8 core; (4) folding of small subunit monomer and (5) association of small subunits with the L_8 core. The GroE proteins could potentially be involved at each step, although evidence suggests that step 5 can occur spontaneously without their involvement (Andrews and Ballment, 1983). The results of Goloubinoff *et al.* (1989b) show that, at the very least, the GroE proteins are involved in dimer formation (steps 1 and/or 2). However, the possibility that the GroE proteins are additionally involved in steps 3 and 4 cannot be excluded.

After Goloubinoff *et al.* (1989b).

rate constant for the chaperonin-dependent reconstitution of activity was the same, regardless of the manner of protein denaturation, indicating that the reconstitution involved a common intermediate referred to as Rubisco-1. If either of the GroE proteins or Mg-ATP was omitted, reconstitution did not occur. Other nucleotide triphosphates, with the exception of CTP, and non-hydrolyzable analogues of ATP did not support reconstitution. Combinations of the yeast mitochondrial and pea plastid cpn60s with the *E. coli* cpn10 were shown to support reconstitution but were, respectively only 10% and 25% as effective as the homologous GroE system (Goloubinoff *et al.*, 1989a).

Omission of GroES or Mg-ATP or both from a reconstitution reaction gave rise to a stable Rubisco-1-GroEL binary complex (Goloubinoff *et al.*, 1989a). Although addition of Mg-ATP to this complex caused dissociation to a lower molecular weight form, no Rubisco activity was recovered. However, when both Mg-ATP and GroES were added the binary complex was discharged with the appearance of native dimer and Rubisco activity.

Experiments in which Rubisco was diluted from denaturant and GroEL added at various times after dilution, showed that the later the addition, the less Rubisco activity was recovered after subsequent addition of Mg-ATP and GroES. The rate at which recoverable activity was lost increased as the Rubisco concentration increased (Goloubinoff *et*

al., 1989a). The authors concluded that when denatured
Rubisco is diluted into solutions containing the
chaperonins, two mutually exclusive processes occur;
aggregation or the formation of a binary complex with
GroEL. This is an important observation, since it
demonstrates that the chaperonins cannot reassemble
aggregated protein, but will bind to and stabilize an
unstable folding intermediate of the substrate protein.

Further work by this group demonstrated that K^+ is
required for both the uncoupled ATPase activity of the
GroEL protein and for the coupled release of active
Rubisco (Viitanen *et al*, 1990). These researchers also
demonstrated that *R. rubrum* Rubisco will fold
spontaneously at 15°C and that the rate of reconstitution
at this temperature was increased tenfold in the presence
of chaperonins, K^+ and MgATP. The fact that Rubisco can
fold spontaneously indicates that the chaperonins
intervene at a kinetic rather than a thermodynamic level
(Viitanen *et al*., 1990).

These papers have clearly identified the components of
the chaperonin system required to mediate Rubisco
assembly. This knowledge has been exploited in the search
for an organellar cpn10 homologue. Lubben *et al*. (1990)
reasoned that, since Rubisco-1 complexed to either plastid
or mitochondrial cpn60 could be discharged by GroES to
give active Rubisco (Goloubinoff *et al*., 1989a), a) there
must be an organellar cpn10 homologue and b) such a cpn10

homologue should be able to replace GroES in a Rubisco
reconstitution system. Using this approach Lubben *et al.*
(1990) have presented evidence of the existence of a cpn10
homologue in beef and rat liver mitochondria. The putative
beef liver mitochondrial cpn10 was shown to assist in the
discharge of Rubisco from GroEL, in a process requiring
MgATP and K$^+$, to form a MgATP-dependent complex with GroEL
and to inhibit the uncoupled ATPase activity of GroEL. The
most likely candidate for the mitochondrial cpn10
homologue was an oligomeric protein of approximately 45
kD, somewhat smaller than the GroES oligomer, that
consisted of 9 kD subunits. The latter appeared to be
immunologically unrelated to the GroES protein. Thus the
authors of this work do not rule out the possibility that
they have identified a functional homologue that is
evolutionarily unrelated to GroES (Lubben *et al.*, 1990).

1.4.2.2. **Role in phage morphogenesis**

The *groE* genes of *E. coli* were originally identified
because some mutations in them block the assembly of
various bacteriophage (Takano and Kakefuda, 1972;
Georgopoulos *et al.*, 1972). Table 1.3. summarizes the groE
requirement for various bacteriophage.

The data in this table outline the role played by the
GroE proteins in bacteriophage assembly. Two of the
bacteriophages, λ and T4, require GroE proteins primarily
at the stage of head assembly, whereas T5 and 186 exhibit

a requirement primarily at the stage of tail assembly. Of
particular interest is the observation that all these
groE-imposed assembly defects can be overcome by mutations
in bacteriophage gene(s), indicating that the GroE
proteins interact directly with these gene products.

Table 1.3. **Effect of *E. coli groE* mutations on bacteriophage**
growth

		groES⁻	*groEL⁻*
λ		Head assembly Genes *B* and *E*	Head assembly genes *B* and *E*
T4		No effect	Head assembly gene *31* and *23*
T5		Tail assembly gene *D19*	Tail assembly gene *D19*
186		Tail assembly gene *H*	Tail assembly gene *H*

This table shows the level at which assembly
is blocked by *groE* mutations and the
bacteriophage genes in which mutations can
overcome this block. Based on Georgopolous and
Ang (1990).

Finally it should be noted that T4 is unique in that the
GroES protein does not appear to play a role in its
assembly. It has been suggested that gp31, a monomeric M_r
12,060 protein encoded by gene *31* of T4, may be a
functional homologue of GroES (Nivinskas and Black, 1988).
There is no evidence for any structural homology between

gp31 and GroES, but it is known that T4 requires gp31 much
more stringently at higher temperatures (Shalniene and
Nivinskas, 1987), and that this requirement is alleviated
by bacterial growth at high temperature prior to
infection. This, along with genetic evidence that gp31 and
GroEL interact has led to speculation that gp31 has a
specialized GroES-like function in modulating the release
of gp23, the major capsid protein of T4, from GroEL
(Georgopolous and Ang, 1990).

1.4.2.3. Role in mitochondrial import

The *S. cerevisiae* mitochondrial cpn60 is one of only
five mitochondrial proteins, all of which are key
components of the protein import system, that are known to
be essential for viability of this yeast (Baker and
Schatz, 1991). Cheng *et al.* (1989) have demonstrated that
the mitochondrial cpn60 protein is required for the
assembly of a number of proteins that are imported into
the mitochondrial matrix. These workers described a
temperature-sensitive nuclear mutation, *mif4*, in which
subunits of mitochondrial enzymes such as subunit β of F_1-
ATPase were completely translocated into the organelle,
were processed to the mature sized forms, but failed to
assemble into active structures (Cheng *et al.*, 1989). The
mif4 mutation was shown to be in the the hsp60 gene and
cells grown at the non-permissive temperature did not form
a normal tetradecameric hsp60 complex (Cheng *et al.*,

1989).

These observations were confirmed and expanded in import experiments using isolated mitochondria from *Neurospora crassa* (Ostermann *et al*., 1989). These studies made use of the fact that dihydrofolate reductase (DHFR), a cytosolic enzyme, retains its active conformation and folds into a highly protease-resistant unit when fused to the presequence of a mitochondrial precursor (Ostermann *et al*., 1989). When the denatured hybrid DHFR protein was diluted into import incubations at 25°C, about 90% of the protein was translocated into mitochondria within 45 seconds. However, only 30% of the imported protein was in a protease-resistant conformation. This figure rose to 70% after 3 minutes (Ostermann *et al*., 1989). When mitochondria were ATP depleted, less than 10% of the imported protein had folded into a protease-resistant form after 6 minutes incubation. In total matrix extracts the protease-sensitive DHFR was physically associated with the hsp60, protein and addition of ATP resulted in the release of the protease-resistant form (Ostermann *et al*., 1989). However, when the DHFR-hsp60 complex was partially purified, subsequent addition of ATP did give rise to a protease resistant form of DHFR, but this was still associated with hsp60. This result indicated that; a) an additional factor, possibly a cpn10 homologue, is required for the complete reaction sequence to take place, and b) some folding takes place at the surface of the hsp60

complex (Ostermann *et al.*, 1989). Ostermann *et al.* (1989) also demonstrated that mitochondrial enzymes such as the β subunit of F$_1$ ATPase and the Rieske Fe/S protein of complex III are associated with hsp60 after import.

1.4.2.4. Interactions with other substrate proteins

Ample genetic and biochemical evidence suggests that the cpn60s interact with a startlingly broad range of substrate proteins. The GroE proteins have been shown to be essential for *E. coli* viability (Fayet *et al.*, 1989). At non-permissive temperatures, temperature-sensitive *groE* mutations have been found to result in a reduction in the rates of synthesis of both DNA and RNA (Wada and Itikawa, 1984), to block cell division with the corresponding formation of aseptate filaments (Miki *et al.*, 1988), and to cause an overall reduction in generalized protease activity in *groEL* but not *groES* mutant bacteria (Straus *et al.*, 1987).

Overexpression of the GroE proteins has been shown to suppress mutations in the *dnaA* gene (Jenkins *et al.*, 1986; Fayet *et al.*, 1986), and to suppress a number of auxotrophic mutations (Van Dijk *et al.*, 1989). The latter observations are particularly interesting, since it was demonstrated that the GroE proteins could supress many temperature-sensitive mutations in the *ilv* and *his* operons of *Salmonella typhimurium*, while suppression of cold-sensitive and temperature-independent mutations (only

studied in the *his* operon) was not observed. Many, but not all, temperature-sensitive mutations in *hisD*, *hisC* and *hisB* could be suppressed by overexpression of the GroE proteins, while two temperature-sensitive alleles of *hisA*, encoding the only monomeric enzyme of the *his* operon, were not suppressed (Van Dijk *et al.*, 1989). These results would seem to indicate that the major role of GroEL *in vivo* is to modulate the assembly of multimeric enzymes. Recent *in vivo* studies of *nif* gene regulation and nitrogenase assembly in *E. coli* have indicated that GroEL is involved in both these processes (Govezensky *et al.*, 1991). *In vitro* studies of the mode of action of GroEL have exploited its interaction with a number of proteins. Substrates include Rubisco (see above), DHFR (see above), rhodanese (Martin *et al.*, 1991), citrate synthase (Buchner *et al.*, 1991) and lactate dehydrogenase (Badcoe *et al*, 1991).

Chaperonins have also been shown to play an important role in protein transport. Bochkareva *et al.* (1988), using a photocross-linking approach in a cell-free translation system, demonstrated that newly synthesized pre-β-lactamase and chloramphenicol acetyltransferase would form a complex with GroEL. In the presence of membranes the cross-linking was sharply lowered for the secreted protein, pre-β-lactamase, but not for the cytosolic protein, chloramphenicol acetyltransferase. These workers went on to demonstrate that the addition of partially

unfolded or denatured proteins to the reaction mixture
significantly lowered the GroEL/pre-β-lactamase cross-
linking efficiency. When the isolated GroEL/pre-β-
lactamase complex was added to a membrane preparation,
pre-β-lactamase translocated in the presence of ATP, but
not in the presence of a non-hydrolyzable analogue
(Bochkareva et al., 1988).

These in vitro observations have been backed up by in
vivo studies in which it was demonstrated that the export
of β-lactamase is less efficient in groE mutants than in
the wild type (Kusukawa et al., 1989). Overexpression of
the GroE proteins has also been shown to improve the
export of a LamB-LacZ fusion protein (Phillips and
Silhavy, 1990). Kusukawa et al. (1989) also studied the
effect of groE mutants on the export of several other
proteins. The export of MalE (maltose-binding protein),
OmpA, OmpF (outer membrane proteins), normally SecB-
dependent, Lpp (lipoprotein), and PhoA (alkaline
phosphatase), SecB-independent, was not impaired in groE
mutants. Thus, SecB and GroEL appear to have different
substrate specificities.

Finally, do chaperones play a role in the assembly of
chaperonins? In vitro studies conducted by Lissin et al.
(1990) indicated that renaturation of GroEL monomers in
the presence of Mg-ATP caused the assembly of a few GroEL
multimers. These then interacted with monomers in an
autocatalytic manner to promote the assembly of more

multimers. On the other hand, Cheng *et al.* (1990), having studied the import of wild type hsp60 into yeast mitochondria isolated from a *mif4*, hsp60 defective, mutant grown at the non-permissive temperature, concluded that folding and assembly of hsp60 cannot occur when there is no pre-existing functional chaperonin. Thus it would appear that chaperonins do promote the assembly of chaperonins, but the biogenesis of the initial chaperonin pool is still unclear. Manning-Krieg *et al.* (1991) have demonstrated that hsp60 transiently associates with hsp70 after import into the yeast mitochondrion. Chemically synthesized GroES protein has been shown to form biologically active oligomeric structures spontaneously (Mascagni *et al.*, 1991). This suggests that no other chaperones are required for GroES assembly.

1.4.3. Mechanistic studies

How the cpn60s recognise unfolded proteins and subsequently modulate their folding is a field of research dominated by much speculation with little, often conflicting, supporting evidence. Goloubinoff *et al.* (1989a) first suggested that GroEL binds to partly folded protein that could correspond to a molten globule state. Studies on the GroEL-mediated refolding of DHFR and rhodanese have also indicated that GroEL seems to stabilise these proteins in a molten globule state (Martin *et al.*, 1991). DHFR is a globular, single domain protein

- 60 -

of 189 amino acids that will refold spontaneously when
diluted from denaturant, while rhodanese is composed of
293 amino acids that form two equal sized domains which
are stabilized by hydrophobic interactions (Martin *et al.*,
1991). Folding reactions of these proteins in the presence
of the GroEL proteins was monitored by measuring intrinsic
tryptophan flourescence, adsorption of a flourescent dye,
protease sensitivity and enzyme activity (Martin *et al.*,
1991). In the absence of ATP both enzymes formed a stable
complex with GroEL. The properties of both bound proteins
were very similar and appeared to correspond to a molten
globule state. Addition of Mg-ATP to the DHFR-GroEL
complex caused the DHFR to fold to a more compact state
that was still associated with GroEL, followed by release
of the active protein (Martin *et al.*, 1991). This process
was retarded in the presence of increased concentrations
of GroEL, indicating that DHFR can re-bind to GroEL before
its folding is complete.

Viitanen *et al.* (1991) have recently reported that
folding and release of DHFR from GroEL will also take
place, albeit more slowly, when non-hydrolysable ATP
analogues were used. When both Mg-ATP and GroES were
present in the reaction mixture, reactivation of the
enzyme occurred much more quickly and no re-binding took
place (Martin *et al.*, 1991). In contrast, folding and
release of rhodanese from the rhodanese-GroEL complex was,
like refolding of Rubisco (Goloubinoff *et al.*, 1989a),

GroES and Mg-ATP dependent; no effect was observed with Mg-ATP alone. Rhodanese was also shown to fold to a more compact state before release from GroEL (Martin *et al.*, 1991). In both cases GroES appeared to couple ATP hydrolysis with folding at GroEL. The amount of ATP hydrolysed by GroEL per rhodanese molecule folded was estimated to be 5-10% of the total required for synthesis (Martin *et al.*, 1991).

Landry and Gierasch (1991) have studied the binding of an N-terminal sequence of rhodanese to GroEL. They proposed that GroEL binds to sequences with the potential to adopt an amphipathic α-helical conformation and that the chaperonin promotes formation of a helix. This interaction could explain the interaction of GroEL with such a wide variety of proteins, since most soluble enzymes and many integral membrane proteins contain internal amphipathic α-helices (see Saier *et al.*, 1989). However, the observation that a single-chain antibody containing no α-helices was able to compete with β-lactamase for binding to GroEL, probably means that substrate recognition is not limited to α-helices (Zahn and Plückthun, 1992).

Separate studies of the GroEL-mediated folding of citrate synthase (Buchner *et al.*, 1991) and lactate dehydrogenase (LDH) (Badcoe *et al.*, 1991) indicated that, like DHFR, there is no absolute requirement for GroES. Badcoe *et al.* (1991) found no evidence to suggest that

GroEL binds the molten globule intermediate of the LDH
folding pathway, and presented evidence that GroEL binds
most tightly to the unfolded protein. In the presence of
Mg^{2+}, both ATP and a nonhydrolyzable ATP homologue were
found to have a near identical effect on the LDH-GroEL
complex, while ADP had no effect. Omission of nucleotide
altogether was observed to increase the yield of recovered
enzyme activity 2.5 fold (Badcoe *et al.*, 1991). These
observations led them to the conclusion that GroEL does
not catalyse folding, but arrests the process. In this
model GroEL acts as a sort of "molecular" sponge that
retains unfolded protein, thus reducing the concentration
of free unfolded protein, and the possibility of incorrect
interactions, and so increasing the yield of active
protein.

1.4.4.   Is there a cytosolic chaperonin?

To date all of the known cpn60s are of either organellar
or bacterial origin. Given the fact that the cpn60s are so
highly conserved and play such an important role in
protein assembly, it is somewhat surprising that no
eukaryotic cytosolic homolgue has been found. One
immunogold-labelling study has indicated that a GroEL
homologue is present in the cytosol of oat leaves, but no
protein was isolated (Grimm *et al.*, 1991). A sequence
similarity search picked out several proteins with some
similarity to the chaperonins (van der Vies, 1989). The

- 63 -

highest similarity is shown by a protein called *t* complex polypeptide 1 (TCP-1). TCP-1 is the product of a gene carried in the *t* locus on chromosome 17 of mice. TCP-1 is an abundant 63 kD protein (Silver and White, 1982), occurring in the cytosol of all cells of mice (Willison *et al.*, 1989). Homologues have been detected yeast and *Drosophila* (Ursic and Ganetzky, 1988). This widespread distribution, cytosolic location and similarity to the chaperonins indicate that this protein might be the missing cytosolic chaperonin. Recent structural and functional studies support the view that TCP-1 is a cytosolic chaperonin (see 5.6.).

1.5. Sequential and overlapping functions of molecular chaperones

Studies of protein import into mitochondria have proved to be critical in revealing the sequential action of certain molecular chaperones. Several studies have demonstrated that newly translocated polypeptides first interact, before translocation is complete, with mitochondrial hsp70 (mhsp70) (Kang *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991). In addition to this requirement, several proteins are also known to interact with mitochondrial hsp60 (mhsp60) before they will fold correctly (Cheng *et al.*, 1989; Ostermann *et al.*, 1989; see 1.4.2.3.). The obvious implication of these results is that these two molecular chaperones act

sequentially. Evidence that this view is correct was
provided by Manning-Krieg *et al.* (1991) who demonstrated
that an imported mitochondrial processing protease (Mas2p)
first bound to mhsp70, then to mhsp60, and only then
assembled with its partner subunit, Mas1p. On the other
hand, imported DHFR was shown to interact with mhsp70, but
no significant association with mhsp60 could be detected
(Manning-Krieg *et al.*, 1991). Thus it seems likely that
some proteins require mhsp60 for post-import folding,
while others can fold independently after discharge from
mhsp70.

Recently, Landry *et al.* (1992) demonstrated that a
specific tridecapeptide is bound by DnaK in an extended
conformation, but is helical when bound to GroEL. The
physiological significance of these observations has been
elegantly demonstrated (Langer *et al.*, 1992). Langer *et
al.* (1992) demonstrated that when rhodanese was diluted
from denaturant, DnaK would retard aggregation only when
present at a high, >20-fold, molar excess, while
surprisingly, DnaJ completely prevented aggregation at a
5-fold molar excess. They then showed that DnaK and DnaJ
acted in a cooperative manner to prevent aggregation of
rhodanese (DnaJ:DnaK:rhodanese 2:5:1), apparently trapping
rhodanese in a nonnative but not fully unfolded state.
Neither the addition of MgATP alone, nor addition of MgATP
followed by GroEL/ES to this mixture, produced active
enzyme. However, when GrpE was included there was rapid

and efficient folding to the active enzyme. Thus GrpE
couples the transfer of rhodanese to GroEL. Using this
system, Langer *et al.* (1992) were able to show that
DnaK/DnaJ allows GroEL to carry out multiple cycles of
rhodanese folding.

The above results clearly show that certain molecular
chaperones act sequentially. What evidence is there that
various classes of molecular chaperone functionally
overlap? *In vitro* studies have indicated that both SecB
and GroEL will stabilize various proteins for
translocation (Lecker *et al.*, 1989). *In vivo* studies have
shown that induction of the heat shock response can
substitute for SecB function in *E. coli* (Altman *et al.*,
1991). Overexpression of DnaK or GroEL/ES did not,
however, rescue cells from the export defect caused when
SecB was limiting or absent. Most suppressors of this
defect mapped to the *rpoH* locus, indicating that the
supressor(s) of the export defect may be as yet
unidentified heat shock proteins, or export factors that
fulfil the normal chaperoning function of SecB (Altman *et
al.*, 1991).

Another possible example of overlapping functions of
molecular chaperones can be inferred from studies of
hsp70. Paek and Walker (1987) demonstrated that *dnaK* null
mutations are inviable at high temperature (42ºC), but
will still grow, albeit slowly, at low temperature (30ºC
and 37ºC). On the other hand, the *S. cerevisiae*

mitochondrial DnaK homologue, mhsp70, is essential for cell viability (Craig *et al.*, 1987).

Presuming that DnaK and mhsp70 perform identical functions, one could interpret these observations to mean that *E. coli* contains another protein that can partially substitute for DnaK, while no such protein exists in yeast mitochondria. The possible presence of a DnaJ homologue in mitochondria (Blumberg and Silver, 1991) may eliminate this protein as a substitute for DnaK. However, there are other possible candidates. Kang and Craig (1990) have identified a gene, *DksA*, encoding a M_r 17,500 polypeptide, that is a dosage-dependent suppressor of a *DnaK* deletion mutant. Another possibility is that the *E. coli* hsp90 homologue, HtpG (see Spence and Georgopolous, 1989), might partially substitute for DnaK function. Recent studies have shown that hsp90 chaperones protein folding *in vitro* (Wiech *et al.*, 1992), and it has been suggested that hsp90, like hsp70, might bind nascent polypeptides (Ang *et al*, 1991). The distribution of hsp90 in eukaryotic cells appears to be confined to the cytosol and endoplasmic reticulum (see Lindquist and Craig, 1988). Even if a mitochondrial homologue is present, it is possible that import defects caused by the absence of mhsp70 do not allow normal import of functional homologues.

Hardy and Randall (1991) have proposed that the binding of ligands by SecB is not only a function of affinity, but is also modulated by kinetic partitioning between the

- 67 -

pathways of folding and association with SecB (see Section
1.3.6.). Perhaps this is true of all the molecular
chaperones and there exists within the cell a hierarchy of
kinetic partitioning/association constants, with hsp70
tending to associate with all polypeptides while cpn60
tends to associate mainly with those destined to become
part of multimeric complexes (see Section 1.4.). Various
molecular chaperones within this hierarchy could
functionally overlap (see above). Cpn60, at the 'bottom'
of this hierarchy, appears to be essential for the
viability of all organisms and is presumed to have no
functional homologues (a reflection of its unique
structural characteristics?). Perhaps the molecular
chaperones broadly define protein folding 'corridors' (for
example some leading to protein export and others leading
to assembly of multimeric complexes) down which protein
folding pathways run. In some cases it is possible that
where a particular 'corridor' is blocked an alternative
one may be accessible.

1.6. Aims and test organisms

The work described in this thesis concerns the isolation
and further study of molecular chaperones in photo-
synthetic organisms. Two organisms are to be studied, *R.
sphaeroides* and *Pisum sativum*.

Rhodobacter sphaeroides is a purple non-sulphur
photosynthetic bacterium that is capable of diverse

nutritional modes ranging from aerobic chemoheterotrophy
to anaerobic photoheterotrophy (Tabita, 1988). The genetic
background of *R. sphaeroides* is probably the best
characterized of all the *Rhodospirilliaceae*. Thus, there
is a wealth of physiological and genetic information to be
drawn upon when studying the chaperonins in this organism.

The second organism to be studied is *Pisum sativum* (pea)
in which the possible existence of a cytosolic cpn60 type
protein is to be investigated.

Chapter 2

Materials and Methods

2.1. Biological materials

2.1.1. Bacterial strains

The following bacterial strains were used in this study.

Escherichia coli MC 1061

Rhodobacter sphaeroides NCIMB 8253

Rhodomicrobium vannielii Rm5 (Whittenbury and Dow, 1977)

2.1.2. Plants

Seeds of *Pisum sativum* (var. Feltham First) were obtained from Charles Sharp, Sleaford, Linconshire.

2.1.3. Animals

New Zealand white rabbits (female) were obtained from Rosemead Rabbits, Waltham Abbey, Essex.

2.1.4. Plasmids

Two plasmids were used in this study.

pBR322 (BRL).

pOF39 (Fayet *et al.*, 1986) a derivative of pBR325 carrying the *groEL* and *groES* genes on a 2.2 kb *E. coli* DNA insert.

2.2. Non-biological materials

All chemicals used were purchased from reputable

companies and were of the highest analytical grade
available. The suppliers of specialist materials, such as
radiochemicals and antibodies, are noted where
appropriate. Solutions were made up in distilled water or
high quality deionised water, referred to as Elga water.

2.3. Growth media

All media were made up in distilled water unless
otherwise stated. All media were autoclaved at 121°C, 15
psi for 15 minutes. Non-autoclavable components were
filter sterilised, through Sartorius Minisart NML (0.2
µm) disposable filter units, and added to media at room
temperature or 45°C when plates were to be poured.
Plates were prepared by adding water agar at a
concentration of 1.5% (w/v) prior to autoclaving. 25 ml
aliquots of the warm molten agar were dispensed into 90
mm diameter sterile plastic petri-dishes.

2.3.1. Pyruvate malate medium

Pyruvate malate (PM) medium for the growth of *R.
sphaeroides* and *R. vannielii* was made up using a
modification of the recipe of Whittenbury and Dow (1977).

Constituents	Amount
10 × MSM stock	100 ml
Pyruvic acid, sodium salt	1.50 g
Sodium hydrogen malate	1.50 g

The pH of the medium was adjusted to 6.8 with 1 M KOH,
and made up to 984 ml with distilled water prior to
autoclaving. Before inoculation 12.5 ml of sterile 0.2 M

phosphate buffer pH 6.8, 2.1 ml vitamin supplement, 1 ml trace elements solution and 0.1 ml $FeSO_4.7H_2O$ solution were mixed together, filter sterilised, and added to the medium.

The constituents of PM medium were made up as follows:

Minimal Salts Medium (MSM) (Whittenbury and Dow, 1977)

Constituents	gl^{-1}
NH_4Cl	0.50
$MgSO_4.7H_2O$	0.40
NaCl	0.40
$CaCl_2$	0.05

The above was made up as a 10 × stock solution and autoclaved.

0.2 M phosphate buffer pH 6.8

Constituents	gl^{-1}
Na_2HPO_4	28.39
NaH_2PO_4	31.20

Vitamin supplement

Constituents	ml
D-biotin (0.15 mg/ml)	0.5
Thiamine hydrochloride (1 mg/ml)	5.0
Nicotinic acid (1 mg/ml)	5.0

The vitamin supplement was made up in Elga water and was dispensed into 1.05 ml aliquots and stored at $-20^{\circ}C$. Each aliquot is sufficient for 500 ml of PM medium.

Trace element solution

Constituents	gl^{-1}
EDTA, disodium salt	5.0
$ZnSO_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.03
H_3BO_4	0.3
$CaCl_2.2H_2O$	0.01
$NiCl_2.6H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.03

Trace element solution was made up in Elga water.

$FeSO_4 \cdot 7H_2O$ was made up as a separate solution of concentration $2.0 \; gl^{-1}$.

2.3.2. Luria-Bertani medium

Luria-Bertani (LB) medium for the growth of *E. coli* was made up as follows (Maniatis *et al.*, 1982):

Constituents	gl^{-1}
Yeast extract	5.0
Tryptone	10.0
NaCl	10.0

Made up to 1 l and the pH adjusted to 7.5 if necessary.

2.3.3. Antibiotics

Ampicillin was made up to a concentration of 100 mg/ml, filter sterilised and stored in 1 ml aliquots at $-20^{o}C$. Plasmid-harbouring *E. coli* strains were grown in the presence of ampicillin at a final concentration of 100 µg/ml.

2.4. Commonly used buffers

2.4.1. Tris buffers

1 M Tris.HCl buffer stocks were made up by dissolving 121.1 g Tris base in 800 ml Elga water. The pH was adjusted to the desired value by adding concentrated HCl at room temperature. The volume of the solution was then made up to 1 l.

2.4.2. 0.5 M EDTA pH 8.0 stock

0.5 M EDTA stocks were made by adding 186.1 g of disodium ethylene diamine tetraacetate.$2H_2O$ to 800 ml Elga water. This was stirred vigorously on a magnetic

stirrer while adjusting the pH to 8.0 by slow addition of
sodium hydroxide pellets (approximately 20 g in total).
The volume was then made up to 1 l. The solution was
dispensed into aliquots and sterilized by autoclaving.

2.4.3. **TE**

TE pH 8.0 (10 mM Tris.HCl pH 8.0; 1 mM EDTA pH 8.0) was
made up using the above stock solutions.

2.4.4. **TEMM and TEMMβ**

These buffers, usually used in the preparation of cell-
free extracts of *R. sphaeroides* and *R. vannielii*, are
modified versions of the buffer used by Cook *et al*.
(1988). The composition of TEMM is shown below. Addition
of 2-mercaptoethanol to TEMM produces TEMMβ

Constituents	
Tris.HCl pH 8.0	20 mM
$MgCl_2.6H_2O$	10 mM
$NaHCO_3$	50 mM
EDTA.disodium salt pH 8.0	10 mM

TEMMβ is made by addition of 2-mercaptoethanol, final
concentration 50 mM, just before use.

2.4.5. **Phosphate buffered saline (PBS)**

Phosphate buffered saline was made up as follows:

Constituents	
NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	11.5 g
KH_2PO_4	0.2 g

This was made up to 1 l with distilled water. The final
pH of the solution is 7.4.

2.5.   Growth and maintenance of biological materials

Growth of bacterial strains in liquid culture was monitored spectrophotometrically (Ultrospec 4050, LKB) at a wavelength of 650 nm.

2.5.1. Growth and maintenance of bacterial strains

2.5.1.1.   *R. sphaeroides* and *R. vannielii*

R. sphaeroides was grown both photoheterotrophically and chemoheterotrophically in PM medium. For photo-heterotrophic growth, the medium was prepared in 500 ml serum bottles containing a 2 cm long stirrer-bar. After inoculation the bottles were sealed with a sterile size 45 suba seal (William Freeman and Co., Barnsley, W. Yorkshire) and flushed with nitrogen, 15 psi for 15 minutes, accompanied by vigorous stirring. Cultures were grown under a bank of 60 Watt electric light bulbs giving an incident light intensity, at the surface of the bottle, of 50 $\mu Em^{-2}s^{-1}$. The stirrer speed was set at about 200 rpm and temperature was maintained at 30ºC in a water-bath. *R. vannielii* was grown in identical media and conditions.

For chemoheterotrophic growth 500 ml medium was dispensed into a 2 l Erlenmeyer with a spring, to increase aeration, in the bottom. Flasks were covered in foil to exclude light and incubated in a rotary shaker set at 200 rpm and 30ºC.

Both strains were grown on PM plates incubated in an illuminated, constant light, 30ºC incubator. For short

term (4-6 weeks) storage, plates were sealed with tape
and stored inverted at 4°C. For long term storage, an
aliquot of a mid-exponential culture was mixed with an
equal volume of sterile glycerol and stored at -20°C.

The purity of cultures was regularly assessed by
streaking out on plates and microscopic examination.

2.5.1.2. *E. coli*

E. coli was grown in LB medium. Cultures were grown in
either 250 ml Erlenmeyer flasks containing 50 ml medium
or 25 ml Universal bottles containing 10 ml medium.
Cultures were incubated in an orbital incubator set at
250 rpm and 37°C. Plasmid-carrying strains were
incubated in the presence of the appropriate antibiotic.

Strains were stored and maintained as for *R.
sphaeroides*.

2.5.2. Growth of *P. sativum*

P. sativum seeds were sown in J. Arthur Bowers potting
compost and normally grown at 20 ± 2°C for 7 to 10 days,
with a photoperiod of 12 hours using "warm white"
fluorescent lights (Philips) giving a light intensity of
photosynthetically active radiation of 50 $\mu Em^{-2}s^{-1}$. Trays
of seeds were well watered after sowing and as required
during growth.

Etiolated *P. sativum* seeds were grown at 20 ± 2°C.
After sowing, trays of peas were placed in large, vented,
wooden cabinets located in a dark-room. The cabinets were

loosely covered in black plastic to ensure that
absolutely no light reached the seedlings.

2.6. Protein analysis methods

2.6.1. Protein concentration determination

Protein concentrations were determined using the Bio-
Rad protein assay system (Bio-Rad Laboratories Ltd).

2.6.2. Polyacrylamide gel electrophoresis

SDS and native gels,both linear and gradient, were used
for the size fractionation of proteins. The following
stock solutions were required:

All acrylamide stocks were filtered through Whatman 3MM
paper.

60% (w/v) acrylamide: high bisacrylamide	
acrylamide	60.0 g
bisacrylamide	1.6 g

Acrylamide and bisacrylamide were dissolved in 30 ml
warm Elga water and the volume then made up to 100 ml.

60% (w/v) acrylamide: low bisacrylamide	
acrylamide	60.0 g
bisacrylamide	0.3 g

Made up as above.

30% (w/v) acrylamide: 0.3% bisacrylamide	
acrylamide	30.0
bisacrylamide	0.3

Made up as above.

10% (w/v) stacking gel acrylamide

acrylamide	10.0 g
bisacrylamide	0.5 g

Made up to 100 ml with Elga water.

Lower gel buffer

Tris base	36.6 g

pH was adjusted to 8.8 with HCl prior to making up to a final volume of 100 ml.

Stacking gel buffer

Tris base	5.98 g

pH was adjusted to 6.8 with HCl prior to making up to a final volume of 100 ml.

10 × running buffer stock: SDS gels

Tris base (25 mM final)	60.4 g
glycine (192 mM final)	288.0 g
SDS (0.1% (w/v) final)	20.0 g

Made up to 2 l with distilled water. Final pH is 8.3.

Running buffer stock: native gels

Tris base	12.0 g
glycine	57.6 g

Made up to 2 l with distilled water.

2.6.2.1. SDS-PAGE

Both linear and exponential gradient gels were used in this study. Slab gels, 1.5 × 160 × 140 mm, were cast in an SE400 Sturdier gel electrophoresis apparatus (Hoefer Scientific Instruments). Plates were scrupulously cleaned before assembly and casting. Gels were run either overnight at 10 mA constant current, or over about 4 hours at 40 mA constant current. Runs were ended just before the marker dye, bromophenol blue, migrated off the end of the gel.

2.6.2.1.1. 10-30% exponential gradient gels

10-30% SDS-polyacrylamide gels were cast according to the procedure described in Section 2.6.2.3. The following solutions are required:

30% (w/v) acrylamide	
60% low bisacrylamide stock	5.00 ml
75% (w/v) glycerol	3.65 ml
lower gel buffer	1.25 ml
10% (w/v) SDS	0.10 ml

This mixture was then degassed on a water pump vacuum line for at least 10 minutes. The mixture was then chilled on ice. Immediately before pouring, 50 µl freshly prepared 10% (w/v) ammonium persulphate (electrophoresis grade, BRL) and 5 µl N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma) was added.

10% (w/v) acrylamide	
60% high bisacrylamide stock	4.15 ml
distilled water	17.45 ml
lower gel buffer	3.125 ml
10% (w/v) SDS	0.25 ml

This mixture was degassed and chilled as above, and 100 µl 10% ammonium persulphate and 10 µl TEMED added immediately before pouring. After casting, the gel was overlayed with water-saturated butan-2-ol and allowed to set. When the gel had set, the butan-2-ol was washed off and the stacking gel layered on top of the gel. The stacking gel was prepared as follows:

3% stacking gel	
stacking gel acrylamide stock	3.0 ml
distilled water	4.4 ml
stacking gel buffer	2.4 ml
10% (w/v) SDS	0.1 ml

This mixture was degassed as above, and 100 µl 10%

(w/v) ammonium persulphate and 5 µl TEMED added
immediately before pouring. The required comb was set
into the solution, with care being taken that no air
bubbles were trapped. If a flat-topped stacker was
required, the solution could be overlayed with water-
saturated butan-2-ol.

2.6.2.1.2.   10% linear gels

In this study two different types linear 10% gels were
used. The main difference between the two systems is the
amount of cross-linking agent, bisacrylamide, as a
percentage of the total acrylamide, bisacrylamide plus
acrylamide. This value is represented by C while the
total acrylamide is represented by T.

10% (w/v) acrylamide (T = 10.27%, C = 2.6%)	
60% high bisacrylamide stock	10.00 ml
distilled water	41.70 ml
lower gel buffer	7.50 ml
10% (w/v) SDS	0.60 ml

This mixture was degassed as above, and 225 µl 10%
(w/v) ammonium persulphate and 15 µl TEMED added
immediately before pouring. After setting, the gel was
overlayed with a stacking gel prepared and cast as for
gradient gels.

10% (w/v) acrylamide (T = 10.1%, C = 1%)	
30% acrylamide stock	12.00 ml
distilled water	19.05 ml
lower gel buffer	4.50 ml
10% (w/v) SDS	0.36 ml

This mixture was degassed as above, and 150 µl 10%
(w/v) ammonium persulphate and 15 µl TEMED added
immediately before pouring. The stacking gel was prepared

and added as before. This gel system gives a much better resolution of polypeptides in the 60 kD size range and is particularly useful for separating the α and β subunits of the pea plastid chaperonin, which differ in molecular weight by less than 1000 (Hemmingsen and Ellis, 1986).

2.6.2.1.3. <u>Sample preparation for SDS-PAGE</u>

Samples were prepared for SDS-PAGE by boiling in 2 × sample buffer which was made up as follows:

2 × sample buffer	
Stacking gel buffer	2.5 ml
Distilled water	0.5 ml
Glycerol	2.0 ml
10% (w/v) SDS	4.0 ml

Just before use, 100 μl 2-mercaptoethanol was added to 900 μl of the above. An equal volume was added to the sample to be analysed and the mixture boiled for 5 minutes, microcentrifuged for a few seconds and then applied to the gel.

2.6.2.2. <u>Non-denaturing-PAGE</u>

4-30% exponential gradient gels were cast according to the procedure described in Section 2.6.2.3.. Slab gels, 2 × 200 × 250 mm, were cast in a home-made gel electrophoresis apparatus. Plates were scrupulously cleaned before assembly and casting. Gels were run overnight at 15 mA constant current until the dye front had migrated off the bottom of the gel.

The following solutions were required:

```
30% (w/v) acrylamide
60% low bisacrylamide stock        10.0 ml
75% (v/v) glycerol                  7.4 ml
lower gel buffer                    2.5 ml
```

This solution was degassed and chilled as described
previously. Immediately before pouring, 100 µl 10% (w/v)
ammonium persulphate and 10 µl TEMED were added.

```
4% (w/v) acrylamide
60% high bisacrylamide stock        3.32 ml
Distilled water                    40.38 ml
lower gel buffer                    6.25 ml
```

This solution was degassed as described previously.
Before pouring 5 ml was removed and retained for later
use as a "tooth-former". Immediately before pouring,
200 µl 10% (w/v) ammonium persulphate and 20 µl TEMED
were added. After casting, the gel was overlayed with
water-saturated butan-2-ol and allowed to set. When the
gel had set, the water-saturated butan-2-ol was washed
off. 100 µl 10% (w/v) ammonium persulphate and 10 µl
TEMED were added to the retained "tooth-former" solution
which was layered onto the top of the gel and an
appropriate comb inserted.

Samples were prepared for electrophoresis by mixing
them with 0.2 volumes of 75% (v/v) glycerol; 0.02% (w/v)
BPB.

2.6.2.3. Casting exponential gradient gels

The apparatus required to cast exponential gradient
gels is that described by Porter (1984). Silicone rubber
tubing which passed through a peristaltic pump was
attached to a 21G × 1.5 hypodermic needle (Sabre, Sabre

```

International Products Ltd.) which was used to pierce a
size 41 Suba seal. The base of a 19G × 2 needle was
broken off and a piece of plastic tubing (1 mm internal
diameter) slid over the shaft of the needle. This plastic
tubing was then forced through the Suba seal and the
needle shaft discarded. The tubing was long enough to
reach the bottom of a universal bottle with the Suba seal
in place, and for the other end to be taped to the
backplate of the assembled casting plates.

To cast gels, the chilled 30% acrylamide solution was
poured into a 25 ml universal bottle containing a small
stirrer bar. Ammonium persulphate and TEMED were added to
the lower percentage acrylamide solution, 4% or 10%,
which was then pumped through to the 21G needle. Ammonium
persulphate and TEMED were then added to the 30% solution
which was vigorously stirred on a magnetic stirrer. The
Suba seal was then inserted into the universal bottle,
causing a few drops of acrylamide to be forced through
the thin plastic tubing onto the backplate of the
assembled casting plates. The pump was then restarted to
deliver the 4% or 10% acrylamide into the mixing chamber
at a rate of about 4 ml/min. Gels were poured until the
acrylamide level was about 4 cm from the top of the
plates. Gels were then overlaid with water-saturated
butan-2-ol and allowed to polymerise for at least 3
hours.

### 2.6.2.4. Molecular weight markers

Electrophoresis calibration kits were supplied by Pharmacia (Uppsala, Sweden) or Sigma.

Pharmacia low molecular weight calibration kit: phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and $\alpha$-lactalbumin (14.4 kD).

Sigma low molecular weight calibration kit: albumin, bovine (66 kD), albumin, egg (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20.1 kD) and $\alpha$-lactalbumin (14.2 kD).

The above kits were used for estimations of molecular weights of polypeptides resolved in SDS-polyacrylamide gels.

Pharmacia high molecular weight calibration kit: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD) and albumin (67 kD), was used for estimations of molecular weights of proteins resolved in non-denaturing-polyacrylamide gels.

### 2.6.2.5. Visualisation of protein bands

Gels were stained in 45% (v/v) methanol/ 10% (v/v) glacial acetic acid containing 2 gl⁻¹ Coomassie blue R-250 for a minimum period of 30 minutes, and destained in several changes of 10% (v/v) propan-2-ol/ 10% (v/v) glacial acetic acid/ 20% (v/v) methanol (Haider *et al.*, 1986). The Coomassie blue stain could be reused several

times.

## 2.6.2.6. Gel drying

Gels were mounted on two sheets of Whatman 3MM
chromatography paper, the upper layer of which was
moistened with distilled water. The gels were then
covered with a sheet of clingfilm which was trimmed to a
size just larger than the gel. Gels were then dried for
1.5 hours under vacuum at 80°C on a Bio-Rad dual
temperature slab gel drier (Model 1125B).

## 2.6.3. Western blot analysis of protein

The method used for western blotting is derived from
that of Burnette (1981) and was carried out as follows:

1) A polyacrylamide gel was run as normal and then
allowed to equilibrate in blotting buffer (20 mM Tris;
150 mM glycine; 20% (v/v) methanol) for about 30 minutes.

2) A sandwich for the blotter was made. This consisted
of:

    A sheet of sponge 1-2 cm thick
    3 thicknesses of Whatman 3MM paper
    The gel
    A sheet of nitrocellulose (Hybond C, Amersham) just
    bigger than the gel
    3 more thicknesses of 3MM paper
    Another sheet of sponge

This was all held together by the plastic holder for
the transblot. The sponges and all other components must
be wetted prior to assembly and great care must be taken
to avoid the entrapment of any air bubbles.

3) Protein transfer: The sandwich was inserted, with

the nitrocellulose closest to the anode, into the transblot, which had already been filled with blotting buffer. Proteins were transferred from SDS-polyacrylamide gels by electrophoresis for 2 hours at 60 V, or overnight at 30 V. Proteins were transferred from non-denaturing gels by overnight electrophoresis at 60 V. From this stage onward the blot was not allowed to dry out.

4) Blocking: The blotted sheet was then soaked in MTP (5% (w/v) Marvel (Nestlé); 0.1% (w/v) Tween 20 in phosphate buffered saline) for 1 hour with gentle agitation.

5) The blot was then incubated with primary antibody appropriately diluted in MTP. This stage was carried out in a sealed plastic bag containing 0.05-0.1 ml antibody solution per $cm^2$ nitrocellulose. The blot was soaked for at least 3 hours, usually overnight, with gentle rocking at room temperature.

6) The blot was washed three times in 200 ml PBS plus 0.1% (w/v) Tween 20 (10 minutes per wash) with gentle rocking.

7) The blot was then incubated for 1 hour at room temperature with shaking in a sealed bag containing 0.05-0.1 ml secondary antibody per $cm^2$ (Amersham, peroxidase-linked anti-rabbit or anti-rat, depending on the sour e of the primary antibody) diluted 1:500 in PBS/0.1% (w/v) Tween 20. In cases were the biotin/streptavidin detection system (Amersham) was used blots were first incubated, as

- 86 -

above, in the presence of biotinylated secondary antibody
(1:330 dilution). The blot was then washed as in step 6,
and incubated as above in peroxidase-linked streptavidin.

8) Washed 2 × 10 minutes in 200 ml PBS plus 0.1% (w/v)
Tween 20.

9) Wash 2 × 10 minutes in 200 ml PBS.

10) The blot was then developed as follows:

Immediately before use, a 30 mg 4-chloro-1-naphthol
tablet (Amersham) was dissolved in 10 ml methanol. When
the tablet had dissolved, 50 ml PBS was added and the
solution mixed by swirling. Then 60 μl 30% (v/v) $H_2O_2$ was
added, the solution mixed by swirling, then poured over
the blot. The blot was then soaked in this solution with
shaking. After the bands had developed, the reaction was
stopped by washing in distilled water (3 × 10 minutes).

11) The blot was dried between two sheets of Whatman
3MM paper and photographed. If desired, the blot can be
rehydrated and probed with a second antibody by following
the above procedure from step 4.

## 2.6.3.1.  Staining of western blots

This method was used to visualise protein bands on
Western blots after immuno-localisation of bands. Blots
were stained in 45% (v/v) methanol/ 7% (v/v) glacial
acetic acid containing 1 gl⁻¹ amido black for a brief
period and as soon as bands became visible, were
destained as in Section 2.6.2.5. The amido black stain
could be reused several times.

## 2.6.4.  N-terminal sequence analysis

N-terminal sequence analyses were conducted at the SERC
protein sequencing facility at Aberdeen University.
Protein preparations for sequencing were lyophilized in a
Speedvac centrifugal freeze-dryer (Model 5PS, Edwards
High Vacuum Ltd) before being taken to Aberdeen.

Protein preparations for sequencing were dissolved and
boiled for 2 minutes in solubilization solution. The
concentration of the polypeptide to be sequenced needed
to be greater than 0.5 mg/ml. This concentration was
required so that it would be possible to load at least
100 pM, approximately 6 µg in the case of a 60 kD
protein, to a single gel lane (15 µl maximum load). The
samples were then separated by SDS-PAGE in a mini-gel
apparatus as described by Yuen *et al.* (1986). After
electrophoresis, polypeptides were transferred onto an
Immobilon membrane (Millipore) by western blotting at 300
mA for 1 hour. The membrane was briefly Coomassie stained
(0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol)
and destained (50% (v/v) methanol; 10% (v/v) glacial
acetic acid). The membrane was washed with water and
dried on a sheet of Whatman 3MM paper. The band of
interest was then excised using a razor blade, and cut
into 2 mm × 4 mm pieces which were then evenly
distributed inside the sequencing cup.

Sequence analyses were conducted by Bryan Dunbar of
Aberdeen University. The polypeptide was sequenced by the

phenylthiohydrantoin (PTH) method using an Applied
Biosystems model 470 A protein sequencer, on-line
connected with an Applied Biosystems 120 A PTH analyser.

## 2.7. Production and purification of antibodies

New Zealand white female rabbits were used to raise
antibodies against the Rs cpn60 protein from *R.
sphaeroides*. The method used was basically that described
by Johnson and Thorpe (1982).

Prior to injecting any animals with the antigen, it was
first necessary ensure that the pre-immune serum did not
cross-react with any of the chaperonins used in this
study. Pre-immune serum was prepared as follows. A blood
sample of 5 ml was removed from the rabbit's ear and
collected in a 25 ml Universal bottle. A Pasteur pipette
was used to streak blood along the sides of the bottle,
to prevent clotted blood sticking to the walls of the
bottle. The blood was allowed to clot at room temperature
for about 1 hour, then left overnight at 4ºC to cause
the clot to contract. Serum was isolated by
centrifugation in a MSE coolspin centrifuge at 3,800 g
for 20 minutes at 4ºC. The pale straw-coloured
supernatant was carefully transferred to a fresh tube,
dispensed into aliquots, 200-500 µl, and stored at
-20ºC. This pre-immune serum was then screened for
antibody activity against GroEL, pea plastid and *R.
sphaeroides* chaperonins as described in Section 2.6.3..

Only rabbits in which no cross-reactive antibodies were detected were used for subsequent immunization.

Rabbits were immunized in the following way: 100 µg of lyophilized protein was dissolved in 200 µl PBS. This was then emulsified in 200-300 µl Freund's incomplete adjuvant (Gibco Ltd.). The emulsified mixture was injected subcutaneously. After 2 weeks a second injection of 100 µg protein was administered as above. 7 days after the second injection, a blood sample was taken and serum prepared and analysed as above. If no strong antibody response was observed, the rabbit was again injected with 100 µg of protein 20 days after the second immunization. When a strong antibody response was observed, a blood sample (10 ml) was taken and serum was prepared and stored as above.

2.8.   Immunocytochemistry

Immunocytochemical studies were conducted on *P. sativum* in an attempt to determine the sub-cellular location of a putative TCP-1 homologue.

2.8.1. Sample preparation

2.8.1.1.   Fixation

Leaves and roots from a seven day old *P. sativum* seedling were placed on a soft surface (dental wax) submerged in fixative solution (2.5% gluteraldehyde in 50 mM sodium cacodylate pH 7.2. The pH of this solution was adjusted to 7.2 after addition of aldehyde) in a 90 mm

petri dish. The plant material was cut into pieces
approximately 1 mm × 4 mm with a new razor blade. Groups
of about five pieces of material were transferred, under
fixative, to small glass vials. Leaf material tends to
float in the fixative; if this was the case, trapped air
was removed under a slight vacuum until the leaf material
sank. The degassed fixative was then replaced with fresh
fixative. The tissue was then incubated under fixative
for 24 hours at room temperature.

## 2.8.1.2.  Dehydration

Dehydration and infiltration of fixed tissue involves a
number of changes of solution that should be done as
rapidly as possible. This was achieved with the aid of a
tissue handling device (Wells, 1985). Figure 2.8.1. shows
how this device was constructed.

Using the tissue handling device, the tissue was
transferred to the following solutions:

1) 50% (v/v) ethanol for 1 hour on ice.

2) 50% (v/v) ethanol for 1 hour at $-20^\circ C$.

3) The material was then tranferred to 70% (v/v)
ethanol at $-35^\circ C$ for 1 hour. This temperature was
maintained in a nitrogen-cooled, low temperature box as
described by Wells (1985). The box contains a rotating
aluminium block in which the tissue handling devices were
mounted.

4) 95% (v/v) ethanol for 1 hour at $-35^\circ C$.

5) 100% ethanol for 1 hour at $-35^\circ C$.

Figure 2.8.1.   Tissue handling device construction



Photograph of tissue handling device.  × 0.8. (1) Plastic transfer pipette (Sarstedt) cut about 15 mm from bulb base. (2) Plastic cap of glass vial drilled with 3 mm hole. (3) Vinyl tubing o.d. 4.1 mm, 8 mm long. (4) Nylon filter, 100 μ mesh, 10 mm square. (5) Vinyl tubing o.d. 5.5 mm, 30 mm long. (6) Nylon filter, 100 μ mesh, 10 mm square. (7) Vinyl tubing o.d. 4.1 mm, 8 mm long. (8) Glass vial, 1 dram size. (9) Assembled device.

After Wells (1985).

## 2.8.1.3. Infiltration

In this step the tissue samples were embedded in an acrylic resin, LR White medium grade (Agar Scientific). The resin was finally polymerized under U.V. light. Benzoin methyl ether at a final concentration of 0.5% (v/v) was added to the resin to catalyse its U.V. polymerization. The process is as follows:

1) Transfer to 1:1 (w/w) mix of resin:ethanol for 1 hour at −35°C. Then:

2) 2:1 resin:ethanol for 1 hour at −35°C.

3) 3:1 resin:ethanol for 1 hour at −35°C.

4) 100% resin for 1 hour at −35°C.

5) 100% resin overnight at −35°C.

6) 100% resin for 8 hours at −35°C.

## 2.8.1.4. Embedding

Samples were transferred to pre-cooled, resin-filled BEEM 00 capsules (Agar Scientific) at −35°C and polymerized by indirect U.V. irradiation for 24 hours at −35°C.

## 2.8.2. Grid preparation

A 6% (w/v) pyroxylin (Agar Scientific) in amyl acetate stock solution was made up by stirring at 4°C for about 3 days in a tightly closed bottle. This was diluted to 4% (w/v) with amyl acetate prior to use. A glass pipette was used to deliver a single drop of the 4% solution onto a 20 cm diameter dish filled with water. This forms a film

over the surface of the water which is then removed thus cleaning the surface of the water. A fresh drop is then delivered to the surface of the water.

Gold electron microscope grids (hexagonal 200 mesh, thin bar gold, 3.05 mm, Agar Scientific) were used. Copper grids are unsuitable because of the relatively high salt concentrations used in immunochemistry. The grids were placed on several sheets of filter paper in a petri dish and sprinkled with a 0.15% (w/v) butwar solution (Agar Scientific). Before the butwar solution dried the grids were picked up and laid, butwar side down, on the pyroxylin film. Butwar aids adhesion between the grids and the pyroxylin film. After approximately 5 minutes, a sheet of absorbent kitchen paper towel was laid over the grids. The towel was allowed to become slightly damp and then lifted off the surface of the water. The paper towel, with grids, was then placed on filter pads in the lid of a petri dish. This was covered with the petri dish bottom and allowed to air-dry overnight at room temperature.

After drying, the piece of absorbent paper holding the grids was cut down to size and laid on Whatman filter paper. The grids were then carbon-coated in a high vacuum coating unit. The carbon film should not be too thick. Thickness could be monitored by turning up one corner of the absorbent paper so that a shadow is cast during coating. As soon as the shadow becomes visible, enough

carbon has been deposited on the grids.

2.8.3. <u>Sectioning</u>

Sections were cut in an ultramicrotome fitted with a
45° glass knife. The block containing the embedded
material was mounted and trimmed with a razor blade to
expose the material for sectioning. When cutting sections
of leaf material, the block was mounted so that the
microtome would cut sections longitudinally. If this is
not done, the tissue tends to be compressed and pulled
away from the resin during the cutting stroke.

For cutting sections, the knife water bath was filled
with water and then the meniscus lowered as far as
possible whilst maintaining a wet edge on the knife.
Sections approximately 90 nm thick were then cut.
Sections of the correct thickness have a silver
appearance when they float off into the water bath.
Sections were picked up by submerging the grid, pyroxylin
face up, in the water bath and then raising the grid
under the floating sections. Excess water was blotted off
the grid by touching its edge against a piece of filter
paper. The grids were then placed on a filter paper disc
in a petri dish, loosely covered with the petri dish
bottom and allowed to air-dry.

2.8.4. <u>Staining</u>

Before attempting any immunogold labelling of sections
it is necessary to check that sample preparation and
sectioning have been successfully accomplished. Material

prepared and sectioned as above was negatively stained as
follows:

Staining and washing materials were dispensed in 15 µl
drops on dental wax or Parafilm. The grids were inverted
and placed on a drop of stain, 2% (w/v) uranyl acetate in
distilled water, for a period of 15 minutes. The grids
were then washed by transferring through 5 drops of
water. After each transfer, the edges of the grids were
touched against a piece of filter paper to dry off most
of the excess liquid. Grids were then transferred to a
drop of 2.8% (w/v) lead acetate for a period of 30
seconds before washing in 5 drops of water as described
above. The grids were finally placed on a filter disc in
a petri dish and allowed to air-dry overnight. Grids were
then examined in the electron microscope.

2.8.5.  **Immunogold staining**

Immunogold staining was carried out using a number of
different stringencies. The lowest and highest stringency
washes are described here. An intermediate stringency
method used the solutions described for western blotting
(see Section 2.6.3.).

Firstly, grids were incubated in the presence of
several dilutions, ranging from 1 in 10 to 1 in 250, of
the primary antibody. Incubations were carried out in 15
µl drops dispensed onto dental wax or Parafilm (American
Can Co.), which was placed on a damp piece of filter
paper in a petri dish.

### 2.8.5.1. Low-stringency labelling

1) Blocking: Grids were blocked by floating them face down in 15 μl blocking solution, 3% (w/v) BSA in TBS (10 mM Tris.HCl pH 7.4, 0.9% (w/v) NaCl, 0.02% (w/v) sodium azide), for a period of 30 minutes.

2) Primary antibody: The grid was removed from the blocking solution, dried by touching the edge against a piece of filter paper, and then floated on primary antibody appropriately diluted in blocking solution. Grids were incubated for either 1 hour at room temperature or overnight at 4°C.

3) Grids were washed 3 times for 10 minutes in distilled water. Washes were carried out, with gentle stirring on an orbital platform, in weighing boats into which about 1 ml of water had been dispensed.

4) Secondary antibody: In these experiments, the secondary antibody was 5 nm gold-labelled goat anti-rat IgG (Auroprobe EM GARa G5, Amersham). The secondary antibody was diluted 1 in 30 in blocking solution and incubations were carried out for 1 hour at room temperature.

5) Grids were washed as in 3).

6) Staining: Grids were stained in 2% (w/v) uranyl acetate for 1 hour at room temperature and washed as described in Section 2.8.4. Grids were then transferred to 2.8% (w/v) lead acetate for 2 minutes and then washed and dried as described in Section 2.8.4.

7) Grids were then examined in the electron microscope.

## 2.8.5.2. High-stringency labelling

The following solutions were required for the high-stringency labelling method:

A) Washing buffer: 0.5% (w/v) BSA, 0.1% (w/v) gelatin, 0.05% Tween 20, 150 mM NaCl, 10 mM Na phosphate pH 7.4.

B) Blocking buffer: Washing buffer plus 5% (v/v) normal goat serum.

C) Incubation buffer: Washing buffer plus 1% (v/v) normal growth serum.

High-stringency labelling was carried out as follows:

1) Blocking: Grids were blocked by incubation in blocking buffer for 30 minutes.

2) Primary antibody: Grids were transferred to primary antibody appropriately diluted in incubation buffer.

3) Washes: Grids were washed 3 times for 15 minutes in washing buffer.

4) Secondary antibody: As in Section 2.8.5.1. but diluted in the blocking buffer described above.

5) Washes: Grids were washed as in 3).

6) Grids were then washed 3 times for 5 minutes in TBS (see Section 2.8.5.1. part i).

7) Grids were fixed in 2.5% (v/v) gluteraldehyde in TBS for 15 minutes.

8) Grids were then washed 3 times for 15 minutes in distilled water.

9) Grids were stained as described in Section 2.8.5.1.

10) Finally grids were examined in the electron microscope.

## 2.9. Protein purification

### 2.9.1. Preparation of cell-free extracts

#### 2.9.1.1. Cell-free extracts of *R. sphaeroides* and *R. vannielii*

500 ml cultures were grown to the mid- to late-exponential phase of growth. Cells were harvested by centrifugation at 4°C, 10,000 g for 15 minutes in a MSE High Speed 18 centrifuge using a 6 × 250 ml rotor. The cells were then resuspended in 10 ml ice-cold TEMM buffer, transferred to a clean Oakridge tube and harvested by centrifugation at 4°C, 12,000 g for 10 minutes. The cells were then resuspended in 3 ml TEMMB containing DNase I (type II, Sigma) and RNase A (Sigma) at concentrations of 40 μg/ml and 80 μg/ml respectively. The cell suspension was then passaged twice through a precooled French pressure cell (American Instrument Company) at 20,000 psi. Intact cells and cellular debris were removed by 5 minutes centrifugation in an Eppendorf microfuge (13,600 g). The supernatant was collected and Nonidet P40 added to a final concentration of 1% (v/v). Chaperonins were then partially purified from this material by sucrose density gradient centrifugation (2.9.2.).

Small scale preparations of cell-free extracts from pulse-labelled cells were prepared by sonication as described in Section 2.10.

2.9.1.2.  <u>Cell-free extracts of *P. sativum*</u>

Both small and large scale preparations were carried
out. In the case of *P. sativum* grown under normal
conditions (12 hour photoperiod) the leaves alone were
harvested. In the case of etiolated *P. sativum* seedlings,
the whole stem was harvested and the rudimentary leaves
discarded.

2.9.1.2.1.  <u>Small-scale preparation</u>

For small-scale preparations, 50 g of pea leaves were
ground up in 20 ml ice-cold grinding buffer (20 mM Tris-
HCl, pH 8.0; 10 mM MgCl$_2$.6H$_2$O; 10 mM EDTA; 50 mM 2-
mercaptoethanol; 1 mM PMSF) in a clean pestle and mortar
in the presence of glass beads (80 mesh, BDH). PMSF was
prepared as a 100 mM stock solution in ethanol immediately
before use. The resulting slurry was then expressed
through eight layers of muslin, giving a total of about 35
ml of crude extract. In initial studies this material was
then subjected to sucrose density gradient centrifugation.
However, this material proved to be difficult to
fractionate; in subsequent studies an appropriate ammonium
sulphate cut was used to partially purify and, in the case
of large scale preparations, concentrate the putative
chaperonins (see 5.4.). Ammonium sulphate cuts were
carried out by slowly adding solid ammonium sulphate to
the crude extract, accompanied by gentle stirring on ice
(England and Seifter, 1990). Amounts of ammonium sulphate

-100-

to be added per unit volume were determined using the
table of values presented by England and Seifter (1990).
Pellets were collected by centrifugation at 27,200 g for
15 minutes at 4°C. The relevant salt cut, 25-60% of
saturation, was resuspended in 14 ml of the grinding
buffer and applied to linear sucrose gradients.

### 2.9.1.2.2.  Large-scale preparation

Large-scale preparations involved the processing of
about 500 g of either normally grown or etiolated pea
material. A total of 250 ml of an ice slurry of grinding
buffer was used per 100 g plant material. The slurry was
decanted into a suitable container. Pea material was added
in 50 g batches and homogenized in 3 second bursts in a
Polytron blender (Kinematica GmbH, Switzerland). The
homogenate was then expressed through muslin and ammonium
sulphate precipitated as before. The 25-60% ammonium
sulphate pellet was resuspended in 60 ml 20 mM Tris.HCl pH
7.5 and dialysed overnight in this buffer. Plenty of space
was left in the dialysis bag, since a considerable amount
of buffer was taken up by the resuspended material.
Dialysis tubing was prepared by the method of Maniatis *et
al*. (1982). After dialysis the material was applied to
linear sucrose gradients.


### 2.9.2.  Partial purification of chaperonins by sucrose density gradient centrifugation

Two types of sucrose gradients were used in this study.

Initially, discontinuous gradients were prepared and centrifuged in a MSE Prespin 65 centrifuge. However, the increasing unreliability of these machines meant that an alternative was required. Paech and Dybing (1986) had used linear sucrose gradients centrifuged in a Beckman VTi50 rotor in a protocol for the rapid purification of Rubisco from soyabean leaves. Non-denaturing gels of Rubisco purified by method clearly showed the presence of a band that seemed likely to be a chaperonin! This method of sucrose density gradient centrifugation was subsequently adopted for both bacterial and plant chaperonin purification.

2.9.2.1. <u>Discontinuous sucrose density gradient centrifugation</u>

The following sucrose solutions were made up in TEMMB (usually 5 ml of each): 60% (w/v) and 0.8 M to 0.2 M in 0.1 M steps. 2 ml of each solution was layered, in order of ascending density, into a threaded 25 ml MSE polycarbonate tube. The crude extract, approximately 2 ml, was then layered on top of this and the gradient sealed by overlaying with paraffin up to the level of the lowest thread. The tube(s) was then balanced, capped and centrifuged in a MSE Prespin 65 centrifuge at 160,000 g, 4°C for 2.5 hours. At the end of the run the rotor was allowed to coast down with the brake off. The tube(s) was punctured at the lowest thread and at the bottom and 1 ml fractions collected by gravity feed.

## 2.9.2.2. Linear sucrose density gradient centrifugation

8-28% linear sucrose gradients were used in the
purification of both the *R. sphaeroides* chaperonin and the
putative *P. sativum* cytosolic chaperonin. In the former
case sucrose solutions were made in TEMM (2.4.4.), while
in the latter they were made up in grinding buffer
(2.9.1.2.1.). 30 ml sucrose gradients, 15 ml of each
solution, were cast, using a gradient-former, in Beckman
VTi50 heat-sealable 39 ml polycarbonate tubes. The tubes
were sealed and centrifuged at 242,000g, 4°C for 2.5
hours. Rotor acceleration was at the slowest rate
possible, 0-500 rpm in 6 minutes, and the run was ended
with the brake off. At the end of the run tubes were
pierced at the top and bottom, and fractions collected by
gravity feed.

Several analyses showed that the bottom 14 ml of the
gradients contained the proteins of interest. Typically
the protein concentration of this fraction was about 1
mg/ml when *R. sphaeroides* cell-free extracts were
fractionated and about 10 mg/ml when *P. sativum* cell-free
extracts were fractionated.

## 2.9.3. Chromatography

All chromatography columns were run at room temperature.
Samples were always filtered through a .22 µm filter
(Millex-GV, Millipore) before being loaded to
chromatography columns. Sucrose gradient fractions from *P.*

*sativum* were pre-filtered through a .45 µm filter (Millex-HV, Millipore). The standard column buffer used was 20 mM Tris.HCl pH 7.5. Where salt gradients were used to elute proteins, gradients were developed with sodium chloride. The salt concentrations and gradient profiles are noted in the results sections. Elution profiles were monitored by measurement of absorbance at 280 nm.

When necessary, particularly for size exclusion chromatography, the volume of protein solutions was reduced by ultrafiltration using an Amicon ultrafiltration cell (Model 8010, 10 ml capacity). The unit was fitted with a 100 kD cut-off filter (Omega series, Filtron) and buffer was forced through the membrane at a pressure of 30 psi.

## 2.9.3.1. ion exchange chromatography

Two ion exchange columns were used in this study. The first, a Pharmacia HR5/5 Mono-Q column, bed volume 1 ml, has a maximum protein binding capacity of approximately 20-50 mg. The second column, a Pharmacia HiLoad 26/10 Q Sepharose high performance column, bed volume approximately 55 ml, has a maximum protein binding capacity of about 1 g, 20 mg/ml. The former column was used for relatively small scale preparations, in which the initial protein content did not exceed the binding capacity of the column. For example, a 500 ml *R. sphaeroides* culture yields 14 ml sucrose density gradient fractionated material at a concentration of about 1 mg/ml.

All of this material could then be fractionated in a single Mono-Q run. The latter column was used exclusively for fractionation of pooled sucrose density gradient material from large scale *P. sativum* preparations. Both columns are strong anion exchangers and have the same functional group, $-CH_2-N^+(CH_3)_3$.

Both columns were run using a Pharmacia FPLC system. Columns were equilibrated by passing at least 5 bed volumes of start buffer through them. Flow rates and gradient profiles for individual runs are noted in the results sections. Cleaning and storage of columns was carried out according to the manufacturer's instructions.

### 2.9.3.2. Dye-ligand chromatography

A number of dye-ligand columns are commercially available. These columns consist of triazinyl dye-ligands covalently coupled via ether linkages to a cross-linked agarose support. The basis of the dye-ligand protein interaction is not well understood, so this type of chromatography could be called unknown affinity chromatography! A Dyematrex screening kit (Amicon) was used to determine which of these columns might be useful in purifying a putative cytosolic chaperonin from *P. sativum* cell-free extracts. The kit consisted of six prepacked columns containing the following media: Green A, Red A, Blue A, Blue B, Orange A and an agarose control. Each column has a 2 ml bed volume. Screening was carried out according to the manufacturer's instructions, with

columns being equilibrated in 20 mM Tris-HCl pH 7.5 and
washed in the same buffer containing 1.5 M NaCl. Screening
indicated that the Green A resin might be useful in these
studies.

### 2.9.3.2.1. <u>Green A dye-ligand chromatography</u>

Green A resin (Amicon) was packed into XK 16/10 columns.
Column bed volume was 20 ml. Columns were packed and
equilibrated according to the manufacturer's instructions.
This column was also run using a Pharmacia FPLC system.
Flow rates and NaCl gradient profiles are listed in the
results section. After use, columns were regenerated
according to the manufacturer's instructions and then
stored at 4°C in deionized water.

### 2.9.3.3. <u>Gel filtration chromatography</u>

Gel filtration was performed using an LKB BROMMA high
performance liquid chromatography system. A 60 cm, 21.5 mm
internal diameter, TosoHaas TSK gel filtration column
(G3000SW) was employed to size-fractionate protein
mixtures. Columns were equilibrated in 100 mM NaCl, 20 mM
Tris.HCl pH 7.5. Protein was loaded through a 2 ml sample
loop. Columns were run at a flow rate of 3 ml/minute.


## 2.10. <u>Radiolabelling of *R. sphaeroides* cultures</u>

The response of *R. sphaeroides*, at the protein synthesis
level, to a number of environmental stresses was studied
by incubating cells in the presence of $^{35}S$ labelled
methionine. $^{35}S$-methionine was purchased from Amersham and

had a specific activity of >37 TBq/mmol (1000 Ci/mmol). A
number of different protocols were used to analyse the
response to various stresses.

2.10.1. **Radiolabelling of *R. sphaeroides* during heat shock**

A 500 ml *R. sphaeroides* culture was grown
chemoheterotrophically at 30ºC as described in Section
2.5.1.1. When the culture reached an OD$_{650}$ of between 1.0
and 1.5, 10 ml aliquots were dispensed into universal
bottles prewarmed to 42ºC. Aliquots were also dispensed
into a pair of control bottles which had been prewarmed to
30ºC. Cultures were incubated in shaking water baths at
the appropriate temperatures. At the appropriate times
$^{35}$S-methionine was added to the aliquots, to a final
concentration of 5 µCi/ml. Cultures were then labelled for
a period of 15 minutes. Control cultures, incubated at
30ºC were labelled at the beginning, t = 0, and end of
the experiment. After labelling for 15 minutes, unlabelled
methionine, 250 mM stock, was added to a final
concentration of 2.5 mM to prevent any significant further
incorporation of isotope, and cultures were chilled on
ice.

Cells were then harvested by centrifugation at 6000 g
for 10 minutes at 4ºC. The pellets were resuspended in
1 ml TEMMB containing 25% (w/v) sucrose, 500 µg/ml
lysozyme and 20 µg/ml each of RNase A and DNase, then
transferred to 1.5 ml Eppendorf tubes. Cell-free extracts
were prepared by sonication. The resuspended cells were

subjected to 5 × 20 second bursts at an amplitude of 12-14 μ in a Soniprep 150 sonicator (MSE) fitted with an exponential microprobe, tip diameter 3 mm. Samples were cooled on ice between each sonication burst. Unbroken cells and cellular debris were then removed by centrifugation in a microfuge, 13,600 g, for 5 minutes at 4°C. The protein concentration of each sample was then determined (see 2.6.1.). Concentrations were then normalized for the presence of exogenous proteins.

Cell-free extracts were analysed in both 4-30% non-denaturing- and 10-30% SDS-polyacrylamide gels. Samples for non-denaturing-PAGE were analysed immediately, while those for SDS-PAGE were boiled in solubilization buffer and stored at -20°C if analysis was not performed immediately. After electrophoresis, gels were stained and dried as described in Sections 2.6.2.5. and 2.6.2.6. In certain cases, gels were dried without prior staining and bands located exclusively by autoradiography.

Autoradiography of dried gels was performed by placing the gel, face up, in an X-ray cassette (X-Ray Accessories Ltd.) and laying an 18 × 24 cm sheet of either X-ray film (Fuji NIF RX X-ray film, Fuji Photo Film Co., Ltd.) or Hyperfilm-βmax (Amersham) on top of the film. The cassette was then closed and stored at room temperature. Films were developed as described in section 2.19.

### 2.10.2. Radiolabelling of *R. sphaeroides* during exposure to chemical stresses

A 500 ml *R. sphaeroides* culture was grown as above. Universal bottles were prepared for labelling by dispensing 1 ml of 10 × stocks of the appropriate chemicals. Control bottles contained 1 ml of distilled water. The bottles were prewarmed to 30ºC. When the starter culture had reached an OD$_{550}$ of 1.0 to 1.5, 9 ml aliquots were dispensed to the prewarmed bottles. At the appropriate times $^{35}$S-methionine was added to the aliquots, to a final concentration of 5 µCi/ml. Cultures were then labelled for a period of 15 minutes. Control cultures were labelled at the beginning and end of the experiment.

After labelling, cultures were treated as described in Section 2.10.1.

### 2.10.3. Radiolabelling of *R. sphaeroides* during a shift from chemoheterotrophic to photoheterotrophic growth conditions

A 500 ml *R. sphaeroides* culture was grown as above. Universal bottles were prepared for labelling by sealing sterile universal bottles, containing 6 mm stirrer bars, with a sterile, size 45 Suba seal. The seal was then pierced with 2 G19 needles and the bottles flushed with oxygen-free nitrogen at a pressure of 15 psi for 5 minutes. Bottles were then prewarmed to 30ºC.

When the starter culture had reached an OD$_{550}$ of 1.0 to

1.5, it was transferred to a sterile 500 ml serum bottle
containing a 2 cm long stirrer bar. The bottle was then
sealed with a size 45 Suba seal, flushed with oxygen-free
nitrogen at 15 psi for 15 minutes and incubated under
lights as described in Section 2.5.1.1. At the appropriate
times, 10 ml aliquots were drawn from the serum bottle and
injected into the flushed, prewarmed Universal bottles.
$^{35}$S-methionine was added to the aliquots to a final
concentration of 5 µCi/ml by injection with a 100 µl
Hamilton syringe. The cultures were then incubated under
lights with stirring for a period of 15 minutes. At the
end of this period, unlabelled methionine was added to a
final concentration of 2.5 mM by injection using a
Hamilton syringe. The cultures were then chilled on ice
and processed as described in Section 2.10.1.

## 2.11. DNA purification

Both plasmid and chromosomal DNA were prepared in this
study. RNase A and phenol/chloroform were routinely used
to degrade RNA and remove protein in DNA preparations.
RNase A was pre-boiled according to the method of Maniatis
*et al.* (1982). Phenol/chloroform was prepared as follows:
100 g phenol crystals were dissolved in 100 ml chloroform
followed by addition of 4 ml isoamyl alcohol, 0.1 g 8-
hydroxyquinoline and 200 µl 2-mercaptoethanol. The phenol
was then neutralized by shaking the above mixture with two
changes of 0.2 volumes 1 M Tris.HCl pH 8.0. The

phenol/chloroform was then stored overnight, under 1 M
Tris.HCl pH 8.0, at 4°C. The phenol/chloroform was then
shaken with two changes of TE buffer and finally stored at
4°C under TE. The pH of the TE overlay should be 7.5-8.0.

## 2.11.1. Preparation of *R. sphaeroides*, *E. coli* and *Sulfolobus acidocaldarius* chromosomal DNAs

Chromosomal DNA preparations from *R. sphaeroides*, *E. coli* and *S. acidocaldarius* were carried in basically the same way. *S. acidocaldarius* cell paste was the kind gift of Dr Paul Norris.

1) In the case of *R. sphaeroides*, 10 ml of a culture
($OD_{650} = 2$) grown under photoheterotrophic conditions was inoculated into 500 ml PM medium, and the culture grown chemoheterotrophically in the dark overnight (see Section 2.5.1.1.). Final $OD_{650}$ was approximately 1.0. In the case of *E. coli*, 5 ml of a starter culture was inoculated into 500 ml prewarmed LB medium in a 2 l Erlenmeyer flask. The culture was grown overnight in an orbital shaker set at 250 rpm and 37°C. The cells were harvested by centrifugation in an MSE High Speed 18 centrifuge (10,000 g, 10 minutes, 4°C), then resuspended in 5 ml ice-cold 25% (w/v) sucrose in 50 mM Tris.HCl pH 8.0 and kept on ice.

2) 1 ml lysozyme solution (10 mg/ml in 10 mM Tris.HCl pH 8.0) was added and the solution gently swirled on ice for 5 minutes.

3) Add 1 ml 0.5 M EDTA pH 8.0 and gently swirl on ice

for 5 minutes.

4) Add 2 ml RNase A solution (20 mg/ml, preboiled) and gently swirl on ice for five minutes.

5) Add 9 ml ice-cold detergent solution (0.1% Triton-X-100; 0.0625 M EDTA pH 8.0; 50 mM Tris.HCl pH 8.0) and swirl gently on ice for 10 minutes. Both *E. coli* and *S. acidocaldarius* were lysed by this treatment.

6) *R. sphaeroides* was more resistant to lysis; thus after the last step the solution was heated at 60ºC with gentle swirling until lysis was complete (the viscosity of the solution suddenly increases).

7) The solution was cooled to 37ºC and 200 µl of proteinase K (5 mg/ml) added. This was then incubated for 30 minutes.

8) 1 g of caesium chloride was added per ml of solution.

9) Place in sealed VTi 65 tubes and spin at 40,000 rpm overnight at 15ºC.

10) Tubes were punctured at the top and bottom and DNA was collected by viscosity of solution, using sterile needles.

11) The chromosomal DNA was then dialysed against TE buffer at 4ºC to remove CsCl.

12) DNA was stored over chloroform in sterile bijoux at 4ºC.

## 2.11.2. Preparation of chromosomal DNA from *R. vannielii*

*R. vannielii* was grown photoheterotrophically to an OD$_{680}$ of about 1.5. Chromosomal DNA preparation was

carried out essentially as above, except that after
resuspending the pellet as in step 1, cells were passed
through a French pressure cell at 1000 psi (Russell,
1984). This step causes minimal lysis, as assessed by
microscopy, but has been reported to render cells
uniformly sensitive to lysozyme/EDTA treatment (Russell,
1984). The preparation was then carried out as above
including the heat treatment in step 6).

### 2.11.3. Preparation of plasmid DNA

Different methods were used for small-scale and large
scale preparation of plasmid DNA.

### 2.11.3.1. Small-scale preparation of plasmid DNA

This was achieved by using the rapid boiling method as
described by Maniatis *et al*. (1982).

1) 10 ml of medium containing the appropriate antibiotic
was inoculated, and incubated overnight in an orbital
shaker set at 37°C and 250 rpm. When screening plasmid
libraries, 25 isolates were patched onto duplicate LB
plates containing 100 µg/ml ampicillin. Plates were
incubated overnight at 37°C. Colonies were washed off one
of the duplicate plates by adding 5 ml LB containing 100
µg/ml ampicillin, and scraping off colonies with a
spreader bar. Colonies were further dispersed by
repeatedly pipetting with a 1 ml pipette.

2) 1.5 ml of the culture was dispensed into an Eppendorf
tube and centrifuged for 1 minute. The remainder of the
overnight culture was stored at 4°C.

3) The medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

4) The cell pellet was resuspended in 0.35 ml of detergent solution (sucrose, 8% (w/v); Triton X-100 0.5% (v/v); 50 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0).

5) 25 µl of a freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris.HCl, pH 8.0) was added to the above and the solutions mixed by vortexing for 3 seconds.

6) This was placed in a boiling water bath for 40 seconds and then immediately centrifuged at room temperature for 10 minutes.

7) The pellet was removed from the tube with a toothpick.

8) 40 µl of 2.5 M sodium acetate and 420 µl of ice-cold isopropanol were added to the supernatant which was then mixed by vortexing.

9) This was then centrifuged for 15 minutes, the resulting pellet washed with 70% (v/v) ethanol and then dried under vacuum.

10) The dry pellet was resuspended in 50 µl of TE containing pre-boiled RNase A (50 µg/ml) and incubated at 37°C for 10 minutes. 10 µl aliquots of this solution could be restricted or analysed on agarose gels without further treatment.

## 2.11.3.2. Large-scale preparation of plasmid DNA

The method used for large scale plasmid purification is a scaled-up version of that of Birnboim and Doly (1979).

1) 500 ml LB medium in a 2 l Erlenmeyer flask was inoculated with a 10 ml seed culture and incubated at 250 rpm, 37°C until late exponential phase. Where the plasmid was pBR322-based 75 mg of chloramphenicol powder was added and incubation continued for a further 14-16 hours. Under these conditions chromosomal DNA replication is halted and plasmid replication continues.

2) Cells were harvested by centrifugation at 10,000 g for 10 minutes in a 6 × 250 ml rotor in an MSE High Speed 18 centrifuge.

3) The supernatant was poured off and the cells resuspended in 6.5 ml solution 1 containing 10 mg/ml lysozyme. Cells were transferred to an Oakridge centrifuge tube and incubated for 10 minutes at 37°C.

| Solution 1 | |
|---|---|
| Glucose | 50 mM |
| Tris.HCl pH 8.0 | 25 mM |
| EDTA | 10 mM |

4) 13 ml of solution 2 was added to the tube and the contents mixed gently by inversion. The tube was then stored on ice for 10 minutes.

| Solution 2 | |
|---|---|
| NaOH | 0.2 M |
| SDS | 1.0% (w/v) |

Solution 2 was made up fresh from stock solutions of 10 M NaOH and 10% (w/v) SDS.

5) 6.5 ml of ice-cold solution 3 (5 M potassium acetate pH 4.8) was added to the tube and the contents mixed gently by inversion. The tube was incubated on ice for 15

minutes.

6) The lysate was cleared by centrifugation in an 8 x 50 ml rotor at 27,200 g, 4°C for 15 minutes.

7) 0.6 volumes of ice-cold isopropanol was added to the cleared lysate. After mixing by inversion, the tube was stored overnight at -20°C to ensure complete precipitation.

8) DNA was pelleted by centrifugation at 27,200 g, 4°C for 15 minutes. The pellet was then washed in 70% ethanol.

9) The pellet was dried under vacuum.

10) The plasmid DNA was made up with TE to a final volume of 4.2 ml.

11) 4.7 g of caesium chloride was added to the solution and mixed by gentle swirling until fully dissolved.

12) 0.5 ml of a 10 mg/ml ethidium bromide solution was added and mixed by gentle swirling.

13) The solution was then sealed in VTi 65 tubes and centrifuged at 40,000 rpm overnight at 15°C.

14) 2 bands were generally visible. Irradiation with long-wave UV light was not always necessary. The lower of the two bands contained covalently closed, circular plasmid DNA. After piercing the top of the tube, this band was removed using a syringe with a G21 needle and transferred to a 1.5 ml Eppendorf tube.

15) Ethidium bromide was removed by repeated extractions against isoamyl alcohol. Generally three extractions were sufficient to remove all traces of the pink coloration.

16) Caesium chloride was removed by dialysis against TE at 4ºC.

17) The DNA was precipitated with two volumes of ice-cold ethanol, washed and dried as above and dissolved in a suitable volume of TE. The DNA solution was stored in aliquots at −20ºC.

## 2.12. Transformation of *E.coli* with plasmid DNA

Competent cells were prepared, stored and transformed in a single solution according to the protocol of Chung *et al.* (1989).

1) A fresh overnight culture of *E. coli* MC 1061 was diluted 1:100 into 50 ml prewarmed LB broth and the cells were incubated at 37ºC with shaking (250 rpm) to an $A_{600}$ of 0.3 of 0.4.

2) Cells were harvested by centrifugation (10,000 g, 10 minutes) and resuspended in 5 ml filter-sterilized TSS (LB with 10% PEG 6000, 5% DMSO and 49 mM $MgCl_2.6H_2O$ at a final pH of 6.5).

3) For long term storage, cells, in 0.5 ml aliquots, were frozen immediately in a dry ice/ethanol bath and stored at −70ºC.

4) For transformation, a 0.1 ml aliquot of the cells was dispensed into a cold Eppendorf tube containing approximately 100 pg of plasmid DNA and the cell/DNA suspension mixed gently by inversion. (When frozen cells were used cells were thawed slowly on ice and used

immediately.)

5) The cell/DNA mixture was incubated for 5-60 minutes at 4°C.

6) A 0.9 ml aliquot of LB broth plus 20 mM glucose was added, and the cells incubated at 37°C with shaking (225 rpm) for 1 hour to allow expression of antibiotic resistance.

7) Transformants were selected on LB plates containing 100 μg/ml ampicillin.

## 2.13. Agarose gel electrophoresis

Preparations of both chromosomal and plasmid DNA were analysed by electrophoresis in 1% (w/v) agarose (type II, Sigma) gels made up in tris-borate electrophoresis buffer, TBE, containing 1 μg/ml ethidium bromide. TBE was made up as a 10 × stock solution as follows:

Tris-borate electrophoresis buffer (TBE)

| Constituents | Amount |
|---|---|
| Tris base | 108 g |
| Boric acid | 55 g |
| 0.5 M EDTA pH 8.0 | 20 ml |

This stock solution was made up to 1 l with distilled water.

Agarose gels were made up by heating the agarose/TBE mixture with constant swirling until the agarose went into solution. This solution was then cooled to 45°C, ethidium bromide added to the required concentration, and then poured into the gel bed.

Samples, in TE or restriction buffer, were prepared for electrophoresis by adding 0.1 volumes of 75% (v/v) glycerol containing 0.2% (w/v) bromophenol blue. The solution was mixed by flicking the side of the Eppendorf tube, which was then given a 2 second microfuge spin to return all of the solution to the bottom of the tube. Samples were then loaded to the gel and electrophoresis was carried out at 50 V if the gel was to be run overnight, or 200 V if run during the day.

DNA bands were visualized over a long-wave UV transilluminator. If gels were to be photographed, they were transferred to a short-wave transilluminator and photographed using Polaroid P665 positive/negative film. Exposure times for good prints were about 10 seconds.

## 2.14. Restriction, electroelution and ligation of DNAs

### 2.14.1. DNA restriction

Both plasmid and chromosomal DNAs were cut with restriction enzymes. Manufacturers of restriction enzymes also supplied 10 × restriction buffer stocks. Digestions were carried out in the presence of 4 mM spermidine, 0.1 mg/ml BSA and 1 mM 2-mercaptoethanol. Approximately 10 units of the appropriate enzyme were added per µg of DNA, and restrictions were carried out for 1 hour at 37ºC. Restriction digests were then analysed by agarose gel electrophoresis as described in section 2.13.

## 2.14.2. Electroelution of DNA from agarose gels

Bands of interest, either plasmid DNA inserts or specific regions of chromosomal DNA restriction ladders, were visualized on a long-wave UV transilluminator and excised with a new razor blade. The time that the DNA is exposed to UV light must be kept to an absolute minumum. DNA was recovered using an IBI electroeluter. The DNA was eluted at 100 V for 30 minutes from TBE into a well containing 7.5 M ammonium acetate. The DNA sample was recovered by ethanol precipitation and stored in TE buffer at -20°C.

## 2.14.3. DNA ligation

Calf intestinal phosphatase (CIP) was obtained from BCL. CIP was used to remove the 5' phosphate from vector DNA molecules prior to ligation. This treatment greatly reduces self-ligation of vector DNA and so dramatically increases the proportion of recombinant molecules obtained.

Restriction digests of vector DNA, normally containing about 2 µg of DNA in a volume of 20 µl, were carried out as usual and then made up to 100 µl with TE. 1 µl of CIP was added and the incubation continued for a further 30 minutes at 37°C. The solution was then extracted with phenol/chloroform and precipitated with ethanol.

T4 DNA ligase and 5 × ligation buffer were supplied by Gibco-BRL andused according to the manufacturers instructions. All ligations were carried out overnight at

15⁰C. Typically tubes containing a range of volumes of insert DNA, including a control tube with no insert DNA, were set up. Ligation reactions were made up as follows:

| | |
|---|---|
| Vector DNA (1 µg/ml) | 1 µl |
| Ligation buffer (5 ×) | 2 µl |
| T4 DNA ligase (2.5 U/µl) | 1 µl |

Either 0, 1, 2 or 5 µl of insert DNA at a concentration of about 40 ng/ml was added and the solution made up to a final volume of 10 µl with sterile distilled water.

## 2.15. Southern blotting of agarose gels

Southern blotting was carried out as described by Maniatis *et al.* (1982). Various dilutions of 20 × SSC are required for this procedure.

### 20 × SSC

| Constituents | g/l |
|---|---|
| NaCl | 175.3 |
| Na₃ citrate | 88.2 |

The above was dissolved in 800 ml Elga water and the pH adjusted to 7.0 with a few drops of 10 M NaOH. Volume was then adjusted to 1 l.

1) After agarose gel electrophoresis, the gel was denatured in enough denaturing solution (1.5 M NaCl; 0.5 M NaOH) to cover the gel. This was left for 15 minutes and the procedure then repeated twice, leaving in the final solution for at least 30 minutes.

2) The denaturing solution was replaced with neutralizing solution (1.5 M NaCl; 0.5 M tris-HCl, pH 7.2; 1 mM EDTA) and leave for at least 30 minutes. Repeat

twice. Remove neutralizing solution and blot off excess
liquid from the gel with tissues.

3) A sheet of filter paper the same width as the gel,
but long enough to form a wick between the buffer
reservoir and the transfer apparatus, was cut. This filter
was carefully placed on top of the gel so that the gel was
centrally located. In this and subsequent steps, care was
taken to avoid trapping any air bubbles.

4) The gel and filter paper were inverted and placed on
the bridge over the reservoir wth the wicks dipping into
the reservoir at each end.

5) A piece of Hybond-N membrane (Amersham) cut to the
same size as the gel was then carefully placed on top of
the gel. Three sheets of Whatman 3MM paper cut to a size
slightly larger than the gel were then laid on top of
this, and a pile of tissues 4-5 cm in height and cut to
the same size was laid on top of the filter papers. The
stack was then compressed using a glass plate and a 1 kg
weight.

6) The buffer reservoir was filled with 10 × SSC and
transfer was allowed to take place overnight.

7) After transfer, the membrane was carefully removed
and washed in 2 × SSC to remove residual gel. The
membrane was then air-dried, wrapped in cling-film and
placed, DNA side down, on a standard UV transilluminator.
The membrane was then irradiated for 5 minutes.

## 2.16. Nick-translation of DNA

The method used to radio-label purified DNA was essentially the same as that described by Maniatis *et al.* (1982). The following stock solutions were required:

### Nick-translation buffer (NTB)

| Constituents | Amount |
|---|---|
| Tris.HCl pH 7.8 (0.5 M) | 5 ml |
| 2-mercaptoethanol | 1 ml |
| $MgCl_2.6H_2O$ (0.5 M) | 1 ml |
| BSA (50 mg/ml) | 100 µl |

### dNTP stock

| Constituents | Amount |
|---|---|
| dGTP (10 mM) | 1 µl |
| dATP (10 mM) | 1 µl |
| dTTP (10 mM) | 1 µl |

This mixture was then made up to a final volume of 30 µl with sterile distilled water.

A dilution of DNase I was prepared to give a final concentration of 0.1 µg/ml and used immediately.

Nick-translations were carried out as follows:

1) The following reaction mixture was prepared on ice:

| Constituents | Amount |
|---|---|
| NTB | 10.0 µl |
| dNTP stock | 7.5 µl |
| DNase I (0.1 µg/ml) | 3.0 µl |
| DNA | 0.5 µg |
| [$\alpha$-$^{32}$P] dCTP | |

[$\alpha$-$^{32}$P] dCTP (Amersham) specific activity 100 TBq/mmol (3000 Ci/mmol).

This mixture was then made up to 90 µl with sterile distilled water and incubated for 10 minutes at 12°C.

2) 1 µl of DNA polymerase I was added and the incubation continued for a further 75 minutes.

3) The reaction was stopped by adding 150 μl of stop solution (10 mM Tris.HCl pH 8.0; 1 mM EDTA pH 8.0; 0.1% (w/v) SDS).

4) The labelled DNA was separated from incorporated material by passing the mixture through a Sephadex G50 (Pharmacia) size exclusion column. The column was prepared in a siliconized glass Pasteur pipette plugged with siliconized glass wool. The Sephadex was allowed to swell overnight in a large volume of TE. The column was equilibrated with TE plus 0.1% (w/v) SDS before use. The reaction mixture was loaded to the top of the column and allowed to run in. 150 μl aliquots of TE plus 0.1% (w/v) SDS were added to the top of the column and the fractions were collected in separate Eppendorf tubes. Peaks of radiolabelled material were detected using a hand-held radiation monitor. The first peak contains the radiolabelled DNA. Fractions from this peak were pooled and stored at -20ºC.

## 2.17. DNA blot hybridisation

Hybridizations were carried out in heat-sealed plastic bags from which all air bubbles were carefully excluded. 20 × SSC and 100 × Denhardt's solution are required for hybridization.

### 100 × Denhardt's solution

Constituents
2% (w/v) bovine serum albumin
2% Ficoll

2% polyvinyl pyrollidone

This was filter sterilized and stored in 10 ml aliquots at -20ºC.

The pre-hybridization mixture was made up as follows:

Pre-hybridization mixture
| | |
|---|---|
| 20 × SSC (6 × final) | 7.5 ml |
| 100 × Denhardt's solution (5 × final) | 1.25 ml |
| 10% (w/v) SDS (0.5% final) | 1.25 ml |

This was made up to 25 ml with sterile, distilled water.

0.5 ml of a 1 mg/ml solution of sonicated calf thymus DNA solution was denatured by heating in a boiling water bath for 5 minutes. This solution was then chilled on ice and added to the pre-hybridization mixture, which was then added to the membrane. Pre-hybridization was carried out in a water bath at 65ºC with gentle shaking for a minimum of 1 hour. After pre-hybridization heterologous DNA was denatured as above, but with nick-translated probe also present. This was added to the membrane and hybridization carried out for at least 12 hours at 65ºC with gentle shaking.

After hybridization, the membrane was removed from the bag and immediately submerged in a tray containing 2 × SSC and 0.5% SDS at room temperature. After 5 minutes the filter was transferred to 2 × SSC containing 0.1% SDS, incubated for 15 minutes at room temperature with gentle agitation. The membrane was then transferred to a solution of 0.1 × SSC and 0.1% SDS and incubated at 65ºC for 2 hours with gentle agitation. The buffer was then changed

and incubation continued for a further 30 minutes.

The membrane was dried at room temperature on a sheet of Whatman 3MM paper. The membrane was then wrapped in clingfilm.

Autoradiography was performed by laying the membrane face up in an X-ray cassette. A sheet of Fuji X-ray film was laid on top of the membrane and an intensifying screen (Dupont Cronex Lightning Plus) was placed face down over the film. The cassette was then placed at -70°C overnight and developed (Section 2.19.) the next day. If the intensity of the bands was not good enough for clear visualization longer exposures were performed.

## 2.18. <u>Photography and light microscopy</u>

With the exception of DNA gels, all photography was performed using Panatomic-X film (Eastman Kodak Co.).

Photographs of bacterial cells were taken using a Leitz Dialux 22/22 EB microscope fitted with a Leitz Vario Orthomat camera assembly. Samples to be photographed were mounted on a slide coated with a thin layer of 1% (w/v) noble agar (Difco), and covered with a cover slip. A sample size of 5 µl was found to be adequate. The microscope was set up for phase contrast and all photographs were taken at maximum magnification.

All other photographs were taken using a Minolta X-300 camera.

Films were developed using D19 developer (Kodak Ltd.)

and Unifix (Kodak Ltd.) according to the manufacturer's
instructions. Prints were made on Kodabrome II RC M
photographic paper (Eastman Kodak). Prints were developed
using Ilford Contrast FF developer and Unifix according to
the manufacturers instruction's.

## 2.19. Development of autoradiograms

Autoradiograms were developed in a 1 in 10 solution LX-
24 X-ray developer (Kodak Ltd.) and fixed in a 1 in 10
solution of FX-40 X-ray fixer (Kodak Ltd.). Fuji X-ray
film was soaked in each solution for 5 minutes. Hyperfilm-
8max was subjected to an extended, 10 minute, period of
fixation.

# Detection, purification and characterization of a
## *Rhodobacter sphaeroides* cpn60

### 3.1. Introduction

At the time that this work was started few chaperonins
had been characterized. The first protein of this type to
be characterized was the GroEL protein from *E. coli*
(Hendrix, 1979; Hohn *et al.*, 1979). This protein was shown
to be composed of 14 identical subunits of $M_r$ 65,000. This
complex was shown to have a seven-fold axis of symmetry
and to be composed of two rings of subunits stacked one on
top of the other (Hendrix, 1979; Hohn *et al.*, 1979). At
this time the GroEL protein was primarily of interest
because of its known involvement in the assembly of a
number of bacteriophage (for examples see Georgopoulos *et
al.*, 1972; 1973; Takano and Kakefuda, 1972; Zwieg and
Cummings, 1973). Mutations in the *groE* locus were also
known to cause defects in cell growth (Georgopolous *et
al.*, 1973; Sternberg, 1973), but the physiological basis
of these defects was unknown.

In 1982, while purifying glutamine synthetase, Pushkin
*et al.* (1982) discovered and purified a protein from pea
leaves that was very similar in terms of structural
properties to the GroEL protein. The authors reported that
the content of this protein varied during the development
of pea leaves and was maximal in young leaves. This

observation led them to speculate that the protein might be involved in the assembly of oligomeric structures (enzymes) in pea leaves. At about the same time Barraclough and Ellis (1980) reported that, in isolated pea chloroplasts, newly synthesized Rubisco large subunits were not immediately incorporated into the holoenzyme, but were found associated with a polypeptide of $M_r$ 60,000. They suggested that this polypeptide may specifically combine with newly synthesized large subunits and somehow play a role in the assembly of the holoenzyme.

Sequence analysis of the *groEL* gene and cDNA encoding the putative Rubisco subunit binding proteins from castor-bean (*Ricinus communis*) and wheat (*Triticum aestivum*), demonstrated that the Rubisco subunit binding protein and the *E. coli* GroEL protein were evolutionary homologues (Hemmingsen *et al.*, 1988). Both of these proteins had been implicated in the assembly of multimeric complexes and were termed chaperonins (Hemmingsen *et al.*, 1988). DNA hybridization studies (Young *et al.*, 1988) as well as immunological studies (see Young, 1990) have indicated that chaperonins might occur universally in prokaryotes and membrane-bound organelles. The importance of chaperonins in protein assembly is now generally accepted, (see 1.4.) and in the last few years chaperonins from a number of sources have been characterized (see 3.5.).

*R. sphaeroides* is capable of growth modes ranging from chemoheterotrophy to photoautotrophy (see Tabita, 1988).

This organism encodes two forms of Rubisco, a form I
($L_8S_8$) enzyme and a form II enzyme that is composed
exclusively of large subunits (see Tabita, 1988). Evidence
has been presented that *R. sphaeroides* carries two
discrete chromosomes of about 3,000 and 900 kb. These are
referred to as chromosome I and chromosome II respectively
(Suwanto and Kaplan, 1989). Copies of several genes from
chromosome II also appeared to be present on chromosome I,
but the extent of the DNA homology between alleles is very
low. Interestingly, genes encoding enzymes involved in $CO_2$
fixation and linked to the gene encoding the form I
Rubisco were located on chromosome I, whereas the form II
Rubisco and associated enzymes were located on chromosome
II. Since Goloubinoff *et al*. (1989b) have presented
evidence clearly showing a role for the GroEL protein in
the formation of $L_2$ dimers, it seems possible that the two
chromosomes of *R. sphaeroides* might encode distinct
chaperonins. Both the nutritional diversity and the
possible expression of multiple chaperonin genes indicated
that *R. sphaeroides* might be an ideal test organism for
the study of the role of chaperonin-type proteins. Before
such a study could be undertaken it was necessary to first
demonstrate that *R. sphaeroides* does indeed express a
GroEL homologue. Initial attempts to isolate and
characterize such a protein were conducted with cell-free
extracts from photoheterotrophically grown cells.

   *Rhodomicrobium vannielii* is, like *R. sphaeroides*, a

member of the α subdivision of the purple bacteria phylum (see Woess, 1987 for phylogenetic tree). This bacterium was under study in other projects being conducted in the group. Western blot analysis using a monoclonal antibody that recognised a *M. tuberculosum* cpn60 epitope had been shown to cross-react with a single $M_r$ 60,000 *R. vannielii* protein (Graeme MacDonald, personal communication). While attempting to isolate and characterize an *R. sphaeroides* cpn60 protein, comparisons were occasionally made with *R. vannielii*.

## 3.2. Growth of *R. sphaeroides*

Throughout this study *R. sphaeroides* was grown in PM medium (2.3.1.). Initial experiments to identify and isolate a *R. sphaeroides* chaperonin were conducted using photoheterotrophically grown cells, while in some later studies chemoheterotrophically grown cells were used. Figure 3.2.1. shows growth curves of cultures grown under both of these sets of conditions. The appearance of cells grown under these conditions as visualized by phase-contrast microscopy is also shown in this figure. Cells grown under both regimes are rods, with those grown photoheterotrophically being about two to three times as long as those grown chemoheterotrophically. The generation time of chemoheterotrophically grown *R. sphaeroides* is approximately 2.8 hours and that of photoheterotrophically grown cells approximately 4.8 hours.

Figure 3.2.1. Growth of *R. sphaeroides* in PM medium under chemoheterotrophic and photoheterotrophic growth conditions at 30°C

PHOTOHETEROTROPHIC    CHEMOHETEROTROPHIC
       ○                      ●

Cultures were grown photoheterotrophically and chemoheterotrophically as described in Materials and Methods (2.5.1.1.). Inset photographs are of cells examined by phase-contrast microscopy.
  A. Photoheterotrophically grown cells.
  B. Chemoheterotrophically grown cells.

## 3.3. Identification of a putative *R. sphaeroides* GroEL homologue

The most striking characteristics of the chaperonins so far examined are their seven-fold axis of symmetry, tetradecameric structure, high native molecular weight of approx. $M_r$ 700,000 and their abundance in cell free extracts (see Hemmingsen *et al.*, 1989). The *R. sphaeroides* form 1 Rubisco, native molecular weight approx. $M_r$ 550,000 (Gibson and Tabita, 1977), is in the same size range as might be expected for a putative chaperonin. For this reason, initial attempts to identify a *R. sphaeroides* chaperonin made use of protocols that had been developed for the purification of *R. sphaeroides* Rubisco. Thus, cell-free extracts, from photoheterotrophically grown cultures, were prepared by the method of Gibson and Tabita (1985) (2.9.1.1.). These extracts were then fractionated in sucrose density gradients and the fractions analysed in non-denaturing 4-30% exponential gradient polyacrylamide gels. Out of necessity (the demise of a make of centrifuge and associated rotor), two methods of sucrose density gradient centrifugation were used. Figure 3.3.1. shows the results obtained using a 0.2-0.8 M discontinuous sucrose gradient centrifuged in an MSE fixed-angle rotor (see 2.9.2.1.). Figure 3.3.2. shows the results obtained when an 8-28% (w/v) continuous sucrose gradient was centrifuged in a Beckmann VT150 rotor (see 2.9.2.2.). The latter method was adapted from a protocol for the rapid

Figure 3.3.1.  Analysis of *R. sphaeroides* cell-free extract fractionated by discontinuous sucrose density gradient centrifugation

1 ml fractions from a discontinuous sucrose gradient were collected. 25 µl aliquots were resolved in a 4-30% native polyacrylamide gel (Coomassie-stained). Tracks are numbered by gradient fraction, the bottom of the gradient being fraction 1.

Figure 3.3.2. Analysis of *R. sphaeroides* cell-free extract fractionated by continuous sucrose density gradient centrifugation

1.5 ml fractions from a continuous sucrose gradient were collected. 25 μl aliquots were resolved in a 4-30% native polyacrylamide gel, (Coomassie-stained). Tracks are numbered by gradient fraction, the bottom of the gradient being fraction 1.

purification of Rubisco from soyabean leaves which also,
from gel photographs presented, co-purified the soyabean
chloroplast cpn60 (Paech and Dybing, 1986).

This approach of sucrose density gradient and native gel
analyses clearly demonstrated the presence of abundant
proteins of apparent $M_r$ 670,000 and 550,000 in extracts of
photoheterotrophically grown *R. sphaeroides* cultures. The
$M_r$ 550,000 protein band was thought likely to be Rubisco.
Upon Green A chromatography this band was shown to be
composed of two proteins; one that did not bind to the
column was identified as glutamine synthetase by N-
terminal sequence analysis, while the other, which was
eluted from the column by washing with 1 M NaCl, was
identified as Rubisco by virtue of its enzymatic activity
(data not shown). This left the $M_r$ 670,000 protein as the
most likely candidate for the *R. sphaeroides* cpn60 and
further attempts to identify a *R. sphaeroides* cpn60
protein focussed exclusively on this protein.

When *R. vannielii* was grown and processed in an
identical manner, no equivalent discrete protein band in
the 700,000 kD range was present (Fig. 3.3.3.). However, a
somewhat diffuse band could be seen in this region (Fig.
3.3.3., lanes 4 and 5). No further attempts were made to
purify this *R. vannielii* protein.

The putative *R. sphaeroides* cpn60 protein was further
purified by pooling the appropriate SDGC fractions and
applying this material to a Mono Q HR 5/5 anion-exchange

Figure 3.3.3.  Comparison of *R. sphaeroides* and *R. vannielii*
cell-free extracts fractionated by discontinuous
sucrose density gradient centrifugation



1 ml fractions from discontinuous sucrose gradients were collected.
50 µl aliquots were resolved in a 4-30% native polyacrylamide gel
(Coomassie-stained). Tracks are numbered by gradient fraction, the
bottom of the gradient being fraction 1. Rm = *R. vannielii*; Rs = *R.
sphaeroides*

column. Proteins were eluted from the Mono Q column with a
0.0-1.0 M NaCl gradient that was developed as shown in
Figure 3.3.4. 1 ml fractions were collected and 50 µl
aliquots analysed in 4-30% non-denaturing exponential
gradient gels (Fig. 3.3.4.). The putative cpn60 eluted as
a broad peak at about 370 mM NaCl. Fractions containing
the partially purified putative cpn60 were collected and
pooled. SDS-PAGE of these fractions indicated that the $M_r$
670,000 protein was composed of a single polypeptide of $M_r$
58,000 (see Fig. 3.3.8.).

Several reports (Bloom *et al.*, 1982; 1983; Hemmingsen
and Ellis, 1986; Musgrove *et al.*, 1987) described the
dissociation of the *P. sativum* chloroplast chaperonin into
monomeric subunits when stromal extracts were incubated in
the presence of MgATP. Similarly, the GroEL tetradecamer
has been shown to dissociate to a species of relatively
low molecular mass with an unknown number of subunits
(Goloubinoff *et al.*, 1989a). The effect of MgATP on the
putative *R. sphaeroides* cpn60 protein was investigated.
The pooled fractions were incubated in the presence of
MgATP under the same conditions used by Musgrove *et al.*
(1987). Figure 3.3.5.A. shows the result of such an
experiment. After 30 minutes on ice in the presence of 5
mM MgATP a new band can be seen in the Coomassie stained
gel (Fig. 3.3.5.A., track 5). It seemed possible that the
appearance of this band was correlated with a slight
reduction in the staining of the putative Rs cpn60 band.

A. Elution profile. Column flow rate of 0.5 ml/min. Fraction size 1 ml. The NaCl gradient was developed as shown.

B. 50 μl aliquots resolved in a 4-30% native polyacrylamide gel (Coomassie-stained). Tracks are numbered by fraction number.

Figure 3.3.5.   MgATP-mediated dissociation of the partially
                purified putative *R. sphaeroides* chaperonin 60

The putative *R. sphaeroides* chaperonin 60, partially purified by
density gradient centrifugation and Mono Q chromatography was
incubated in the presence of MgATP, at the concentrations noted, for
30 minutes on ice. 50 µl aliquots were resolved in a 4-30% native
polyacrylamide gel. Proteins were then either Coomassie-stained (A)
or western blotted and probed with anti-Rs cpn60 (B) (see Section
3.5.).

Furthermore, the new band has an $M_r$ of about 120,000,
indicating that it could be the dimeric form of the *R.
sphaeroides* protein. This observation did, however,
further encourage the thought that the protein of apparent
$M_r$ 670,000 was in fact the *R. sphaeroides* cpn60.
Subsequent production of antisera (see 3.5.) that
recognized this protein, allowed this experiment to be
repeated and Western blot analysis of the native gel to be
conducted. This analysis reveals that the $M_r$ 120,000 band
cross-reacts strongly with this antibody thus
demonstrating that this band was indeed derived from the
putative cpn60 (Fig. 3.3.5.B.). The circumstantial
evidence gathered at this point suggested that the $M_r$
670,000 protein was indeed an *R. sphaeroides* chaperonin.

The protein was further purified by gel filtration using
a G3000SW column connected to a LKB BROMMA HPLC system
(2.9.3.3.). The column has an upper exclusion limit of
500,000 kD. It was hoped that this column, from which a
protein of $M_r$ 700,000 would be excluded, would separate
the smaller protein contaminants and perhaps even Rubisco
from the protein mixture. Figure 3.3.6. shows the results
of this analysis. The only visible contaminant in the
protein preparation at this point appears to be Rubisco.
However, the low overall protein concentration in
fractions eluted from this column means that any minor
contaminants would probably not be detected.

Fractions containing the putative *R. sphaeroides*

-141-

**Figure 3.3.6.** **Further purification of Rs cpn60 by gel filtration**

A



B



Pooled fractions from Mono Q chromatography (Figure 3.3.4.) were concentrated by ultrafiltration then applied to a TosoHaas G3000SW column.

A. Elution profile. Column flow rate 3 ml/min. Fraction size 3 ml.

B. 50 µl aliquots resolved in a 4-30% native polyacrylamide gel (Coomassie-stained). Tracks are numbered by fraction number.

chaperonin were pooled and then subjected to a final
purification step by anion exchange chromatography on a
Mono Q column. Proteins were eluted from this column using
a slightly flatter NaCl gradient. Since Rubisco elutes
from this column slightly later than the chaperonin, it
was hoped that the flatter gradient would produce a good
enough separation of Rubisco and the putative chaperonin.
Figure 3.3.7. shows the results of this analysis. The
protein elution profile shows that three peaks are visible
in the region where the putative chaperonin would be
expected to elute. Native gel analysis showed that
fraction 36 appeared to contain pure chaperonin protein.

Figure 3.3.8. shows the results of SDS-PAGE analysis of
protein from various stages, not including the gel
filtration step, in the purification of the putative
chaperonin. Tracks were loaded with 5 μg of protein from
each of the steps. The final purification step clearly
shows that the $M_r$ 670,000 protein (Fraction 36, Figure
3.3.7.) is composed of a single polypeptide of $M_r$ 58,000.
This subunit composition is what would be expected of a
cpn60 type molecule.

The yield of pure protein from a 500 ml *R. sphaeroides*
culture was approximately 250 μg. This was calculated to
be approximately 50 μg per g dry weight of cells. Purified
protein was stored in 50% glycerol at -20ºC. Further
proof that this protein is an *R. sphaeroides* chaperonin

Figure 3.3.7. **Further purification of Rs cpn60 by a second round of Mono Q chromatography**

Pooled fractions from the gel filtration column were applied to a second Mono Q column.

A. Elution profile. Column flow rate of 0.5 ml/min. Fraction size 1 ml. The NaCl gradient was developed as shown.

B. 50 μl aliquots resolved in a 4-30% native polyacrylamide gel (Coomassie-stained). Tracks are numbered by fraction number.

Figure 3.3.8.   SDS-PAGE analysis of protein from various steps in the Rs cpn60 purification procedure

Each was track loaded with a constant amount of protein, 5 μg/track. Track 1, *R. sphaeroides* cell-free extract; track 2, pooled sucrose density gradient material; track 3, pooled material from the first Mono Q column; track 4, fraction 36 from the second Mono Q column. Polypeptides resolved in 10% SDS-polyacrylamide gel (T = 10.1%, C = 1%)

was sought by determining the N-terminal amino acid
sequence of the $M_r$ 58,000 polypeptide.

## 3.4. Amino-terminal sequence determination

The purified protein was lyophilized prior to resolution
in a 10% SDS polyacrylamide mini-gel, followed by
electrophoretic transfer onto an Immobilon membrane
(2.6.4.). After staining with Coomassie blue in methanol
(2.6.4.), the band of interest was excised and the protein
sequenced using an Applied Biosystems 470A protein
sequencer. Phenylthiohydrantoin amino acids were analysed
on line using an Applied Biosystems 120A analyser. The
sequence of the first 19 amino acids, presented using the
one letter amino acid code (IUPAC-IUB, 1970; 1984), was
determined to be:


A A K D V K F D T D A R D X M L(R)G V


This sequence is identical to GroEL at 15 of the 19
residues determined, i.e. 79% identity (see Fig. 3.4.2.).
This degree of identity between the sequences, combined
with the circumstantial evidence already presented,
confirmed that the protein isolated is a member of the
cpn60 family of proteins. For the rest of this work, the
protein is referred to as Rm cpn60. Figure 3.4.1.
compares the above sequence with the N-terminal sequences

Figure 3.4.1. __Comparison of N-terminal sequences of cpn60__
__proteins from various prokaryotic and eukaryotic__
__sources__

```
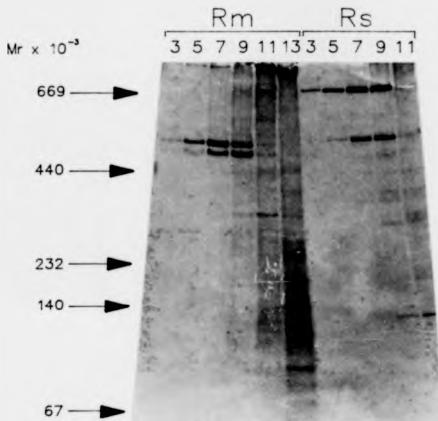Rs cpn60   A A K D V K F D T D A R D X M L(R)G V

GroEL   M A A K D V K F G N D A R V K M L R G V

Mtb   M A K T I A Y D E E A R R G L E R G L

Syn   M A K R I I Y N E N A R R A L E K G I

Cbu   M A A K V L K F S H E V L H A M S R G V

Hsp60   --S S H K E L K F G V E G R A S L L K G V

WBPα   G A D A K E I A F D Q K S R A A L Q A G V

PBPα   A A K D I A F D Q H S R S A M Q A G I

PBPβ   A K E L H F N D K G S A I R K L Q N
```

Sources of sequences: Rs cpn60 (protein), this study; GroEL (*E. coli*, gene) (Hemmingsen *et al.*, 1988); Mtb (*M. tuberculosis* 65 kD antigen, gene) (Shinnick, 1987); Syn (*Synechococcus* P1 related protein) (Cozens and Walker, 1987); Cbu (*Coxiella burnetii* 62 kD antigen, gene) (Vodkin and Williams, 1988); Hsp60 (*S. cerevisiae* mitochondrial hsp60, gene) (Reading *et al.*, 1989); WBPα (*T. aestivum* plastid chaperonin α subunit, gene) (Hemmingsen *et al.*, 1988); PBPα and PBPβ (*P. sativum* plastid chaperonin, α and β subunits, protein) (Musgrove *et al.*, 1987). Underlined residues are identical to the Rs cpn60 residue at the same position.

of cpn60s from a number of prokaryotic and eukaryotic
organisms.

Terlesky and Tabita (1991) have also published an N-
terminal sequence of an *R. sphaeroides* cpn60. Save for an
extra N-terminal alanine, the sequence determined in this
study is identical to that presented by Terlesky and
Tabita (1991). The strain of *R. sphaeroides* used by
Terlesky and Tabita (1991) is a streptomycin-resistant
derivative of the same strain used in this study.

## 3.5. Immunological studies

The sequence similarity of GroEL and Rs cpn60 was
considered to provide proof that the Rs cpn60 protein was
a member of the cpn60 family of proteins. This conclusion
suggested the likelihood that these proteins would be
immunologically related. Van der Vies (1989) had reported
that antibodies raised against the *Pisum sativum* plastid
cpn60 detected a related protein in a total cell extract
of *R. sphaeroides*. Polyclonal antisera raised against
GroEL and the *P. sativum* plastid cpn60, were used in an
attempt to determine whether the Rs cpn60 protein was
immunologically related to these proteins. Western blot
analysis of purified GroEL, Rs cpn60 and a pea stromal
extract probed with these antisera, revealed that neither
antibody cross-reacted with Rs cpn60, anti-pea plastid
chaperonin cross-reacted with GroEL and anti-GroEL did not
cross-react with either protein (Fig. 3.5.1.). The lack of

-148-

**Figure 3.5.1.** Cross-reactivity of Ra cpn60 with antisera raised against the GroEL and pea plastid chaperonins

Polypeptides were resolved in SDS-polyacrylamide gels (T = 10.27%, C = 2.6%). Gels were either Coomassie stained (A) or blotted onto nitrocellulose membranes and probed with anti-GroEL (B) or anti-pea plastid chaperonin (C). Track 1, pea stromal extract; 2, purified GroEL; 3, partially purified Ra cpn60. Western blots were developed using the biotin/streptavidin detection system.

cross-reactivity between anti-pea plastid chaperonin and Rs cpn60 was somewhat unexpected, since van der Vies (1989) had detected a protein in a total cell extract of *R. sphaeroides*. In these experiments, the detection system (biotin/streptavidin) used was the same as that used by van der Vies (1989). However, I have been unable to confirm this observation (data not shown).

To further investigate the immunological properties of the Rs cpn60, it was decided to raise antisera to the purified Rs cpn60 protein by injecting this antigen into New Zealand white rabbits. Because of the suspected universal occurrence of cpn60 type proteins in prokaryotes and eukaryotes, two critically important precautions were taken when raising antisera against the Rs cpn60 protein. Firstly, preimmune sera were obtained from rabbits and used to probe SDS-PAGE western blots of Rs cpn60 preparations, to ensure that no antibodies that recognized this protein were present. Secondly, the standard procedure for raising antibodies involves the emulsification of antigen in Freund's complete adjuvant prior to injection (Johnson and Thorpe, 1982). Freund's complete adjuvant contains dead *Mycobacterium tuberculosis* which in turn contain a cpn60 protein (Shinnick, 1987). Any antisera raised by this method would thus be expected to contain antibodies to the *M. tuberculosis* cpn60, which could in turn cross-react with the *R. sphaeroides* cpn60. For this reason Freund's incomplete adjuvant, which

**Figure 3.5.2.**    **Cross-reactivity of anti-Rs cpn60 with Rs cpn60**

 **and GroEL**



Polypeptides were resolved in SDS-polyacrylamide gels (T = 10.27%, C = 2.6%). Gels were either Coomassie stained (A) or blotted onto nitrocellulose membranes and probed with anti-Rs cpn60 (B). Track 1, Rs cpn60; 2, GroEL. Western blot was developed using peroxidase-linked anti-rabbit Ig.

contains no mycobacterial proteins, had to be used for
emulsification of the antigen prior to injection. Rabbits
were injected and immune sera prepared as described in
Section 2.7. Sera were then used to probe western blots of
purified GroEL and Rs cpn60 (Fig. 3.5.2.). This figure
clearly shows that hyperimmune serum prepared from a
rabbit injected with Rs cpn60 recognizes both of the
purified proteins, thus demonstrating that these proteins
are serologically related. This result provides further
evidence that Rs cpn60 is a GroEL homologue.

## 3.6. Structural studies

This work was carried out in collaboration with Helen
Saibil of Birkbeck College, London. Previous image
analyses of electron micrographs of the GroEL protein had
shown that storage of purified GroEL led to significant
changes in the ultrastructure of the GroEL tetradecameric
complex (Saibil, personal communication). Thus, in
preparing Rs cpn60 for ultrastructural analysis, speed was
of the essence whilst sample purity, although desired to
be as high as possible, was of secondary importance.

To achieve this end *R. sphaeroides* was grown
photoheterotrophically to an $OD_{680}$ of about 1.8 and then
transferred to chemoheterotrophic growth conditions for a
period of 4 hours (to suppress Rubisco synthesis). At this
stage the culture had reached an $OD_{680}$ of about 2.3. Cells
were harvested and cell-free extracts prepared as normal

(2.9.1.1.). The cell-free extracts were fractionated in sucrose gradients in a VTi50 rotor (2.9.2.2.). The bottom 12 ml of the gradient was collected, filtered (Section 2.9.3.) and immediately fractionated by FPLC Mono-Q chromatography (2.9.3.1.). Fractions from the region where Rs cpn60 was expected to elute were dialysed overnight against 20 mM Tris-HCl pH 7.5, while aliquots of these fractions were simultaneously analysed in 4-30% non-denaturing polyacrylamide gels (Fig. 3.6.1.). The next morning, gels were Coomassie-stained and peak fractions identified. Appropriate fractions were immediately taken to London for electron microscope analysis. Typically the time between the start of a preparation and fixation was less than 30 hours.

Rs cpn60 prepared in this manner was negatively stained with uranyl acetate on a carbon film support and examined in the electron microscope under low electron dosage (1000-2000 e/nm$^2$) conditions; conditions of high electron dosage being known to cause substantial damage to this type of specimen (Saibil, personal communication).

Under these conditions the protein preparations gave rise to excellent images (Fig. 3.6.2.). Two views of the molecule predominate in these images. The 'top' views clearly show a rosette-like structure with a seven-fold axis of symmetry, whilst 'side' views are rectangular in

Figure 3.6.1. **Rs cpn60 preparation for electron microscope analysis**

The bottom 12 ml from a continuous sucrose gradient was collected and immediately applied to an ion exchange, Mono Q, column. Proteins were eluted and fractions collected as described in Figure 3.3.4. 50 µl aliquots were resolved in a 4-30% native polyacrylamide gel (Coomassie-stained). Tracks are numbered by fraction number.

Figure 3.6.2.  **Negative stain electron micrograph of Rs cpn60**



200 nm

A 0.1 mg/ml solution of Rs cpn60 in 20 mM Tris.HCl pH 7.5. were negatively stained in 2% uranyl acetate on thin carbon support films and photgraphed under low electron dose conditions at a magnification of 40,000 x. Microscopy was performed by Helen Saibil's group at Birkbeck College, London.

appearance with four equally spaced, approximately equally dense, transverse striations. These views suggest a cylindrical shape for the protein. The diameter of the top view is approximately 12 nm, whilst the side views are approximately 12 nm x 9 nm. This geometry is common to virtually all the cpn60 type proteins examined so far. These include cpn60s isolated from *E. coli* (Hohn *et al.*, 1979; Hendrix , *Bacillus subtilis* (Carazo *et al.*, 1991), P. sativum chloroplasts (Pushkin *et al.*, 1982; Tsuprun *et al.*, 1991), mitochondria from *Tetrahymena thermophila* (McMullin and Hallberg, 1987), *Neurospora crassa* (Hutchinson *et al.*, 1989), *Zea mays* (Prasad and Hallberg, 1989) and *Treponema pallidum* (Houston *et al.*, 1990). Hutchinson *et al.* (1989) studied tilted views of the *N. crassa* mitochondrial cpn60 and inferred that the striations are perpendicular to the axis of the cylinder. It seems likely that the same arrangement prevails for the Re cpn60 molecule.

An interesting feature of the images produced in this study is that far more side views are seen than are commonly observed in other cpn60s (Saibil, personal communication). The reasons for this are unclear, but happily the large number of side views should facilitate the deduction of a three-dimensional model from tilted views of the protein.

Top views from Figure 3.6.2 were image processed using the SEMPER programme (Synoptics Ltd, Cambridge). The image

shown (Fig. 3.6.3.) was derived from 22 individual top
views and shows details to a resolution of 20 Å. This
treatment strikingly enhanced the impression of seven-fold
symmetry and revealed several interesting features. An
outer ring of seven electron-dense areas corresponds to
the vertices of the seven-fold structure. Within this ring
are another seven, smaller, electron dense regions. The
centre of the structure shows an area of intermediate
electron density.

Figure 3.6.4. compares the *R. sphaeroides* cpn60 with an
image of the *E. coli* GroEL protein. The latter protein was
prepared from *E. coli* cells harbouring a plasmid that
over-expresses the GroEL protein (Jenkins *et al.*, 1986).
As expected, this protein shows seven-fold symmetry.
However, the finer details of the structure are
significantly different from those of the *R. sphaeroides*
protein. Single regions of high electron density are
located at the vertices of the heptagonal structure, while
the centre of the structure is not occupied by any
electron-dense material.

The temptation to speculate on the possible functional
significance of these structural features is irresistible.
A potentially crucial difference in the biogenesis of
these proteins, is that the *R. sphaeroides* protein is
chromosome-encoded while the GroEL protein used in these
studies is plasmid-encoded. One could argue that in a

Figure 3.6.3.  Rotational analysis of Ra cpn60 electron

microscope images



├─ 5 nm ─┤

Rotational analysis was carried out using the SEMPER programme
(Synoptics Ltd, Cambridge). The image shown was derived from 22
individual top images. Blue = high electron density; yellow = low
electron density; red = intermediate electron density. Analysis was
performed by Helen Saibil's group at Birkbeck College, London.

**Figure 3.6.4.  Comparison of rotational analyses of Rs cpn60 (chromosome-encoded) and the _E. coli_ GroEL protein (plasmid-encoded)**



Rotational analyses were carried out using the SEMPER programme (Synoptics Ltd, Cambridge). Upper image: Rs cpn60 (chromosome-encoded). Lower image: GroEL (plasmid-encoded). Note that the magnification of the two images is slightly different. The actual diameters of the are within 1 nm of each other. Blue = high electron density; yellow = low electron density; red = intermediate electron density. Analyses were performed by Helen Saibil's group at Birkbeck College, London.

normally growing cell the synthesis of chaperonin proteins
is regulated by the availability of substrate proteins
(see 1.3.8.; 1.3.9.). Thus it is possible that the
equilibrium state of chaperonins in the cell is one in
which the major portion of the cpn60 protein is complexed
with substrate proteins. On the other hand, in the case of
the plasmid-encoded *E. coli* protein it is possible that
over-expression of GroEL titrates out substate proteins,
leaving the majority of the GroEL tetradecamers free of
substrate. There is no evidence to support this scenario.
However, it seems possible that the electron dense 'blob'
at the centre of the *R. sphaeroides* structure is a bound
substrate protein. Furthermore, one could speculate that
in the absence of a substrate protein the Rs cpn60 might
look similar to the GroEL protein, and that the presence
of a substrate protein has given rise to a conformational
change that results in the appearance of the inner ring of
electron dense patches. The possibility that such a
conformational change could take place is currently being
investigated at Birkbeck by comparing images of plasmid
and chromosomally expressed GroEL protein.

3.7. **Preliminary attempts to characterise the Rs cpn60 gene**

pOF39 is a derivative of pBR325 that carries a 2.2 kb *E.
coli* DNA insert containing the *groEL* and *groES* genes along
with their promoter (Fayet *et al*., 1986). It was decided
to make use of this plasmid as a heterologous probe to

screen digests of chromosomal DNA from several sources by
Southern hybridization. The insert carrying the *GroE* genes
was purified from pOF39 in the following manner. pOF39 DNA
was digested with *Eco*RI and *Hind*III and subjected to
agarose gel electrophoresis. A region of the gel
containing the 2.2 kb fragment was then excised and the
fragment electroeluted from the gel. DNA prepared in this
manner was labelled as described in Materials and Methods
(2.16.) and used to probe Southern blots of DNA from
several sources.

Initially this probe was used to interrogate digests of
chromosomal DNA from *E. coli*, *R. sphaeroides*,
*Rhodomicrobium vannielii* and an archaebacterium,
*Sulfolobus acidocaldarius*, under high stringency
conditions (0.1 × SSC; 0.1% SDS at 65ºC. Autoradiography
revealed that the probe hybridized to five *E. coli Pst*I
DNA fragments (Fig. 3.7.1.; lane 9). This is the number of
fragments that one would expect in a complete *Pst*I digest
of the *groEL/ES* operon (see Hemmingsen *et al.*, 1988). The
probe appeared to hybridize to a single fragment from *R.
vannielii* (Fig. 3.7.1.; lanes 3, 7, 11) while no
hybridization with *S. acidocaldarius* DNA was detected. In
the case of *R. sphaeroides* there appeared to be some
hybridization with an *Eco*RI fragment (Fig. 3.7.1.; lane
2). However, no hybridization was detected in other
digests (Fig. 3.7.1.; lanes 6, 10). Upon repetition of

**Figure 3.7.1. Hybridization of a groEL/ES probe to various chromosomal DNA digests**

Digested DNAs were resolved in a 1% (w/v) agarose gel and then tranferred to a Hybond-N membrane (Amersham). The blot was then probed with gel-purified ³²P-labelled *groEL/ES* at high-stringency (0.1 × SSC; 0.1% SDS at 65⁰C). Digests: E = *Eco*RI; H = *Hind*III; P = *Pst*I. DNAs: Ec = *E. coli*; Rs = *R. sphaeroides*; Rm = *R. vannielii*; Sa = *S. acidocaldarius*.
   A. Ethidium bromide (1 μg/ml) stained gel.
   B. Autoradiogram of ³²P-labelled *groEL/ES* probed Southern blot.

this experiment with increased loadings of *R. sphaeroides*
DNA, no hybridization to the probe could be detected (data
not shown). Even when lower stringency washes (2 x SSC,
0.1% (w/v) SDS) were performed, only a very weak signal
could be detected with digests of *R. sphaeroides*
chromosomal DNA (data not shown).

Immunological data had indicated that *R. vannielii*
expressed a cpn60 homologue (Graeme MacDonald, personal
communication). However, native polyacrylamide gel
analysis of cell-free extracts of *R. vannielii*, carried
out in exactly the same manner as for *R. sphaeroides* cell-
free extracts, did not reveal the presence of a discrete
high molecular weight putative *R. vannielii* cpn60 (see
Fig. 3.3.3.). Nonetheless, it was decided that, since the
hybridization signal given by *R. vannielii* chromosomal DNA
was relatively strong compared to the *R. sphaeroides*
signal, the *R. vannielii* gene should be cloned initially
and then used as an intermediate heterologous probe to
interrogate *R. sphaeroides* DNA. The grounds for this
decision were that *R. vannielii* and *R. sphaeroides* are
more closely related than *R. sphaeroides* and *E. coli* (see
Woese, 1987).

A *Hind*III-digested *R. vannielii* chromosomal DNA fragment
of about 2.4 kb hybridised with the *groEL/ES* probe (Fig.
3.7.1.). A partial *R. vannielii Hind*III DNA library was
constructed by *Hind*III digesting total *R. vannielii* DNA,
resolving the fragments in an agarose gel followed by

excision and electro-elution of fragments in the 2-3 kbp size range. The electro-eluted DNA was then ligated into *Hind*III-digested, phosphatase-treated pBR322. This library was then used to transform *E. coli* strain MC 1061, and transformants were selected on Luria-Bertani agar plates containing 100 µg/ml ampicillin. Transformants were picked and replica-plated in groups of twenty five. Plasmid DNA was prepared from transformants as described in Materials and Methods (2.11.3.1.). One of these sub-libraries gave a fairly strong hybridization signal when interrogated with the labelled *groEL/ES* probe (data not shown).

Plasmid DNA from each member of this sub-library was prepared, resolved in agarose gels and probed as above. Figure 3.7.2. shows the result of such an experiment. The probe hybridized weakly to pBR322 (Fig. 3.7.2.B.; lane 18). However, a much stronger signal, from a smaller DNA loading (Figure 3.7.2.A.), was detected in lane 17. Faint bands were detected in several other lanes, but were not thought to be strong enough to warrant further investigation. Although the plasmid, pGW17, loaded to lane 17 gave an encouragingly strong hybridization signal, it appeared to be much too small to contain a 2-3 kb insertion. Subsequent *Hind*III restriction of pGW17 failed to demonstrate the presence of any insert whatsoever (data not shown) although a very small insert might have been overlooked. The most likely explanation of these observations is that a large DNA deletion had taken place.

-164-

Figure 3.7.2. Hybridisation of *groEL/ES* to members of a
pBR322/*R. vannielii* chromosomal DNA sub-library

Undigested plasmids were resolved in a 1% (w/v) agarose gel and
then tranferred to a Hybond-N membrane (Amersham). The blot was then
probed with gel-purified ³²P-labelled *groEL/ES* at high-stringency
(0.1 × SSC; 0.1% SDS at 65°C).
A. Ethidium bromide (1 µg/ml) stained gel.
B. Autoradiogram of ³²P-labelled *groEL/ES* probed Southern blot.

No further attempts to characterize the *R. vannielii* and *R. sphaeroides* genes were made. Since this work had been started, several sequences of chaperonin genes had been published and it was thought that more novel work could be done in other areas. Attention was subsequently focussed on structural studies (see 3.6.) and the regulation of Rs cpn60 in response to various enviromental stresses. In addition, a collaboration with Keith Willison of the Chester Beatty Laboratories had presented the opportunity of investigating whether TCP-1 might be a cytosolic chaperonin.

## 3.8. Conclusions

A chaperonin cpn60 protein, dubbed Rs cpn60, was isolated from *R. sphaeroides*. This protein was shown to have a native $M_r$ of about 670,000 and is composed of subunits of $M_r$ 58,000. The N-terminus of the $M_r$ 58,000 polypeptide was sequenced and shown to be identical to the N-terminus of the GroEL protein (Hemmingsen *et al.*, 1988) at 15 of the 19 amino acids identified. Native-PAGE analysis of cell-free extracts from *R. vannielii* did not reveal the presence of a discrete protein band in the $M_r$ 700,000 region of these gels. A broad smear of protein detected in this region could represent a partially dissociated form of a *R. vannielii* cpn60 tetradecamer. This possibility has not been investigated in this study.

Initially it was thought that the expression of two

forms of Rubisco in *R. sphaeroides* (see Tabita, 1988),
probably expressed from separate chromosomes (Suwanto and
Kaplan, 1989), might presage the presence of more than one
cpn60-type protein. In the course of this study no
evidence to support this hypothesis was forthcoming.
Recently evidence of the occurrence of multiple cpn60
proteins in *Streptomyces* species has been presented
(Guglielmi *et al.*, 1991). These proteins are very similar
in size and were not well resolved by SDS-PAGE, and the
presence of more than one cpn60 protein was demonstrated
by sequencing material from the leading and trailing edge
of the cpn60 band. In this study the whole of the cpn60
band was excised and then subjected to N-terminal
sequencing. There was no evidence of the presence of a
second protein in this band. This observation does not
exclude the possibility that an N-terminally blocked
protein was present, or that there is a second very minor
component of the band. It is also possible that a second,
minor, cpn60 could have been separated from the major
species during purification. However, any such protein has
escaped detection during a large number of preparations.
Overall these results suggest that a single major cpn60
protein is expressed in this strain of *R. sphaeroides*.

Antisera raised against the *E. coli* GroEL protein and
the pea plastid chaperonin did not cross-react with the Rs
cpn60. Purified Rs cpn60 was emulsified with Freund's
incomplete adjuvant and injected into rabbits to raise

antisera to this antigen. Hyperimmune sera was shown to cross-react with the purified GroEL protein. This result provided further evidence that Rs cpn60 was a true chaperonin protein.

Musgrove *et al.* (1987) had reported that the pea plastid chaperonin dissociated into its monomeric form when incubated in the presence of Mg-ATP at low temperature. Rs cpn60 was also shown to dissociate under similar conditions. However, in this case a dimeric form appeared to be the final product. This difference may be a reflection of the different subunit compositions of the proteins. The plastid chaperonin is composed of $\alpha$ and $\beta$ subunits while Rs cpn60, like the GroEL protein, appeared to be composed of a single type of subunit. The GroEL protein has also been shown to dissociate in the presence of Mg-ATP (Goloubinoff *et al.*, 1989a). Although the subunit composition of the dissociated form is unknown, like Rs cpn60 it does not become monomeric.

Structural studies on partially purified Rs cpn60 have shown that this protein is superficially (raw electron microscope data) very similar to the other cpn60 proteins so far examined. Top views of individual particles clearly display seven-fold rotational symmetry, while side views show the characteristic brick-like appearance of cpn60s with four equally spaced striations perpendicular to the axis of the particle. The dimensions of the particles are also very similar to those of other cpn60 proteins so far

examined. Carazo *et al.* (1991) have suggested that six-
and seven-fold forms of the cpn60 proteins from *E. coli*
and *B. subtilis* exist in a temperature-regulated
equilibrium, with the six-fold form being dominant at
lower temperatures. Carazo *et al.* (1991) also demonstrated
that when preparations of predominantly seven-fold
symmetry particles were dialyzed at pH 5, the number of
six-fold views were dramatically increased. Careful
examination of electron micrographs of the Rs cpn60
preparation does not reveal the presence of any particles
with an obvious six-fold axis of symmetry. It is, however,
possible that such particles might emerge if *R.
sphaeroides* is grown at a lower temperature or Rs cpn60
preparations dialyzed against acidic buffers.

When the raw electron microscope data was subjected to
image processing, structural details at a resolution of
20 Å could be seen. When these images were compared to
similar ones of the GroEL protein, differences in the fine
structure could be discerned. Of particular interest is
the appearance of an inner ring of electron dense areas in
the Rs cpn60 particle. In section 3.5, I have speculated
that the biogenesis of these proteins, plasmid-encoded
GroEL versus chromosmally encoded Rs cpn60, may be an
important contributing factor to these differences.
However, the obvious point, that these are different
proteins, must not be overlooked. Before any firm
conclusions can be drawn it will be necessary to compare

the appearance of plasmid and chromosomally encoded cpn60 that originates from a single species. To this end I have prepared *E. coli* chromosomal—encoded GroEL. Analysis of this material at Birkbeck has revealed differences between the plasmid— and chromosomal—encoded proteins. However, the differences are not as great as those between Rs cpn60 and plasmid—encoded GroEL.

Finally, preliminary attempts have been been made to clone and characterize the cpn60 genes from *R. vannielii* and *R. sphaeroides*. When digests of chromosomal DNA from these species were probed with a DNA fragment containing the *E. coli groEL* and *groES* genes at high stringency, only the *R. vannielii* DNA gave a signal that was considered to be suitable for subsequent cloning of a cpn60 gene. The strategy adopted was to initially clone the *R. vannielii* gene and then to use this as a probe for the *R. sphaeroides* gene. Unfortunately, the only positive clone detected appeared to have undergone a large deletion and no further attempts to characterize the cpn60 genes were made for the reasons already outlined (see 3.7.). DNA from an archaebacterium, *S. acidocaldarius*, was also interrogated but failed to hybridize to the *groEL/ES* probe. The recent findings that archaebacteria express a TCP-1 homologue could explain this result (Trent *et al.*, 1991; see Chapter 5).

In summary: *R. sphaeroides* grown under photoheterotrophic conditions appears to express a single

-170-

cpn60 type protein. Structural, immunological and biochemical data identifying this protein as a GroEL homologue has been presented.

# Chapter 4

## The stress response of *Rhodobacter sphaeroides*

### 4.1. Introduction

In this chapter the response of *R. sphaeroides* to a number of stresses is described. These studies focus exclusively on the alterations in protein synthesis that occur when this bacterium is exposed to an environmental stress. In particular, the effects on the synthesis of Rs cpn60 have been investigated.

The stress response was discussed, briefly, in the Introduction (1.3.10) and has been reviewed extensively in recent years (Neidhardt *et al.*, 1984; Gottesman, 1984; Lindquist, 1986; Lindquist and Craig, 1988; Gross *et al.*, 1990; Morimoto *et al.*, 1990; Schlesinger, 1990). To recapitulate; this response involves a transient increase in the expression of a small number of genes, with the consequent production of a small number of proteins. These proteins are commonly referred to as the heat shock proteins (hsps) or stress proteins. Typically, the first signs of a heat shock response are detected within a minute of the temperature increase, with the response reaching a peak after 20–60 minutes depending on the organism and the type of stress (Neidhardt *et al.*, 1984). In *E. coli* there are about 20 known heat shock proteins (Kamath-Loeb and Gross, 1991) which vary greatly both in size and abundance. Representatives of the heat shock

proteins are amongst the most acidic and basic proteins found in *E. coli* (Neidhardt *et al.*, 1984).

Experimental evidence indicates that environmental stresses that cause the denaturation of intracellular proteins also induce the synthesis of heat shock proteins (Ananthan *et al.*, 1986). The table below lists agents that are known to induce hsp synthesis in eukaryotes.

Table 4.1. Agents or treatments that activate hsp genes.

| Inducing agent or treatment | Proposed effects |
|---|---|
| **Group I** | |
| Ethanol | Translation errors |
| Amino acid analogues, puromycin | Abnormal proteins |
| **Group II** | |
| Heat shock | Increased unfolding of proteins |
| Heavy metals, copper-chelating groups, arsenite, iodoacetamide, *p*-chloromercuribenzoate | Binding to sulphydryl groups, conformational changes in proteins |
| Return from anoxia, hydrogen peroxide, superoxide ions and other free radicals | Oxygen toxicity, free radical fragmentation of proteins |
| Ammonium chloride | Inhibition of proteolysis |
| Amytal, antimycin, azide, dinitrophenol, rotenone, heptyl-hydroxy-quinoline N-oxide, ionophores | Inhibition of oxidative phosphorylation, changes in redox state, covalent modifications of proteins |
| Hydroxylamine | Cleavage of asparagine-glycine bonds in proteins |

Group I treatments cause the denaturation of newly made proteins.
Group II treatments cause the denaturation of preexisting proteins.

After Ananthan *et al.* (1986)

All of the treatments described above cause some sort of modification of the normal state of proteins within the cell. Ananthan *et al.* (1986) co-injected purified proteins and *hsp* genes into frog oocytes and demonstrated that if

the proteins were denatured prior to injection, the *hsp*
genes were subsequently activated. The observation that
abnormal proteins elicit a stress response has been
repeated several times in both eukaryotes (Munro and
Pelham, 1985; Kozutsumi *et al.*, 1988) and prokaryotes
(Goff and Goldberg, 1985; Parsell and Sauer, 1989), with a
similar range of stresses being known to induce hsp
synthesis in prokaryotes (see Morimoto *et al.*, 1990).
Thus, it appears that the primary elicitor of the stress
response is the presence or production of abnormal
proteins.

Craig and Gross (1991) have proposed that hsp70 acts as
a cellular thermometer. In *E. coli* DnaK, the hsp70
homologue, along with the the GrpE and DnaJ proteins, are
involved in every process known to regulate the levels of
$\sigma^{32}$. These proteins have been shown to negatively regulate
$\sigma^{32}$ in three ways: they are required for translational
regulation of $\sigma^{32}$ synthesis at high temperature, for
inactivation of $\sigma^{32}$ after shift to low temperature and
they facilitate degradation of $\sigma^{32}$ at all temperatures
(Strauss *et al.*, 1990; Tilly *et al.*, 1989). The abnormal
proteins produced when cells are exposed to an
environmental stress interact with, and deplete, the
levels of free hsp70 (DnaK and other homologues) (see
Craig and Gross, 1991). The reduced level of free hsp70 is
thought to interfere with the ability of the cell to
regulate the degradation, and repress, the synthesis of

-174-

$\sigma^{32}$. The $\sigma^{32}$ protein then enhances the synthesis of the whole range of heat shock proteins (Craig and Gross, 1991).

The heat shock or stress response cannot be clearly separated from other stress systems. For example, deletion of the *rpoH* gene from *E. coli* leads to the sensitization of these cells to oxidative stress (Kogoma and Yura, 1992), and the *groE* genes are known to play an important role in SOS repair (Liu and Tessman, 1990). Furthermore, various stresses have been shown to cause differential induction of the heat shock, SOS and oxidative stress regulons in *E. coli* (VanBogelen *et al.*, 1987).

In the series of experiments presented here, chemoheterotrophically grown cultures of *R. sphaeroides* have been subjected to a variety of stresses, during which cells were pulse-labelled with $^{35}$S methionine. Alterations in protein synthesis have been visualized by autoradiography of proteins resolved in polyacrylamide gels. In this study, attention has been focussed on the effects of these stresses on the synthesis of the *R. sphaeroides* chaperonin.

## 4.2. Response of *R. sphaeroides* to heat shock

*R. sphaeroides* cultures were grown at 30°C throughout this study. In these heat shock experiments, cultures were subjected to a temperature shift to 42°C during the mid-exponential phase of growth. Temperature shifts of this

magnitude are known to produce a clear heat shock response in a variety of organisms including *E. coli* (see Neidhardt *et al*., 1984). Aliquots of a culture, grown at 30ºC, were transferred to Universal bottles prewarmed to 42ºC, and labelling was carried out and cell-free extracts prepared as described in Materials and Methods (2.10.). Proteins were analysed by both native- and SDS-PAGE. Figures 4.2.1. and 4.2.2. show the results of these analyses. The times noted in the figures represent the time at which label was added to the culture after commencement of the shock. It should be noted that cells were labelled for 15 minutes so that, for example, the t = 0 sample represents protein synthesis over the subsequent 15 minutes.

As expected, the heat shock causes a marked alteration in protein synthesis. Native gel analysis shows transient increases in the rate of synthesis of about 10 proteins, while SDS-PAGE reveals a transient increase in the synthesis of about the same number of proteins. Obviously a two-dimensional analysis would be expected to identify several more heat shock-inducible proteins. As expected, these proteins are induced to varying extents at different times during the heat shock. Some general comments will first be made regarding the general pattern of protein synthesis after heat shock, before a more detailed discussion of the regulation of Rs cpn60 under these conditions.

**Figure 4.2.1.** **Native gel analysis of protein synthesis in _R._**

_sphaeroides_ after heat shock



Mr x 10⁻³

669
440
232
140

67

_R. sphaeroides_ cells were pulse-labelled with [³⁵S] methionine for 15 minutes at various times after a shift in growth temperature from 30°C to 42°C. Cell-free extracts were prepared by sonication and equal amounts of soluble cell protein, 40 µg, were loaded to each lane of a 4-30% native gel. Tracks are numbered according to the time, in minutes, when label was added. Control samples, 0con and 60con, from a culture aliquot maintained at 30°C were taken at the beginning and end of the experiment.

A. Coomassie-stained gel.
B. Autoradiograph of dried gel.

**Figure 4.2.2.**  **SDS-PAGE analysis of protein synthesis in _R. sphaeroides_ after heat shock**

_R. sphaeroides_ cells were pulse-labelled with [³⁵S] methionine for 15 minutes at various times after a shift in growth temperature from 30°C to 42°C. Cell-free extracts were prepared by sonication, boiled in sample buffer, and equal amounts of soluble cell protein, 20 µg, were loaded to each lane of a 10-30% SDS-polyacrylamide gel. Tracks are numbered according to the time, in minutes, when label was added. Control samples, 0con and 60con, from a culture aliquot maintained at 30°C were taken at the beginning and end of the experiment.

  A. Coomassie-stained gel.
  B. Autoradiograph of dried gel.

Protein synthesis induced by the heat shock appears to fall into three broad expression groups:

1. those proteins whose synthesis is induced briefly during the first 20 minutes of the heat shock, followed by a rapid decline in synthesis.

2. those proteins whose synthesis appears to peak somewhat later and to be followed by a more gradual decline in synthesis (Rs cpn60 falls into this group)

3. proteins whose synthesis is maintained at elevated levels after induction by heat shock.

Table 4.2.1. lists the heat shock proteins identified after native and SDS-PAGE and places them in the groups described above.

Table 4.2.1. Heat shock proteins identified after native and SDS-PAGE.

| Native gel analysis | | | SDS gel analysis | | |
|---|---|---|---|---|---|
| Protein number[†] | $M_r$ × $10^{-3}$ | Group | Protein number[†] | $M_r$ × $10^{-3}$ | Group |
| 1 | 670 | 2 | 1 | >94 | 1 |
| 2 | 104 | 2 | 2 | >94 | 1 |
| 3 | 87 | 2 | 3 | 84 | 2 |
| 4 | 69 | 1 | 4 | 72 | 2 |
| 5 | <67 | 3 | 5 | 58 | 2 |
| 6 | <67 | 3 | 6 | 44 | 2 |
| 7 | <67 | 3 | 7 | 38 | 2 |
| 8 | <67 | 3 | 8 | 33 | 2 |
| 9 | <67 | 3 | 9 | 26 | 1 |
| | | | 10 | 18 | 3 |
| | | | 11 | <14.4 | 3 |

† Protein number refers to the numbers used to identify proteins in Figures 4.2.1. and 4.2.2.

The information presented in Table 4.2.1 is notable for the quite different view of the heat shock response presented by the native- and SDS-PAGE analyses. Most of the heat shock proteins detected by native gel analysis fall into group 3, while most of those detected by SDS gel analysis fall into group 2. Both gel systems have a maximum acrylamide concentration of 30%, and would be expected to retain low molecular weight proteins of approximately the same size. Thus, all the heat shock proteins detected by SDS-PAGE would also be expected to be resolved on native gels, although these proteins could be found in higher molecular weight multimeric complexes. Bearing this in mind, it was expected that the expression group profile would be similar in each gel system. However, this has not proved to be the case. One possible explanation for the increased occurrence of group 3 heat shock proteins on native gels, is that members of group 3 detected by SDS-PAGE (proteins 10 and 11) could be members of a range of multimeric complexes *in vivo*. This would also explain the decreased relative intensity of the group 3 proteins on native gels. The group 1 and several of the group 2 proteins detected by SDS-PAGE are fairly faint and may simply have escaped detection on native gels. In retrospect a much longer exposure of the native gel may have yielded some additional information.

No attempt has been made to identify heat shock proteins other than Rs cpn60 and I would generally consider

speculation as to the identity of these proteins on the basis of molecular weight alone to be a fruitless exercise. However, the $M_r$ 72,000 protein detected after SDS-PAGE (protein 4, Figure 4.2.2.) is worthy of some comment. The stained gel indicates that this is a fairly abundant protein and its apparent molecular weight, along with its elevated rate of synthesis after heat shock, indicate that it could be the *R. sphaeroides* DnaK (hsp70) homologue. This protein does not, however, appear to be detected after native gel electrophoresis. A heat shock protein of $M_r$ 69,000 is detected on native gels, but this protein falls into a different expression group and it is by no means certain that this is the same protein detected by SDS-PAGE. If any substantial amount of this protein exists in a monomeric form *in vivo*, one would expect it to be readily detectable on native gels. The hsp70 proteins are known to interact with a wide variety of normal and abnormal proteins and to be involved in a number of cellular processes (see Pelham, 1986; Deshaies *et al*., 1988; Chiroco *et al*., 1988; Georgopolous *et al*., 1990; Gross, *et al*., 1990; Flynn *et al*., 1991). Furthermore, although hsp70s are purified predominantly as monomers, the functional form of this protein is believed to be a multimer of unknown size (see Craig and Gross, 1991). This plethora of interactions would be expected to abolish any single hsp70 band on native gels. Overall, these observations provide circumstantial evidence that the $M_r$

72,000 protein is likely to be the *R. sphaeroides* hsp70 homologue.

The most striking feature of both analyses is a large increase in the synthesis of Rs cpn60. This induction of Rs cpn60 synthesis reaches a peak in the first 25 minutes after commencement of the shock (see track 4, t = 10, Figures 4.2.1. and 4.2.2.) and then gradually diminishes over the next 50 minutes, with the final sample, at t = 60, showing a level of synthesis that is similar to that of unshocked cells. Unshocked control cultures at t = 60 show a small increase in Rs cpn60 synthesis compared to those at t = 0. This could be a consequence of transferring the cells to a less highly aerated growth regime for labelling purposes. The relative increase in Rs cpn60 synthesis was quantified by scanning densitometry and these results are summarized in Table 4.2.2.

Table 4.2.2. **Relative increase in synthesis of Rs cpn60 during heat shock.**

| Time after start of shock (minutes) | Relative increase in Rs cpn60 synthesis | |
|---|---|---|
| | Native gel[‡] | SDS-PAGE[‡] |
| 0 (control)[a] | 1.0 | 1.0 |
| 0 | 2.9 | 4.2 |
| 5 | 12.4 | 10.3 |
| 10 | 16.1 | 7.8 |
| 15 | 8.3 | 5.3 |
| 30 | 6.1 | 3.0 |
| 60 | 1.5 | 1.2 |
| 60 (control) | 1.6 | 1.1 |

‡ Bands quantified by integrating volumes.
‡ Bands quantified by integrating areas.
a The control tracks were not subjected to heat shock and

-182-

the t = 0 (control) value was used to normalize the relative
increase of Rs cpn60 synthesis.

The overall view presented by both methods of
quantification is of a rapid induction of Rs cpn60
synthesis, with the increase peaking after about 20-25
minutes and followed by a gradual decline. However, the
details are somewhat different, with the apparent peak of
induction as measured from an SDS-polyacrylamide gel
taking place slightly earlier, and being about two thirds
of that calculated from native gels. These differences are
thought to be largely artefacts arising from methods of
measurement. Quantification of the Rs cpn60 band on native
gels is thought to be somewhat more accurate, since this
band is well separated from any other protein bands
(possible variations in background were minimized by
determining the background in each track just below the
chaperonin band). However, it is important not to forget
that the native chaperonin would be expected to be
associated with other proteins *in vivo*, and that such
proteins would tend to slightly inflate the apparent
induction of Rs cpn60 synthesis. On the other hand, when
attempting to quantify the Rs cpn60 band intensity in SDS
gels, one needs to bear in mind the likelihood of
interference from other proteins of a similar relative
mobility. Any such protein would be expected to grossly
distort the measurements of relative band intensity.

The likelihood that other proteins would be associated with the native Rs cpn60 band was investigated. This was done by cutting out a strip containing the Rs cpn60 protein from a dried, unstained native gel, then submerging this in solubilization buffer layered on a flat-topped stacker of a 10-30% SDS polyacrylamide gel. The strip was allowed to equilibrate in solubilization buffer for one hour at room temperature before electrophoresis. After electrophoresis the gel was stained, destained, dried and then subjected to autoradiography with Hyperfilm (Amersham) for a period of 104 days.

The stained gel (Figure 4.2.3.) shows a somewhat puzzling polypeptide profile. Besides the expected Rs cpn60 band, there is also a band of $M_r$ 15,000. Initially it was thought that this could be the Rs cpn10 polypeptide. However, this seems unlikely, since Terlesky and Tabita (1991) have isolated a cpn10 protein from *R. sphaeroides* and shown that it has a subunit $M_r$ of 13,000. This leaves the possibility that the $M_r$ 15,000 polypeptide is lysozyme, used during preparation of the cell-free extracts, which has a molecular weight of 14,400 (Merck Index, 1984). Unfortunately, the amounts of the polypeptides on the stained gel could not be quantified; thus it is not possible to determine whether the $M_r$ 15,000 polypeptide is present in a quantity that suggests the

Figure 4.2.3. **SDS-PAGE analysis of pulse-labelled Rs cpn60**

**excised from a native gel**



Cell-free extracts from heat shocked, pulse-labelled *R. sphaeroides* cells were resolved in a 4-30% native gel, 40 µg/track, which was dried without prior staining. After autoradiography the Rs cpn60 band was excised, submerged in 2 × sample buffer on top of a flat-topped 10-30% SDS-polyacrylamide gel for a period of 1 hour, and then electrophoresed as usual. Tracks are numbered according the time, in minutes, when label was added. Control samples, 0con and 60con, from a culture aliquot maintained at 30°C were taken at the beginning and end of the experiment.

A. Coomassie-stained gel.
B. Autoradiograph of dried gel.

formation of a stable binary complex with Rs cpn60. Furthermore, it is impossible to say whether the $M_r$ 15,000 polypeptide could have displaced other polypeptides from Rs cpn60, bound to a subpopulation of "empty" Rs cpn60 or interacted non-specifically with the chaperonin.

Two major observations can be made as a result of the autoradiographic analysis (Figure 4.2.3.). Firstly, no polypeptides of $M_r$ greater than 58,000 appear to be associated with the Rs cpn60 protein. Secondly, the profile and amount of protein found in association with Rs cpn60 varies during the course of the heat shock. There are two possible interpretations of these observations. One is that the ladder of polypeptides below the Rs cpn60 band is the product of proteolysis of the Rs cpn60 protein and any other polypeptide(s) that are associated with it. The other is that there is a limit on the size of polypeptide that can form an association with Rs cpn60 protein. Neither possibility can be eliminated on the basis of this result alone. These observations will be discussed in more detail in the concluding part of this chapter.

4.3. **Response of *R. sphaeroides* to a variety of other stresses**

The response of *R. sphaeroides* to a variety of stresses was studied, again with particular emphasis on the synthesis of Rs cpn60. The stresses imposed were exposure to 5% (v/v) ethanol; 100 µM hydrogen peroxide; 0.5 M

sodium chloride; 600 µM cadmium sulphate and a transition from aerobic to anaerobic growth. Each of these stresses had quite distinct effects on the synthesis of Rs cpn60. Exposure to 100 µM hydrogen peroxide led to a virtual abolition of all protein synthesis at this concentration, sodium chloride and ethanol caused a fairly long and gradual induction of Rs cpn60 synthesis, and cadmium sulphate appeared to upregulate synthesis to a new basal level (Figures 4.3.1.; 4.3.2.). Examination of the autoradiographs presented in these figures shows that besides the different effects of these stresses on Rs cpn60 synthesis, each treatment gives rise to a unique protein synthesis profile. The relative increases in Rs cpn60, measured from autoradiographs of native gels, over the course of these stresses are shown in Table 4.3.1.

Table 4.3.1. **Relative increase in synthesis of Rs cpn60 after exposure to a variety of stresses.**

| Stress | | Relative increase[†] in Rs cpn60 synthesis after time in minutes | | | |
|--------|--------|-----|-----|-----|-----|
| | | 5 | 15 | 45 | 90 |
| Ethanol | 5% (v/v) | 2.1 | 2.3 | 5.3 | 3.9 |
| Cadmium | 600 µM | 2.0 | 1.7 | 2.3 | 1.8 |
| NaCl | 500 mM | 0.8 | 2.2 | 4.9 | ND |
| H₂O₂ | 100 µM | Protein synthesis reduced | | | |

† Increase was normalized relative to synthesis of an unstressed control sample taken 5 minutes into the experiment. ND Not determined.

Again significantly different views of the stress response are presented by native- and SDS-PAGE analyses.

Figure 4.3.1.   **Native gel analysis of protein synthesis in _R._**

_sphaeroides_ **after exposure to a variety of**

**stresses**



*R. sphaeroides* cells were pulse-labelled with [35S] methionine for
15 minutes at various times after exposure to various stresses. Cell-
free extracts were prepared by sonication and equal amounts of
soluble cell protein, 30 µg, were loaded to each lane of a 4-30%
native gel. The gel was then Coomassie-stained, dried and
autoradiographed. Tracks are numbered according to the time, in
minutes, when label was added. Et = 5% (v/v) ethanol; Cd = 600 µM
cadmium; Hp = 100 µM H2O2; Na = 500 µM NaCl. Control samples, 5con
and 90con, from a culture aliquot not exposed to stress were taken at
the beginning and end of the experiment.

**Figure 4.3.2.** **SDS-PAGE analysis of protein synthesis in *R.
sphaeroides* after exposure to a variety of
stresses**

*R. sphaeroides* cells were pulse-labelled with [35S] methionine for
15 minutes at various times after exposure to various stresses. Cell-
free extracts were prepared by sonication, boiled in sample buffer,
and equal amounts of soluble cell protein, 15 µg, were loaded to each
lane of a 10-30% SDS-polyacrylamide gel. The gel was then Coomassie-
stained, dried and autoradiographed. Tracks are numbered according to
the time, in minutes, when label was added. Et = 5% (v/v) ethanol; Cd
= 600 µM cadmium; Hp = 100 µM H₂O₂; Na = 500 µM NaCl. Control
samples, 5con and 90con, from a culture aliquot not exposed to stress
were taken at the beginning and end of the experiment.

In particular cadmium induced synthesis of Rs cpn60 appears to fall off more rapidly when visualized in SDS-polyacrylamide gels. At the same time a polypeptide of $M_r$ 24,000 is fairly strongly induced (Figure 4.3.2, Cd t = 45 and 90). Perhaps this polypeptide could form an association with Rs cpn60. Unfortunately time did not permit a repeat of a "cutout" experiment to determine whether such an association exists.

In the case of a shift from chemoheterotrophic to photoheterotrophic growth, protein synthesis appeared to cease immediately, compared to the unshifted control, and only resumed after a period of about 18 hours (Figure 4.3.3.). Colony counts were determined before and after stress. Only ethanol treatment led to a count reduction, of about 15%. In all the other cases there was an inhibition of growth but no apparent loss of viability (data not shown).

The pattern of protein synthesis observed when *R. sphaeroides* was shifted from chemoheterotrophic to photoheterotrophic growth was not unexpected. Chory *et al*. (1984) studied the effects of a rapid switch from chemoheterotrophic to photoheterotrophic growth on cultures of *R. sphaeroides*. They found that this abrupt change in growth conditions resulted in an immediate cessation of cell growth and whole cell protein, DNA and phospholipid accumulation. Detectable cell growth and whole cell protein accumulation resumed approximately

**Figure 4.3.3.** **Native gel analysis of protein synthesis in *R.* *sphaeroides* after a shift from chemohetero- trophic to photoheterotrophic growth conditions**

*R. sphaeroides* cells were pulse-labelled with [³⁵S] methionine for 15 minutes at various times after a shift from chemoheterotrophic to photoheterotrophic growth conditions. Cell-free extracts were prepared by sonication and equal amounts of soluble cell protein, 30 µg, were loaded to each lane of a 4-30% native gel. The gel was then Coomassie-stained, dried and autoradiographed. Tracks are numbered according to the time, in hours, when label was added. A control sample, 0con, was taken at the beginning of the experiment.

12 hours later. It was hoped that an examination of protein synthesis over this period might provide some insights concerning the synthesis of Rs cpn60. The data presented in Figure 4.3.3. shows a slight increase in Rs cpn60 synthesis over and above that of other proteins when protein synthesis resumes. However, the two hour gaps between samples may have been too long to pick up any large induction when growth resumed.

## 4.4. Conclusions

In this chapter the effect of various stresses on *R. sphaeroides* protein synthesis, Rs cpn60 synthesis in particular, has been studied. The stress response is thought to be universal and so it is not at all surprising to have observed such a response in *R. sphaeroides*.

Of the conditions studied here, a shift from 30ºC to 42ºC elicited by far the strongest and most rapid increase in Rs cpn60 synthesis. This response reached a maximum at 15 to 20 minutes after the temperature upshift. Using scanning densitometry quantification of $^{35}S$ methionine incorporation, synthesis of Rs cpn60 at the peak of the response was calculated to increase approximately 16-fold when resolved in native gels, or about 10-fold when SDS-polyacrylamide gels were used in the analysis. This magnitude of Rs cpn60 synthesis is similar to that observed when *E. coli* cultures are subjected to an identical temperature shift, from 30ºC to 42ºC (Neidhardt *et al.*, 1982; see Gross *et al.*, 1990).

After reaching this peak the response rapidly decayed, so that levels of Rs cpn60 synthesis had returned to near background levels about one hour after induction of the response. Again, the response is similar to that observed in *E. coli* (Straus *et al.*, 1987), but the time course of the response is somewhat slower. This is not unexpected since the doubling time for *R. sphaeroides* grown under chemoheterotrophic conditions at 30ºC is approximately 3 hours, while that of *E. coli* grown at 30ºC in a rich medium is approximately 30 minutes (Herendeen *et al.*, 1979).

It was the demonstration of an interaction between newly synthesized Rubisco large subunits and the pea plastid chaperonin (Barraclough and Ellis, 1980), that was pivotal in the formulation of the molecular chaperone concept (Ellis, 1987). This interaction was demonstrated by excising the plastid chaperonin from native gels and then resolving this material in SDS polyacrylamide gels. However, these observations related to a special case: that of protein synthesis in illuminated, isolated chloroplasts (Barraclough and Ellis, 1980). Since then, the role of chaperonins in preventing improper interactions between proteins has become well established (see Goloubinoff *et al.*, 1990a, b; Martin *et al.*, 1991). Furthermore, it has been shown that the GroEL protein is a heat shock protein that is essential for *E. coli* viability at all growth temperatures (Fayet *et al.*, 1989). Thus, it

would seem likely that cpn60s are normally associated with a variety of substrate proteins *in vivo*.

To date, very little data is available concerning the physical interaction of substrate proteins with chaperonins *in vivo*. It was thought that the material generated in the heat shock experiments conducted here, could be used in an attempt to determine the range of proteins that normally interact with Rs cpn60, and determine how this range might be influenced during heat shock. The strategy used was similar to that used by Barraclough and Ellis (1980) to uncover the association between the large subunit of Rubisco and the plastid chaperonin. The results of this analysis are somewhat equivocal. After an extended period of autoradiography (104 days), a ladder of polypeptides with $M_r$s <60,000 was detected. The profile of polypeptides in this ladder varied during the course of the heat shock (Figure 4.2.3.). It is not possible to say with any certainty whether these polypeptides represent bound substrate proteins or are the result of proteolysis of Rs cpn60. An added complication is the presence of an $M_r$ 15,000 polypeptide that appears to be a component of the native Rs cpn60 band. It is probable that this polypeptide is lysozyme. The nature of the interaction between Rs cpn60 and lysozyme, and any effect this may have had on the binding of other substrate proteins, is not at all clear.

The total absence of any polypeptides of $M_r$ >60,000,

even after such a long exposure, could indicate that
proteins of this size do not interact with the chaperonin
in the normal course of events. Electron microscope data
presented in Section 3.6. indicate that substrate proteins
might be bound in the central cavity of the Rs cpn60
tetradecamer. If a protein must be sequestered within the
Rs cpn60 molecule for the chaperonin to aid in its
folding, this would be expected to place a size constraint
on substrate proteins. It should be noted, however, that
significantly fewer high molecular weight proteins were
synthesized after heat shock. If the size constraint
observed here represents a correct view of the size of
chaperonin substrate proteins, it would provide strong
evidence for the Anfinsen cage model of chaperonin action
proposed by John Ellis (personal communication). In this
model the final stages of protein folding would take place
within a cage formed by the central pore of the cpn60
tetradecamer. This cage is thought to provide an
environment in which the substrate protein is effectively
sequestered from other proteins, that is, is infinitely
diluted, and can thus continue to fold without
interference from other proteins. Obviously, evidence for
a role of the chaperonins in the folding of large ($M_r$
>70,000) proteins would seriously challenge the validity
of this model. Such evidence, however, has not been
forthcoming.

The largest protein that has been shown to be a

substrate for GroEL is α-glucosidase from yeast with an $M_r$ of 68,000 (Höll-Neugebauer and Rudolph, 1991). However, none of the substrate proteins were selected with the aim of testing the Anfinsen cage hypothesis. Recently Viitanen *et al.* (1992) reported that when $^{35}S$-labelled, soluble proteins from *E. coli* were diluted from 5 M guanidine-HCl into a solution containing GroEL, about 50% of the labelled protein was found in association with the chaperonin. These workers also state, but do not present the data, that >90% of the bound proteins can be discharged by the addition of ATP. In personal communications with John Ellis they have reported that high molecular weight species are bound to the chaperonin. This work suggests that the Anfinsen cage model is wrong. However, several alternative explanations of these observations are possible. Firstly, although the chaperonins are reputed to prevent improper interactions between proteins, it is not inconceivable that some proteins could interact improperly with the chaperonin. Diluting a denatured cell-free extract into a chaperonin solution would surely provide ideal conditions for such improper interactions to take place. Secondly, although the discharge of bound proteins by ATP addition would appear to indicate a specific interaction, previous studies by the same group have shown that addition of ATP to GroEL, whether it is complexed to a substrate protein or not, causes the complex to dissociate into a form of

unknown, multimeric structure (Goloubinoff *et al.*, 1989a).
Thus the apparent discharge could simply be the result of
disruption of the native GroEL protein.

The fact that the polypeptide profiles of the t = 0 and
t = 5 heat shock samples are so different, could indicate
that the flux of polypeptides passing through the
chaperonin is increased after heat shock. On the other
hand, heat shock is known to upregulate the synthesis of
at least one protease, ClpB, in *E. coli* (Squires *et al.*,
1991). Such a protease in *R. sphaeroides* might cause an
increased level of degradation of Rs cpn60 during the
course of the heat shock. It would, however, be quite
strange for a heat shock protease thought to target
abnormal proteins, to also target what is one of the most
important proteins in the cell under any conditions. The
intensity of the bands in the ladder does not, however,
seem to be correlated with that of the Rs cpn60 band (see
in particular lanes 2 and 3, Figure 4.2.3.). This
observation may support the argument for an increased flux
of proteins through Rs cpn 60 after heat shock. This
increased flux appears to be maintained even though there
is a progressive reduction in protein synthesis throughout
the period of the shock (Figures 4.2.1. and 4.2.2.).

The regulation of protein synthesis in response to other
stresses showed characteristics that were unique for each
type of stress. Hydrogen peroxide at 100 mM was found to
rapidly repress all protein synthesis in *R. sphaeroides*.

-197-

VanBogelen *et al.* (1987) had previously shown that
hydrogen peroxide only weakly induced the synthesis of
heat shock proteins. However, protein synthesis was not
completely suppressed since oxidative stress proteins were
strongly induced. Unfortunately, VanBogelen *et al.* (1987)
did not report the concentration of hydrogen peroxide
added to their cultures. Hebert *et al.* (1991) had shown
that addition of hydrogen peroxide, final concentrations
50 mM and 100 mM, to cultures of the archaebacterium
*Methanococcus voltae* caused a marked alteration in the
pattern of protein synthesis. However, protein synthesis
was elevated rather than inhibited in these experiments.
Thus it would appear that *R. sphaeroides* is more sensitive
than *M. voltae* to oxidative stress. It is, nevertheless,
possible that reduced concentrations of hydrogen peroxide
might induce the synthesis of a subset of proteins in *R.
sphaeroides*.

The other stresses all caused an increase in the
synthesis of Rs cpn60. Both 5% (v/v) ethanol and 500 mM
NaCl caused an approximately 5-fold induction of Rs cpn60,
as measured from native gels. In both cases a relatively
slow induction of Rs cpn60 synthesis took place, with the
peak of synthesis occurring more than 45 minutes after
exposure to stresses. The slow induction of protein
synthesis after exposure to ethanol is similar to that
observed in *B. subtilis* (Arnosti *et al.*, 1986),
*Pseudomonas aeruginosa* (Allan *et al.*, 1988) and *E. coli*

-198-

(Neidhardt *et al.*, 1984). While 500 mM NaCl caused a similar increase in the rate of synthesis of Rs cpn60, both quantitatively and temporally, the overall protein synthesis profile is quite different from that observed after ethanol stress. There appears to be little repression of synthesis of proteins after treatment with NaCl and nothing like the heat shock response is induced. High salt is known to not induce a heat shock response in *E. coli* (Neidhardt *et al.*, 1984).

After exposure to cadmium, a quite different response was observed. Relative Rs cpn60 synthesis very rapidly doubled, and was then maintained at this level until the final sample was harvested after 105 minutes. The cadmium treatment causes a substantial repression in overall protein synthesis that is first detected after the t = 45 minute sample. The synthesis of several proteins is induced during the early part of the stress (t = 5 and t = 15 minutes), with a protein of $M_r$ 50,000 being particularly strongly induced (Figure 4.3.2.). This protein is likely to be monomeric, since native gel analysis reveals the presence of a strongly induced $M_r$ <67,000 protein in these samples (Figure 4.3.1.). It is possible that the same protein is also mildly induced after exposure to ethanol.

VanBogelen *et al.* (1987) reported that cadmium did not induce the synthesis of the GroEL protein, although it did induce the synthesis of several other heat shock proteins.

The induction detected in the experiments presented here, although relatively low, is quite clear. It is possible that VanBogelen *et al*. (1987) did detect this level of induction but did not consider it to be significant.

Comparison of native- and SDS-PAGE analyses of protein synthesis after imposition of a stress again show possibly significant differences. In particular the induction of Rs cpn60 synthesis by cadmium appears to be somewhat different in these analyses. It would be interesting to analyse stress-induced, labelled, Rs cpn60 from dried native gels in SDS polyacylamide gels. This type of analysis might reveal differences in the profile of associated polypeptides which might be important in determining whether the polypeptide ladder seen in the heat shock "cutout" experiment is the result of specific association of polypeptides with Rs cpn60 or degradation of Rs cpn60.

A switch from chemoheterotrophic to photoheterotrophic growth caused a complete halt in protein synthesis. Protein synthesis was arrested for a period of approximately 18 hours before pre-shift levels of synthesis were re-established. These observations confirm those of Chory *et al*. (1984), who reported that the accumulation of whole cell protein in *R. sphaeroides* was halted for a period of approximately 12 hours after a similar switch in growth conditions. When protein synthesis resumed, synthesis of Rs cpn60 was slightly

elevated relative to other proteins and the chemo-
heterotrophically grown control.

These studies on the response of *R. sphaeroides* to a
variety of stresses have not revealed any major surprises.
The general pattern of protein synthesis after heat shock
and other stresses, except for exposure to hydrogen
peroxide, is similar to that observed in other organisms.
*R. sphaeroides* appears to be very sensitive to oxidative
stress with hydrogen peroxide greatly inhibiting protein
synthesis.

The investigation into the nature of the proteins found
in association with Rs cpn60 has produced some thought-
provoking observations. It does appear that there is a
size constraint substrate on proteins. This constraint
provides the first physical evidence supporting the
Anfinsen cage model of chaperonin action. However, the
evidence presented is open to other interpretations;
further studies are required to clear up the proteolysis
versus bound substrate argument.

## Chapter 5

## Attempts to identify and purify a putative cytosolic chaperonin
### from the leaves of *Pisum sativum*

### 5.1. Introduction

In the introduction (1.4.4.) it was noted that all the cpn60 proteins so far identified are of either bacterial or membrane-bound organellar origin. The chaperonins are a highly conserved family of proteins with typically >40% of the amino acids along the entire length of chaperonin sequences being identical (Hemmingsen *et al.*, 1988). Chaperonins clearly play an important role in protein assembly and have been shown to be essential for viability of *E. coli* (Fayet *et al.*, 1989). Given these observations on the nature of the chaperonins it seems extremely likely that a functional cpn60 homologue must be present in the cytosol of eukaryotic cells. At the time that this research was started it was somewhat surprising that no such homologue had been identified.

Van der Vies (1989) used 11 chaperonin sequences to search the PIR (Protein Identification Resource) database, using the computer program FASTP. This program uses an algorithm developed by Lipman and Pearson (1985) to identify regions of similar sequence, then scores the aligned identical and differing residues in those regions by means of an amino acid replaceability matrix. An initial score calculated from the best sub-sequence

alignment is used to rank sequences found in the database.
An optimized score, which allows for insertions and
deletions, is then calculated for sequences with high-
ranking similarities. Optimized scores of related
sequences will often be more than double the initial score
while the scores remain much the same for unrelated
proteins. When the biological context of a potential
relationship is unknown, an estimate of the statistical
significance, $z$, of a relationship is useful. Calculation
of $z$ is as follows:

$$z = \frac{(\text{similarity score} - \text{mean of random scores})}{\text{standard deviation of random scores}}$$

Random scores are generated by comparing the query
sequence with randomly permuted versions of the
potentially related sequence. Lipman and Pearson (1985)
suggested that a $z$ value >3 indicates possible
biologically significant sequence similarity, $z$ >6
probably significant and $z$ >10 significant.

The results of the search conducted by van der Vies
(1989) are reproduced in Table 5.1. The numbers in this
table represent $z$ values calculated using the optimized
similarity scores. Table 5.1. clearly shows that only one
protein, $t$-complex polypeptide 1b (TCP-1b), consistently
scores >10 when compared with a number of cpn60 sequences.
We decided to investigate whether this protein might be a
cytosolic chaperonin. Keith Willison and Victoria Lewis,
members of a group that has studied the $t$-complex in

Table 5.1.

**Table 5.1.  Proteins related to the chaperonins**

| Protein | Z value | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | M1 | M2 | P1 | P2 | P3 | P4 | P5 | C1 | C2 | C3 | C4 |
| DNA-directed RNA polymerase | | | | | 5 | 5 | 4 | | 7 | 7 | 4 |
| Myosin heavy chain | | | | 7 | | 7 | | 9 | | 12 | 9 |
| Insulin receptor precursor | | | 3 | 3 | 2 | 3 | | | | | |
| TCP-1 | | | 16 | 16 | | 13 | 11 | | | 15 | |
| 30S ribosomal protein A | 6 | 7 | | | 9 | 8 | | | 4 | | |
| Apolipoprotein B100 precursor | | 6 | | | 6 | | | | 8 | | 7 |
| Cytochrome b | | | 4 | | | | | 5 | 6 | 5 | |
| Fusion glycoprotein | | | 9 | 10 | | | 10 | | | | - |
| Heat shock p70 (*Trypanosoma*) | | 4 | | | | | | | | 6 | 3 |
| DNA K protein | | 6 | | | 4 | 5 | | | | | |

z values calculated using optimized similarity scores (Lipman and Pearson, 1985). z value >3 indicates possible biologically significant sequence similarity, z >6 probably significant and z >10 significant. Chaperonins: M1, human mitochondrial; M2, yeast mitochondria; P1, *Mycobacterium leprae*; P2, *Mycobacterium tuberculosis*; P3, *E. coli*; P4, *Coxiella burnetii*; P5, *Anacystis nidulans*; C1, *Ricinus communis* plastid α; C2, *Triticum aestivum* plastid α; C3 *Brassica napus* plastid α; C4, *Brassica napus* plastid β.

After van der Vies (1989).

mammals for many years, kindly supplied us with monoclonal
antibodies that recognise a murine TCP-1 protein and
agreed to allow us to use these antibodies to search for a
TCP-1 homologue in higher plants.

In the rest of this introductory section I shall briefly
outline the main features of the *t*-complex with particular
emphasis on TCP-1 and then describe why *Pisum sativum* was
chosen for this study.

## 5.1.1. The *t*-complex

Before entering into any description of the *t*-complex,
one should be aware that some of the terms commonly used
to describe the *t*-complex have meanings that differ from
the conventional definitions. A gene complex or complex
genetic locus is usually defined as a cluster of two or
more closely linked and functionally related genes (see
Silver, 1985). The *t*-complex, however, does not represent
a particular family of genes and is only identified as
being the region of a chromosome that can be occupied by a
complete *t*-haplotype. A haplotype is usually defined as a
particular set of alleles present at closely linked (and
often functionally related) loci on any one chromosome
(see Silver, 1985). In contrast, the *t*-haplotype
represents a variant region of the chromosome rather than
a set of alleles (see Silver, 1985; see below).

The mouse *t*-complex has attracted the attention of
geneticists for some 60 years (see Silver, 1985). The

study of the *T*-locus began with the detection of a
dominant mutation referred to as *Brachyury* (*T*) (see
Bennett, 1975). This spontaneous mutation produced short
tails in heterozygous mice and was shown to be lethal in
homozygotes (see Bennett, 1975). Shortly after the
discovery of *T*, it was shown that apparently normal mice
of two different wild origins had a gene that interacted
with *T* to produce a new phenotype, taillessness. Initially
the genetics of the system appeared to be remarkably
simple. Tailless mice of any one stock bred true,
suggesting that taillessness was produced by a balanced
lethal system. In this system two allelic genes, one
dominant (*T*) and one recessive (*t*), each lethal when
homozygous, were responsible for the tailless condition.
Such a system is illustrated below:

| Parents | *T/t* | x | *T/t* |
|---|---|---|---|
| Offspring | *T/T* | *T/t* | *t/t* |
| | (dead as embryos) | tailless | (dead as embryos) |

   This suggested a simple locus model for the interaction
of these two mutations. There is indeed a well defined and
simple *T*-locus near the centromere on mouse chromosome 17
(see Silver, 1985). However, it soon became clear that the
*t*-haplotypes are not single-locus mutations.

   A large body of evidence has shown that *t*-chromosomes
are variants of mouse chromosome 17 that exist naturally
in wild mouse populations. The principal variation in *t*-

chromosomes consists of at least four inversions spanning approximately 30 Mb of DNA. It accounts for most of the proximal one third of the chromosome, that is, approximately 1% of the mouse genome (Herrmann *et al.*, 1987; Hammer *et al.*, 1989). This region is known to contain around 100 genetic loci (Lyon *et al.*, 1988) and includes the *T*-locus as well as the entire major histocompatibility complex and expresses pleiotropic effects on tail length, fertility, embryogenesis, male transmission ratio and meiotic recombination (Dunn and Glueksohn-Schoenheimer, 1950; see Glueksohn Waelsch, 1989). Sequence comparison of *Tcp-1a* and *Tcp-1b* cDNAs and their introns indicated that *t*-haplotypes arose within the genus Mus one to two million years ago (Willison *et al.*, 1986).

The *t*-haplotype appears to have maintained itself as a well defined genomic entity by suppression of recombination along its length, and propagation of itself through mouse populations by means of a male-specific transmission ratio distortion (TRD) in its favour (Silver, 1985). The suppression of recombination appears to be the result of a basic non-homology in the genomic organization of the wild type and *t*-haplotype forms of chromosome 17 (Silver and Artzt, 1981). The TRD of heterozygous *t*/+ males results in the recovery of the *t*-bearing chromosome among progeny in vast excess of the Mendelian expectation, so that wild males that carry a complete *t*-haplotype will

transmit it to nearly all (often greater than 99%) of
their progeny (see Silver, 1985). Males doubly
heterozygous for complementing *t*-haplotypes are completely
sterile while homozygous animals are embryonic lethals
(see Bennet, 1975). The unusual properties of the *t*-
complex have generated so much interest that its area on
chromosome 17 has become "one of the most completely
analyzed regions of any mammalian chromosome" (see
Glueckschn Waelsch, 1989).

While the genetics of the *t*-complex have been studied
for over 60 years, it is only in the last 13 years that
any significant progress has been made in identifying
proteins specified by genes within the *t*-complex. Since
the *t*-haplotype has profound effects on fertility and
causes the TRD, it was likely that a comparative analysis
of testicular cell proteins from wild-type and *t*-bearing
mice would identify *t*-specific proteins. In the first
unambiguous analyses of this type, 2-dimensional gel
electrophoresis was used to identify a series of *t*-encoded
polypeptides (Silver *et al.*, 1979; 1983). Silver *et al.*
(1983) were able to identify at least nine *t*-haplotype-
specific proteins which they named *t*-complex protein-1
(TCP-1) through to *t*-complex protein-9 (TCP-9). Today
these proteins are usually referred to as *t*-complex
polypeptides. To date, very little is known of the
biochemical and functional properties of these proteins.

## 5.1.2. TCP-1

TCP-1 is by far the most intensively studied of the *t*-complex polypeptides. It was initially identified in mice as an abundant, testicular *t*-specific protein of $M_r$ 63,000 with an isoelectric point of pH 6.9 (Silver *et al*., 1979). Cells from animals that did not carry a *t*-haplotype expressed a slightly more basic, 0.03 pH units, form of this protein (Silver *et al*., 1979). These proteins are now known as TCP-1A and TCP-1B respectively (Danska and Silver, 1980).

TCP-1 has been shown to be expressed in all mouse cell lines, tissues and developmental stages analyzed to date (Silver *et al*., 1979; Silver and White, 1982; Nozaki *et al*., 1986), with the exception of two-cell embryos (Nozaki *et al*., 1986) and mature sperm (Willison *et al*., 1989). The highest level of expression is observed in testicular cells (Silver *et al*., 1979). Dudley *et al*. (1984) have used a *Tcp-1* cDNA clone to demonstrate the appearance of stable *Tcp-1* transcripts throughout spermatogenesis, with *Tcp-1* mRNA levels reaching a peak of accumulation in spermatids. The level of transcripts was shown to be 22.4 times higher in whole adult mouse testis RNA than in liver RNA (Dudley *et al*., 1984).

TCP-1 is a well conserved molecule. *Tcp-1* DNA sequences from mouse, *Tcp-1ᵃ* and *Tcp-1ᵇ*, (Willison *et al*., 1986), human (Willison *et al*., 1987), *Drosophila melanogaster* (Ursic and Ganetzky, 1988) and *S. cerevisiae* (Ursic and

Culbertson, 1991) have been determined, and shown to share extensive identity at both the DNA and deduced amino acid sequence level. Willison *et al.* (1989) used bacterially expressed TCP-1B to produce a panel of monoclonal antibodies, which recognized TCP-1 homologues in both somatic and germ cells from a large variety of vertebrate species. The widespread distribution and strong conservation of both the primary structure and antigenic epitopes, strongly indicate that TCP-1 proteins are required for an essential cellular function. The demonstration that *Tcp-1* is an essential gene in *S. cerevisiae* (Ursic and Culbertson, 1991), and the failure to identify viable mice homozygously deleted for the *Tcp-1* gene (Willison *et al.*, 1989), have confirmed this view.

Identification of the sub-cellular location of TCP-1 has proved to be somewhat difficult. Silver *et al.* (1979) suggested that TCP-1 must be a cell surface protein, since limited proteolysis of viable testicular cells caused selective cleavage of TCP-1 relative to other cell proteins known to be internal. Subsequently the subcellular distribution of TCP-1 was examined in F9 teratocarcinoma cells and testicular cells (Silver and White, 1982). Cells were fractionated into four compartments (nuclear, mitochondrial, crude membrane pellet and soluble cytoplasmic). This analysis revealed the presence of significant levels of TCP-1 in all fractions and that there were striking differences in the

distibution of TCP-1 in these cell lines (Silver and
White, 1982).

Although these results present a rather confusing
picture, Silver and White (1982) suggested that a portion
of the extracellular matrix might be trapped into co-
purification with nuclei. The differences in distribution
between the two cell lines are ascribed to fact that F9
teratocarcinoma cells do not have a well-defined
extracellular matrix, while testicular cells have a highly
organized extracellular matrix. It was suggested that the
extracellular form of TCP-1 can thus be easily purified
away from the former type of matrix, but not the latter
type. Silver and White (1982) went on to compare the
extraction properties of TCP-1 under a variety of
treatments with those of proteins of known subcellular
location, and concluded that TCP-1 was indeed associated
with the cell surface matrix. The presence of 50% of TCP-1
in the F9 teratocarcinoma soluble cytoplasmic fraction is
largely passed over without comment, although they do
suggest that the nature of TCP-1 intra- and/or
intermolecular interactions might be under some form of
tissue-specific control.

Willison *et al.* (1989) used a bank of monoclonal
antibodies that recognise TCP-1 in an attempt to determine
its subcellular location. Both indirect immunofluorescence
and immunogold localization studies indicated that TCP-1
was peripherally associated with the cytoplasmic aspect of

membranes of the *trans*-Golgi network. Analysis of
microsomal and cytosolic fractions prepared from
postnuclear supernatants demonstrated that, while most of
the TCP-1 was associated with the membrane fraction, 5-10%
was consistently found in the cytosolic fraction (Willison
*et al.*, 1989). These workers also examined the subcellular
distribution of TCP-1 in spermatids, and observed that
there appeared to be extensive alterations in the
distribution of TCP-1 at different stages of development.
In early spermatids immunoflourescence was detected in
structures resembling the enlarged Golgi apparatus
characteristic of these cells, as well as in a structure
close to the nucleus that was thought to be the pro-
acrosomal granule. This observation is interesting since
enlargement of the Golgi apparatus during the early
spermatid stages plays a key role in acrosome formation.
Furthermore, coated vesicles budding from the *trans* face
of the Golgi and fusing with the developing acrosome are
thought to convey the lytic enzymes that comprise the
acrosome content (see Willison *et al.*, 1989). Willison *et
al.* (1989) also observed that the distribution of TCP-1
was significantly altered in noninterphase cells. The
Golgi apparatus is known to fragment during mitosis, and
this fragmentation can be reproduced experimentally by
treatment with the microtubule depolymerizing drug,
nocodazol. When cells were treated with this drug, the
distribution of TCP-1 was altered in a manner that

suggested to the authors that TCP-1 associated with
membranes which are dependent on microtubules for their
perinuclear organization (Willison *et al.*, 1989). The true
significance of this observation has recently become
apparent and will be discussed at the end of this chapter.

Willison *et al.* (1989) were consistently unable to
detect TCP-1 on the surface of tissue culture or germ
cells. The deduced amino acid sequence of TCP-1 reveals a
protein that lacks a signal sequence and is devoid of
hydrophobic domains (Willison *et al.*, 1986), and is thus
unlikely to be a secretory or integral membrane protein.
Willison *et al.* (1989) were unable to reconcile their
results with the biochemical data suggesting that TCP-1 is
associated with the extracellular matrix (Silver *et al.*,
1979; Silver and White, 1982). More recently Willison
(personal communication) has suggested that TCP-1 is
largely found in the cytosol, and that only a much smaller
proportion the protein is associated with the *trans*-Golgi
network. While localization studies have not given clear-
cut answers it is clear that at least a portion of the
TCP-1 protein is located in the cytosol. We decided to
determine whether a homologue of this protein is present
in *P. sativum*.


5.1.3. *Pisum sativum as a test organism*

The *P. sativum* plastid chaperonin was first identified
by Barraclough and Ellis (1980) in work carried out at the

-213-

University of Warwick. This discovery was crucial in the
formulation of the molecular chaperone concept. In the
years since the discovery of this protein, considerable
expertise and knowledge has been gained regarding the
purification and biochemical properties of this protein.
*P. sativum* is easy to grow, purification of the plastid
chaperonin is relatively simple and antibodies to this
protein are available. Thus, *P. sativum* appeared to be a
good model system to investigate the presence of a
putative cytosolic chaperonin in a photosynthetic
eukaryote.


## 5.2. Identification of a putative TCP-1 homologue in
### *P. sativum*

This investigation would not have been possible without
Keith Willison's kind gift of TCP-1-specific monoclonal
antibodies. 91a is a monoclonal antibody that recognizes
an epitope residing between residues 306-492 of the murine
TCP-1 sequence, while 84a recognizes a more C-terminal
epitope (Willison *et al.*, 1989). Both of these monoclonal
antibodies were used to probe proteins from several
sources (Fig. 5.2.1.). In these experiments a biotinylated
sheep anti-rat antibody/streptavidin detection system was
used. Controls in which the western blots were incubated

Figure 5.2.1. Identification of a putative TCP-1 homologue in *P. sativum*



TCP-1-specific monoclonal antibodies 91a and 84a were used to probe polypeptides from several sources. Polypeptides were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 91a, 84a or no antibody. Tracks are labelled according to the extract loaded. E. coli total = *E. coli* total; C. bean mito = castor bean mitochondria; Pea chloro = *P. sativum* chloroplast; Pea total = *P. sativum* total leaf; Mouse mito = mouse mitochondria. Mouse extracts were the kind gift of Dr Martin Connock and castor bean mitochondrial extracts the kind gift of Dr Sharon Alldrick. Western blots were developed using the biotin/streptavidin detection system.

-215-

in the absence of primary antibody, demonstrated that this
detection system non-specifically interacted with
polypeptide bands in all the preparations analyzed. While
this detection system was adequate for the detection of
specific antigens the number of non-specific interactions
was undesirable and also gave the developed blots an
untidy and confusing appearance. In later experiments (see
Figure 5.4.1.) a peroxidase-linked secondary antibody was
used for detection. This system is simpler, the blots
being developed after incubation with secondary antibody
instead of first being incubated with streptavidin. It is
also faster, it costs no more than the biotin/streptavidin
system and produces fewer non-specific interactions. Given
these observations I find it somewhat surprising that the
biotin/streptavidin system is used at all!

  Probing with the 91a antibody and using the
biotin/streptavidin detection system clearly detected a
polypeptide of $M_r$ 57,000 in a *P. sativum* total leaf
extract (Fig. 5.2.1.). This polypeptide is slightly
smaller than the polypeptide detected in a mouse extract
and no such polypeptide was detected in bacterial,
mitochondrial or chloroplast extracts. 84a detected a
single band in a mouse extract and, surprisingly, a large
number of bands in a mouse mitochondrial extract. This is
presumably a result of the increased loading of
mitochondrial proteins relative to those in the total
extract. 84a also interacted weakly with several bands in

*P. sativum* total and chloroplast extracts. However, none
of these bands corresponds to the one detected by 91a in
the *P. sativum* total cell extract.

The results obtained with 91a indicated that there may
be a TCP-1 homologue in *P. sativum*, and that this protein
is only detected in total cell extracts and is apparently
absent from mitochondrial and chloroplast extracts. These
observations are consistent with, but by no means proof
of, a cytosolic location of this protein. Attempts to
determine the subcellular location of this protein in *P.
sativum* cells were then made, using 91a as a probe. The
multiplicity of polypeptides detected in various extracts
using 84a as a probe suggested that this monoclonal would
not be suitable for use in immunocytochemical studies, and
no further work was done using this antibody.

## 5.3. Attempts to determine the subcellular distribution of a
## putative *P. sativum* TCP-1 homologue

The data presented above clearly demonstrated that 91a
detected a single protein in total *P. sativum* leaf
extracts, and that this protein did not appear to be
present in the chloroplasts of this tissue. It was
important in this study to determine the precise
subcellular distribution of this protein. To this end, pea
leaf, root and anther preparations were studied in
immunogold localisation experiments, using 91a as a probe.

In initial experiments a low stringency method was used

in which sections of gluteraldehyde-fixed plant material
mounted on electron microscope grids were first blocked in
the presence of 3% (w/v) BSA, then incubated in the
presence of 91a for either 1 hour at room temperature or
16 hours at $4^{\circ}C$ was used. After washing, grids were
incubated in the presence of secondary antibody, 5 nm
gold-labelled goat anti-rat IgG, for 1 hour at room
temperature. After staining electron microscopy revealed
gold particles in most compartments of the cell, including
the chloroplasts. Figure 5.3.1. shows typical results
obtained using leaf material. No gold particles were
observed in the cell wall, vacuoles and intracellular
spaces. Since the biochemical data presented in Section
5.2. had indicated that the putative TCP-1 homologue was
not present in chloroplasts, this result was treated with
some scepticism. On control grids incubated in the absence
of primary antibody, few if any gold particles were seen
in any one field (data not shown), indicating that 91a was
indeed mediating the binding of the secondary antibody.
These observations raised the possibility that the
observed binding of 91a was non-specific.

This was investigated by varying the conditions under
which antibody binding took place. All of these conditions
involved the use of more stringent binding and washing
conditions, including conditions identical to those used
for western blots. As the stringency of the binding and
washing conditions was increased, there was a general

**Figure 5.3.1.**  <u>Attempted immunogold localisation of a putative</u>
<u>TCP-1 homologue in pea leaves</u>

This figure contains two micrographs that show most
compartments of the *P. sativum* leaf cell. Sections were
incubated in the presence of 9la (dilution 1 in 50) for 16
hours at 4ºC. Sections were then labelled with secondary
antibody, 5 nm gold-labelled goat anti-rat IgG, for 1 hour
at room temperature. Sections were negatively stained with
2% (w/v) uranyl acetate.

An electron-dense gold particle is highlighted with an
arrow in each micrograph. Cell components are identified
as follows:

>       C = chloroplast
>
>       S = stroma
>
>       G = granum
>
>       M = mitochondrion
>
>       N = nucleus
>
>       NE = nuclear envelope
>
>       Ch = chromatin
>
>       CW = cell wall
>
>       L/P = lysosomes/peroxisomes

BW GW PEA1 91A16H
H21421  80 0KV  X20K  200nm

L/P

BW GW PEA1 91A16H
H21426  80.0KV  X20K  200nm

reduction in the number of gold particles seen in any field (data not shown). Under the most stringent conditions, binding of secondary antibody was virtually abolished, so that controls incubated in the absence of 9la were indistinguishable from those incubated in the presence of 9la (data not shown).

These results indicated that the binding of 9la observed under low stringency conditions was non-specific and that high stringency conditions abolished this binding. Furthermore, the observation that 9la clearly recognizes an SDS-denatured protein on western blots suggests that the epitope recognized by 9la is buried in the native protein and is thus inaccessible to this antibody. Immunoprecipitation of the native murine TCP-1 protein by 9la (Willison *et al.*, 1989) indicates that in this protein the epitope recognized by 9la is present on the surface of the protein. The significance of the difference between the native putative *P. sativum* TCP-1 homologue and the native murine protein is unknown. Whatever the reasons for the non-specific binding of 9la to plant tissue, it was clear that an unequivocal determination of the subcellular location of the putative TCP-1 homologue would be impossible using this antibody.

The results of this study also clearly demonstrated that immunocytochemical data on their own must be treated with extreme caution. Immunogold localization techniques are a powerful tool for studying the distribution of individual

cellular proteins. However, without supporting evidence
from traditional biochemical subcellular fractionation and
purification techniques, results obtained using this
particular methodology are of little, if any, value.

Attempts to establish the intracellular distribution of
the putative TCP-1 homologue using the 91a monoclonal
antibody had failed. Therefore, the only way forward
appeared to be to attempt to purify this protein and then
produce antisera that would, hopefully, recognise the
native form of this protein. This antiserum would then be
used to repeat the localization studies. It should be
noted that Willison *et al*. (1989) have already attempted
to raise antibodies to the native murine TCP-1 protein,
but these attempts were unsuccessful.

## 5.4. Attempts to purify a putative TCP-1 homologue from the leaves of *P. sativum*

In attempting to purify a putative TCP-1 homologue, a
single consideration has strongly influenced the strategy
used. This is that the putative TCP-1 homologue is a
cytosolic chaperonin and thus might be expected to share
many features, particularly high native molecular weight,
with the known cpn60 proteins.

Two methods were used to prepare leaf extracts (see
2.9.1.2.). In the first method pea leaves (50 g) from 10
day old plants were harvested and ground in a pestle and
mortar in a small volume (20 ml) of TEMM$\beta$ buffer

containing 1 mM PMSF. After expression through muslin and removal of cellular debris by centrifugation, this extract was applied directly to an 8-28% (w/v) sucrose density gradient. Initial results were promising. However, these preparations were often contaminated with debris that tended to peel off the walls of the centrifuge tubes during fractionation (data not shown). Even after centrifugation this material was almost impossible to filter prior to column chromatography. This problem was solved by subjecting the crude leaf extract to ammonium sulphate precipitation. Figure 5.4.1. shows the results of a series of ammonium sulphate fractionations. Clearly, most of the putative TCP-1 homologue is contained in the 40-50% (of ammonium sulphate saturation) cut. In subsequent experiments a fairly broad cut, 25-60%, was used. The material from such a cut contained relatively little cellular debris and was easily filtered after SDGC. Preparations conducted in this manner are referred to as grind preparations.

The grind preparation method was, however, not suitable for large scale (>200 g) leaf extract preparations. The main reason for this was that considerable physical strength was required to grind the pea leaves to a smooth paste. It soon became apparent that such a procedure was time-consuming, physically demanding and, for the latter reason, could not easily be repeated by other researchers. For these reasons a different approach was taken: leaves

-222-

Figure 5.4.1.   <u>Ammonium sulphate fractionation of a total *P.*</u>

   <u>*sativum* leaf extract</u>



A total leaf extract, 17.8 ml from 50 g leaves, was subjected to ammonium sulphate fractionation as described in Materials and Methods. Pellets from each cut were resuspended in 10 ml grind buffer. Tracks are labelled according to the range of the cut as percentages of ammonium sulphate saturation. 20 µl aliquots were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

(usually 500 g) were homogenized in a large volume of
TEMMβ buffer in a Polytron blender. The leaf extract was
then treated in the same way as extracts from grind
preparations. This second method of preparation is
referred to as a blender preparation.

Figure 5.4.2. shows the results of an analysis of a pea
leaf extract prepared by the grind method. Fractions from
sucrose density gradients were analysed by SDS-PAGE,
followed by western blot analysis using 91a as the primary
antibody. This analysis clearly demonstrated that the
putative TCP-1 homologue sediments slightly faster than
the Rubisco peak. The distribution of the putative TCP-1
homologue relative to Rubisco is very similar to that of
Rs cpn60 relative to *R. sphaeroides* Rubisco in SDGC
fractionated cell-free extracts of *R. sphaeroides* (see
Figure 3.3.2.). Thus, in its native form, the putative
TCP-1 homologue is a component of a multimeric complex
which is approximately the same size as the Rs cpn60
native protein. Two other bands of higher and lower
molecular weight than the putative TCP-1 homologue were
detected. These bands were not detected consistently, from
batch to batch of peroxidase-linked antibody. However,
control blots indicated that this binding was non-specific
(data not shown).

Willison *et al*. (1989) had suggested that the murine
TCP-1 protein was rather labile under various extraction
conditions. The stability of the putative TCP-1 homologue

Figure 5.4.2. **Analysis of *P. sativum* leaf extract fractionated by continuous sucrose density gradient centrifugation**

1 ml fractions from a continuous sucrose gradient were collected. 20 μl aliquots were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%). Tracks are numbered by gradient fraction, the bottom of the gradient being fraction 1.

A. Western blot probed with 9la (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

B. Coomassie-stained gel.

was tested by incubating aliquots of pooled sucrose gradient fractions at 18°C, in the presence of a variety of protease inhibitors recommended for use during isolation of plastid proteins (Gray, 1982). The inhibitors used and their target proteases are listed below:

| Protease inhibitor | Target protease |
| --- | --- |
| Phenylmethylsulphonyl fluoride (PMSF) | Serine proteases and thiol proteases |
| Mercuric chloride ($HgCl_2$) | Thiol proteases |
| Diazoacetyl nor-leucine methyl ester (DAN) + $Cu^{2+}$ | Carboxyl proteases |
| Ethylenediaminetetra-acetic acid (EDTA) | Metallo-proteases |

The pooled sucrose gradient fractions were prepared in TEMM buffer (see 2.4.4.). Normally, leaf extracts were prepared in TEMM containing 2-mercaptoethanol (TEMMB). The possibility that the 2-mercaptoethanol might activate thiol proteases was investigated by incubation in the presence of this reagent. Another aliquot was incubated in the presence of added magnesium since Willison had suggested that added magnesium, might aid in the purification of TCP-1 (Willison, personal communication). A control sample was boiled in solubilisation buffer at the start of the experiment.

Figure 5.4.3. shows the results of these stability studies. It should be noted that the material used in this

**Figure 5.4.3.   Stability of the *P. sativum* putative TCP-1**

**homologue**



Putative
TCP—1
homologue

Putative
TCP—1
homologue

Pooled sucrose density gradient fractions were incubated in the presence of various protease inhibitors for the times noted. Track 1, 1 mM PMSF; 2, 5 mM HgCl₂; 3, 5 mM DAN + 5 mM copper (II) acetate; 4, 10 mM EDTA; 5, 50 mM MgSO₄; 6, 50 mM 2-mercaptoethanol; 7, no addition; Con = control sample boiled in solubilization buffer at the start of the experiment. 10 μl aliquots were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

experiment had already been exposed to PMSF during preparation of the leaf extract. No significant degradation of the putative TCP-1 homologue occurred in any of the samples during the first two days of incubation. After four days, the aliquot containing DAN/$Cu^{2+}$ appeared to contain significantly less of the putative TCP-1 homologue relative to the control sample. After nine days, barely any TCP-1 homologue could be detected in the aliquots containing added DAN/$Cu^{2+}$ or $MgSO_4$. The aliquot containing 2-mercaptoethanol produced a weak signal relative to the control, while aliquots containing $HgCl_2$, additional PMSF or additional EDTA were very similar to the control. No degradation products were detected in any of the incubations. However, it is quite possible that higher protein loadings may have revealed the presence of such products. Interestingly, aliquots that were incubated in the presence of $HgCl_2$ appeared to contain a lower mobility species of the TCP-1 homologue. Since the higher mobility species seen in all the other incubations is not present, it is possible that $HgCl_2$ inhibits a protease that is activated during sample preparation or that binding of $HgCl_2$ to thiols reduces mobility of the polypeptide. There is no evidence that this protease, should it exist, is active under normal purification conditions. In view of these observations no protease inhibitors, other than PMSF, were included in the extraction buffer, and no protease inhibitors were

included in subsequent purification steps.

Having demonstrated that the putative TCP-1 homologue
had similar characteristics to Rs cpn60 during the initial
stages of purification, it was decided to continue the
purification in the same manner as used for Rs cpn60
purification. Thus, pooled fractions from sucrose density
gradients were then subjected to anion exchange
chromatography on a Mono Q column. Figure 5.4.4. shows the
western blot analysis of fractions eluted from the Mono Q
column. The putative TCP-1 homologue eluted from the
column on the shoulder of the Rubisco peak at a NaCl
concentration of approximately 370 mM. Again this
behaviour is reminiscent of the Rs cpn60. When fractions
from the Mono Q column were resolved in a 4-30% native
polyacrylamide gel (Fig. 5.4.5.), a number of high
molecular weight protein bands of relatively low abundance
were detected on the edge of the Rubisco peak. These bands
were considered to be likely candidates for a putative
TCP-1 homologue containing native protein.

Several attempts were made to identify which of these
proteins, if any, might contain the putative TCP-1
homologue. This was done by excising bands from unstained
native gels and then analysing them in SDS-polyacrylamide
gels, followed by western blot analysis. Unfortunately,
this method failed to reveal the presence of the putative
TCP-1 homologue in any of these bands (data not shown).
This failure was not taken to mean that this polypeptide

Figure 5.4.4. **Fractionation of pooled *P. sativum* sucrose density gradient material by ion exchange, Mono Q. chromatography**

A. Elution profile. Column flow rate of 0.5 ml/min. Fraction size 1 ml. The NaCl gradient was developed as shown. Total protein loaded 35 mg.

B. 20 μl aliquots were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 9la (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

Figure 5.4.5.   Native gel analysis of Mono Q fractions



Native gel analysis of fractions from a typical Mono Q fractionation of pooled sucrose density gradient material (see Figure 5.4.4.). 50 μl aliquots were resolved in a 4-30% native polyacrylamide gel (Coomassie stained). Tracks are numbered by fraction number.

definitely was not present in any of these bands, since
the resolution of polypeptides after this type of analysis
was very poor and the putative TCP-1 homologue might have
smeared down the gel to such an extent that it escaped
detection.

An abundant species of $M_r$ 670,000 that was reasonably
well separated from the Rubisco peak was thought to be the
plastid cpn60. In separate experiments western blot
analysis of Mono Q fractions from the shoulder of the
Rubisco peak across the presumed plastid cpn60 peak was
performed. Figure 5.4.6. shows the results of such an
analysis after blots had been probed with 91a and anti-
plastid chaperonin. The anti-plastid chaperonin polyclonal
antibody predominantly detects two major proteins of $M_r$
58,000 and 57,000. These proteins represent the $\alpha$ and $\beta$
subunits respectively of the native plastid chaperonin
(Musgrove *et al.*, 1987). The blots clearly demonstrate
that 91a does not cross-react with the plastid chaperonin,
thus confirming and extending the observation that 91a
does not recognise the chloroplast protein (see Fig.
5.2.1.). However, the possibility that 91a is recognizing a
chaperonin or other protein from another membrane-bound
organelle, for example the mitochondrion, cannot be
excluded at this stage. Again the observed properties of
the putative TCP-1 homologue appeared to be very similar
to those of a chaperonin, in this case the plastid
chaperonin.

Figure 5.4.6.   Selected Mono Q fractions probed with 91a and
*P. sativum* anti-plastid chaperonin

When the putative TCP-1 homologue-containing fractions
were pooled, diluted two-fold and subjected to a second
bout of anion exchange chromatography, the protein of
interest still eluted with the Rubisco peak even though a
step, designed to elute the Rubisco peak alone, was
included in the gradient (Fig. 5.4.7.). It was hoped that
alteration of the NaCl gradient parameters would allow the
putative TCP-1 peak to be separated from the Rubisco peak.
Flattening the gradient did not achieve any significant
improvement. Attempts to introduce a step in the gradient
that would elute the Rubisco peak first followed by the
putative TCP-1 homologue on restarting the gradient also
failed (data not shown).

## 5.5. Further purification of the putative TCP-1 homologue

It was obvious from these initial steps that
purification of the putative TCP-1 homologue was going to
be more difficult than purification of either the plastid
chaperonin or the Rm cpn60 protein. The main problem to be
overcome was to separate the putative TCP-1 homolgue from
the Rubisco protein.

A number of other chromatographic techniques were used
in attempts to attain this goal. Amicon dye-affinity
columns were screened for their potential usefulness in
purifying the putative TCP-1 homologue (data not shown).
Of these columns, the Green A matrix appeared to most
promising. Figure 5.5.1. shows the fractionation of pooled

Figure 5.4.7. **Fractionation of pooled *P. sativum* Mono Q fractions by a second round of ion exchange, Mono Q, chromatography**

A. Elution profile. Column flow rate of 0.5 ml/min. Fraction size 1 ml.

B. 20 μl fractions were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 9Ia (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

Figure 5.5.1. **Fractionation of pooled *P. sativum* sucrose density gradient material by dye-ligand, Green A, affinity chromatography**



A. Elution profile. Column flow rate of 2 ml/min. Fraction size 2.5 ml. Total protein loaded 160 mg.

B. 20 µl aliquots were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

sucrose density gradient material on a Green A column. This matrix appeared to bind very few proteins other than Rubisco and the putative TCP-1 homologue. However, once again, the putative TCP-1 homologue eluted as a shoulder on the Rubisco peak. Although Rubisco was present, this treatment represented a substantial purification step and use of the Green A column was further investigated. Again, attempts to separate the putative TCP-1 homologue from the Rubisco peak by varying gradient parameters were unsuccessful (data not shown).

The most promising way to remove the Rubisco appeared to be by affinity chromatography, using anti-Rubisco cross-linked to a suitable matrix. It seemed likely that any polyclonal antisera raised against the *P. sativum* Rubisco would not be suitable for this purpose, since the antigen used to raise such antisera would almost certainly be contaminated with the putative TCP-1 homologue protein. A monoclonal antibody raised against the *P. sativum* Rubisco protein appeared to be the ideal solution to this problem. Unfortunately no such antibody was at hand and time constraints did not permit the production of a bank of anti-*P. sativum* Rubisco antibodies. Alfred Keys of the Rothamstead plant research institute was kind enough to provide a sample of a Sepharose-linked anti-wheat Rubisco monoclonal antibody. However, when the relevant fractions obtained from Green A chromatography were pooled and applied to the Rubisco affinity column, no binding of the

Rubisco could be detected at all (data not shown). This appeared to indicate that the affinity column carried a monoclonal that did not recognise the *P. sativum* Rubisco. No further work was done with this antibody.

Another approach to the Rubisco problem was to reduce the amount of Rubisco in the starting material. This was achieved by growing peas in the dark for seven days. The etiolated peas were then harvested by removal of the leaves and the stem material was treated by the blender method of extract preparation. Extracts prepared in this way were fractionated by sucrose density gradient centrifugation and the appropriate fractions analysed on a Mono Q column. Figure 5.5.2. shows the western blot analysis of pooled fractions retained after sucrose density gradient centrifugation and Mono Q chromatography. Although the quantity of Rubisco in these extracts was greatly reduced, this method of preparation did not appear to be promising. The main reason for this was that so little material was recovered from the ground-up stems; only 350 g stem material was harvested from six trays of peas. After sucrose density gradient centrifugation, approximately 44 mg of material was collected. The putative TCP-1 homologue was then further purified by FPLC Mono Q chromatography. Peak fractions were pooled, concentrated by ammonium sulphate precipitation, and resuspended in 0.6 ml buffer. When 25 µl of this material was subjected to western blot analysis, an immunoreactive

Figure 5.5.2. Attempted purification of the *P. sativum* putative TCP-1 homologue from root tissue

Polypeptides were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%). Track 1, pooled sucrose density gradient material from leaf (12.5 μl); 2, pooled sucrose density gradient material from root (25 μl); 3, pooled Mono Q fractions from root (25 μl).

A. Coomassie-stained gel

B. Western blot probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

band could be detected but the quantity of protein in this band was so low that it was not possible to identify the corresponding band in a stained gel. Such low yields of only partially purified protein, combined with the difficulty of growing etiolated peas (these were often contaminated with fungi and large amounts of the starting material had to be discarded), led to the abandonment of this line of investigation.

It seemed obvious from the preceding results that the putative TCP-1 homologue was not a very abundant protein in extracts of *P. sativum*. This in itself was somewhat worrying since bacterial and organellar chaperonins are characteristically abundant proteins. In an attempt to produce enough of the TCP-1 homologue to at least attain an N-terminal sequence, it was decided to use far greater amounts of pea leaves as the starting material for these purifications. In such experiments, 500 g of pea leaves was harvested and processed using the blender method. The material gathered after ammonium sulphate precipitation was dialysed overnight against grind buffer and then loaded onto eight standard sucrose gradients (see 2.9.2.2.). After centrifugation the gradients were fractionated as usual and, after filtration, the TCP-1 homologue-containing material was loaded onto a HiLoad (26/10) Q Sepharose High Performance column (2.9.3.1.).

Material was eluted from the column by developing a similar salt gradient to that used for elution from Mono Q

columns. Fractions were analysed by western blot analysis (Figure 5.5.3.). This type of gradient on the HiLoad Q column appeared to give a slightly better resolution than that obtained with the Mono Q column. In particular, the resolution of the putative TCP-1/Rubisco peak from the plastid chaperonin peak was considerably improved. This latter property has been exploited in the preparation of very high purity plastid chaperonin protein (Linda Barnet, personal communication).

The fractions containing the putative TCP-1 homologue were pooled, ammonium sulphate precipitated and resuspended in 20 mM Tris-HCl pH 7.5. This material was then subjected to chromatography on a variety of columns, including Green A, Mono Q and S-400 (size exclusion). Regardless of the nature of the second column used the subsequent recovery of the putative TCP-1 homologue was either much lower than expected or, in the case of the S-400 column, no immunoreactive material could be detected at all (data not shown). These observations indicated that something was happening to the native protein during the purification. Since previous results had suggested that the polypeptide was reasonably stable (see Fig. 5.4.3.), it was thought that the most likely change was the dissociation of the native protein into lower molecular weight forms with different chromatographic properties. Previous attempts at western blot analysis of sucrose density gradient fractions resolved in native gels had not

-241-

Figure 5.5.3. **Fractionation of pooled *P. sativum* sucrose density gradient fractions by ion exchange, HiLoad Q, chromatography**



A. Elution profile. Column flow rate of 10 ml/min. Fraction size 15 ml. The NaCl gradient was developed as shown. Total protein loaded 800 mg.

B. 25 μl fractions were resolved in 10% SDS-polyacrylamide gels (T = 10.1%, C = 1%) and western blotted onto nitrocellulose membranes. Blots were probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

revealed the presence of any immunoreactive material. This appeared to confirm the view gained from immunogold studies that 9ia does not cross-react with the native form of the putative TCP-1 homologue. The possibility that the native form was dissociating was investigated by western blot analysis of HiLoad Q anion exchange fractions resolved in non-denaturing polyacrylamide gels. This analysis clearly shows the appearance of a number of immunoreactive bands after anion exchange chromatography, while none were detected after the initial SDGC fractionation (Fig. 5.5.4.). Western blot analysis of SDGC fractions resolved in SDS-polyacrylamide gels (Fig. 5.4.2.) had indicated that the putative TCP-1 homologue was present in a high molecular weight complex. This analysis did not reveal the presence of any significant amounts of immunoreactive material in forms of lower molecular weight than Rubisco. After anion exchange chromatography these, lower molecular weight complexes were observed, indicating that this procedure either enriches previously undetected lower molecular weight species or causes the dissociation of the native complex. The failure of 9ia to detect any of the putative TCP-1 homologue in immunogold localization experiments (see Section 5.3.) indicates that the lower molecular weight forms, detectable by 9ia in western blot analyses, do not occur naturally, and that their appearance is a direct consequence of the chromatographic technique used.

Figure 5.5.4.  **Western blot analysis of partially purified putative TCP-1 homologue resolved in a native gel**

Mr x 10$^{-3}$

669 ———▶

440 ———▶

232 ———▶

140 ———▶

67 ———▶

*P. sativum* leaf extracts were fractionated by sucrose density gradient centrifugation, and then by ion exchange, Mono Q. chromatography. Fractions containing the putative TCP-1 homologue were pooled in each case. Track 1, 50 µl pooled Mono Q material; 2, 50 µl pooled sucrose gradient material. Proteins were resolved in 4-30% native polyacrylamide gels and western blotted onto nitrocellulose membranes. Blots were probed with 91a (1:500 dilution) and immunoreactive bands detected using peroxidase-linked anti-rat Ig.

-244-

Presumably the salt required to elute the TCP-1 protein
from the column caused the dissociation of the naturally
occuring complex. It does not seem likely, however, that
an increase in ionic strength alone can be responsible for
this dissociation, since a complex containing the putative
TCP-1 homologue did not appear to be dissociated after
ammonium sulphate precipitation (see 5.4.1.).

It was thought that an increase in the hydrophobicity of
the elution buffers might stabilize the protein. To this
end, several runs were conducted in the presence of 10%
(v/v) ethylene glycol. This procedure did not, however,
improve the recovery of the putative TCP-1 homologue (data
not shown). Willison (personal communication) had
suggested that the addition of magnesium might facilitate
the purification of a TCP-1 homologue. A series of
experiments were conducted in which magnesium was added to
the elution buffers at variety of concentrations. Again no
noticeable improvement in recovery of the protein was
noted, although it seems possible that an impairment of
the column's binding properties might be at least in part
responsible for this result. Unfortunately time did not
permit a repeat series of these experiments. The inclusion
of Triton X-100 in buffers was also investigated, again
without achieving any noticeable improvements (data not
shown).

## 5.6. Conclusions

The main objectives of this part of the research, to purify a putative TCP-1 homologue from *P. sativum* and determine its subcellular location, have not been achieved. In the attempt to realize these aims, several properties of the putative TCP-1 homologue have been identified.

The putative TCP-1 homologue was shown to be a component of a high molecular weight complex which sediments in sucrose gradients in a manner similar to that observed for the *P. sativum* plastid chaperonin and the *R. sphaeroides* chaperonin. The complex also eluted from ion exchange columns (Mono Q and HiLoad Q) at a sodium chloride concentration (about 370 mM) similar to that required for elution of other chaperonins prepared in the course of this study. These include the pea plastid chaperonin, the *R. sphaeroides* chaperonin (Rs cpn60) and the *E. coli* chaperonin (GroEL). Thus the gross physical features of the complex, molecular weight and net charge of the native protein, were similar to those expected for a chaperonin-type molecule. It was initially thought that it would be quite simple to purify this protein sufficiently to obtain sequence and structural data. This has not turned out to be the case. Two major problems were encountered while trying to purify this protein. Firstly, the putative TCP-1 homologue co-purified with Rubisco in every purification technique used. Secondly, it became apparent at a fairly

late stage in the study that the native protein appeared
to dissociate into smaller multimeric forms upon elution
from ion exchange columns. Attempts were made to stabilize
the native form but unfortunately none of these appeared
to be successful.

A major drawback has been the failure of the 91a
monoclonal antibody to recognise the native protein. An
antibody that did recognize the native protein would have
permitted the identification of the complex on native
gels. This in turn might have allowed the amount and
subunit composition of the protein to be quantified. It
would almost certainly have allowed the subcellular
distribution of the putative TCP-1 homologue to be
determined. This was also a major aim of the work
undertaken, since the interest in the TCP-1 protein was
initially stimulated by the thought that it might
represent an example of a cytosolic chaperonin. Given what
is known of the functions of the prokaryotic and
organellar chaperonins, it seemed strange that no
cytosolic homologue had been identified. The quest for a
cytosolic chaperonin is of fundamental biological
significance. Failure to find a cytoslic chaperonin would
pose fundamental questions about protein folding in the
cytosol, and indeed about the presumed role in protein
folding of the chaperonins in prokaryotes and organelles.
At the same time, the detection of a cytosolic chaperonin-
like molecule with limited sequence similarity to the

known chaperonins might have revealed important
information regarding the functional significance of
various parts of the native protein.

Although it has not been possible to demonstrate that
the *P. sativum* putative TCP-1 homologue was a cytosolic
chaperonin, several recent reports have tended to support
the idea that TCP-1 may indeed be a cytosolic chaperonin.
There are several lines of evidence suggesting that
components of the archaebacteria are evolutionarily
related to proteins in the eukaryotic cytosol (Dahlmann,
*et al.*, 1989; Iwabe *et al.*, 1989; Auer *et al.*, 1989).
Gupta (1990) had postulated that the divergence of TCP-1
from the cpn60 group of proteins suggested that TCP-1 may
have directly descended from a universal ancestor (or
progenote) via the eukaryotic lineage.

Proteins antigenically related to the cpn60s have been
detected in archaebacteria (Thole *et al.*, 1988), but until
recently nothing was known of the structure and primary
amino acid sequence of these proteins. Phipps *et al.*
(1991) have described a protein isolated from an
archaebacterium, the hyperthermophile *Pyrodictium*
*occultum*, which, like the cpn60s, is a large cylindrical
protein that exhibits an ATPase activity. This protein is
composed of equimolar amounts of two polypeptides of $M_r$
56,000 and 59,000. The ATPase activity of this protein is
extremely stable and has a temperature optimum of 100°C.
Like the cpn60s, this complex is a major cellular

component and selectively accumulates in cells after heat shock (Phipps *et al.*, 1991). Unlike the cpn60s, this protein complex appears to have an eight-fold axis of symmetry. Antiserum against the *P. occultum* protein was shown to react with polypeptides of similar size in a wide range of archaebacteria, as well as two proteins in *E. coli*. However, no cross-reaction with proteins from a yeast cell extract was detected, and this report makes no mention of the protein possibly being related to the TCP-1 protein.

More recently, Trent *et al.* (1991) have presented evidence that a major heat shock protein isolated from thermophilic archaebacterium *Sulfolobus shibatae* is related to TCP-1. This protein, referred to as TF55 (thermophilic factor 55), was shown to be composed of a single $M_r$ 55,000 polypeptide, and to have a similar double-ring structure to the cpn60s and the *P. occultum* protein. However, in this case most end views of the protein had nine-fold symmetry, while a few views with eight-fold symmetry were also seen. This group went on to demonstrate that the TF55 protein complex is an ATPase with an activity very similar to that reported for the GroEL protein (Trent *et al.*, 1991). Most importantly, they were able to demonstrate that the native TF55 protein could bind to unfolded proteins, although the discharge of the substrate proteins in an active form was not demonstrated. So far no relationship between the TF55

protein and that isolated from *P. occultum* has been
demonstrated. However, given what is known of the
structure and enzymatic activities of these proteins, it
would be somewhat surprising if they turned out to be
unrelated.

The progress made regarding a potential archaebacterial
TCP-1 homologue with properties similar to the cpn60s, has
been matched by recent developments in the eukaryotic
field. The TCP-1 homologue from *S. cerevisiae* has been
shown to share 72% amino acid sequence identity with the
mouse protein (Ursic and Ganetzky, 1988). A cold-impaired
recessive mutation, *tcp-1-1*, was constructed in the yeast
gene. Cells carrying this mutation were shown to grow
linearly rather than exponentially at the restrictive
temperature (15ºC) (Ursic and Culbertson, 1991). Further
analysis of the phenotypic effects of this mutation
demonstrated that both multinucleate and anucleate cells
accumulated with time at the restrictive temperature, and
that these cells were more sensitive to drugs which
inhibited microtubule polymerization than were the wild
type. Indirect immunofluorescence with α-tubulin-specific
antibodies revealed that abnormal microtubular structures
accumulated when cells were grown at the restrictive
temperature (Ursic and Culbertson, 1991). The clear
implication from these studies is that the TCP-1 protein
plays a role in microtubule-mediated processes.

These observations have been strongly supported and

expanded upon in a recent report from Yaffe *et al.* (1992).
This group examined the biogenesis of α- and β-tubulin in
rabbit reticulocyte lysates, observing that newly
synthesized tubulin subunits entered a 900 kD complex in a
protease-sensitive conformation. The 900 kD complex was
shown to be composed of a major species of 58 kD as well
as several other minor species of approximately the same
size. The 58 kD species was shown to cross-react strongly
with 91a, the monoclonal antibody used in this study. 91a
did not, however, interact with the TF55 protein (Yaffe *et
al.*, 1992). Yaffe *et al.* (1992) also demonstrated that the
α- and β-tubulin subunits could be discharged from the 900
kD complex by the addition of MgATP, but not by
nonhydrolyzable analogues. They have also presented
evidence that these proteins are discharged from the
complex in an assembly-competent form. Yaffe *et al.* (1992)
reported that these *in vitro* observations will soon be
supported by data gathered from *in vivo* experiments which
also demonstrate an association between the 900 kD complex
and newly translated tubulin. Finally, Yaffe *et al.* (1992)
have claimed to have preliminary data suggesting that the
TCP-1 complex associates with other newly synthesized
proteins besides tubulin.

These new studies on eukaryotic TCP-1 homologues
strongly support the argument that this protein is a
cytosolic chaperonin. However, structural information
about the native TCP-1 proteins is still rather scanty.

Lewis *et al*. (1992) have purified mouse TCP-1 containing protein complex, and have suggested that in the native state, TCP-1 is associated with at least four other unidentified proteins of about the same size as the TCP-1 polypeptide, and with two members of the hsp70 family. Lewis *et al*. (1992) have argued that the hsp70 proteins might interact directly with the TCP-1 complex *in vivo* but are not part of the complex itself. The other proteins associated with the TCP-1 protein have been dubbed TAPs (TCP-1 associated polypeptides) and Lewis *et al*. (1992) suggest that the possible protein folding capability of the TCP-1-containing complex may be regulated in part by their unique associations with other polypeptides. If it does transpire that there is a family of TCP-1-containing complexes whose subunit compositions determine their substrate specificities, this will pose intriguing questions about the manner in which these proteins recognise different, presumably unfolded, substrates. Electron microscopy of the purified TCP-1-containing protein(s) revealed the now familiar, stacked ring structure. Top views indicated that the subunits were arranged in rings of eight or nine subunits and most closely resembled the putative TCP-1 homologues discussed above, rather the cpn60 proteins.

The widespread cytosolic occurrence of TCP-1 homologues in eukaryotic cells and the emerging consensus regarding their role in protein folding, indicates that there is

likely to be a TCP-1 homologue in the cytosol of higher plants. The gross physical characteristics of the protein that I have attempted to purify in this study would seem to indicate that this protein is indeed a TCP-1 homologue. However, it does appear that there might be significant differences between this protein and the other TCP-1 homologues so far described. The basic purification protocols used in all the studies discussed above involved sucrose and/or glycerol gradient centrifugation, followed by ion exchange chromatography. In none of these studies was there any indication of fundamental instability of the complex during purification.

The way forward in the purification of the putative TCP-1 homologue from *P. sativum* is far from clear. If the instability problem cannot be overcome, it might be possible to purify the protein by immune precipitation using one of the antibodies, excepting 91a, used in the above studies. On the other hand, more information might be gathered by attempting to clone a TCP-1 homologue gene from *P. sativum*, or perhaps by using a cDNA to express the protein in *E. coli* to construct a bank of monoclonal antibodies that might be useful for purification.

# Chapter 6

## Discussion and Conclusions

### 6.1. Overview

The chaperonins are a class of sequence-related proteins belonging to the molecular chaperone family. The members of this family are thought to function by preventing improper interactions taking place between partially folded and unfolded proteins. Chaperonins have been identified in all the eubacteria and membrane-bound organelles so far studied and have been shown to be essential for cell viability (Fayet *et al.*, 1988). Given that the chaperonins show such high sequence similarity and appear to be so important in modulating protein folding, it was somewhat mysterious that no cytosolic homologue of these proteins has been identified in eukaryotes.

In this study two subjects have been investigated. The first topic entailed the identification of and further studies on a GroEL (cpn60) homologue in the purple non-sulphur bacterium *R. sphaeroides*. The second topic has concerned attempts to identify and purify a putative cytosolic chaperonin from *P. sativum*. After summarizing the work conducted in the experimental sections I will consider the possible modes of actions of the chaperonins. Finally, I will briefly discuss experimental strategies for carrying this work forward.

### 6.1.1. Identification and characterisation of an
#### R. sphaeroides cpn60 protein

The cpn60 proteins have a characteristic tetradecameric structure composed of two stacked rings of seven subunits each. Besides having a high molecular weight, typically about 800,000 kD, they are also very abundant proteins; usually about 1% of total cell protein under normal growth conditions. Native gel analysis of *R. sphaeroides* cell-free extracts revealed the presence of just such a protein. This protein was purified using a combination of size fractionation and anion exchange chromatography. The purified native protein has an $M_r$ of 670,000, as determined by native-PAGE, and was shown to be composed of a single polypeptide of $M_r$ 58,000, as determined by SDS-PAGE. N-terminal sequence of this polypeptide was shown to be identical to the *E. coli* GroEL polypeptide at 15 of the 19 positions determined. Antisera raised against this polypeptide were shown to cross-react with both the GroEL and pea plastid chaperonins.

Structural analysis of the partially purified native protein was conducted in collaboration with Helen Saibil and coworkers of Birkbeck College, London. Electron micrographs of protein preparations clearly showed the seven-fold symmetry that is characteristic of 'top' views of the chaperonins. 'Side' views, in common with other chaperonins, have a rectangular appearance with four equally spaced, approximately equally dense, transverse

striations. These views are consistent with a cylindrical shape for the protein. This cylinder is approximately 12 nm in diameter and about 9 nm in height. Image processing of electron microscope images has enhanced the resolution such that structural features as small as 20 Å across can be visualized. This treatment revealed several interesting features. An outer ring of seven electron-dense areas corresponds to the points of the seven-fold structure. Within this ring are another seven, smaller, electron-dense regions, whilst the centre of the structure shows an area of intermediate electron density. Images of the *E. coli* plasmid-encoded GroEL protein do not contain the inner ring of electron density, and the centre of the GroEL protein does not appear to contain any electron-dense material. Speculations as to the possible functional significance of these differences were presented in Section 3.8.. Later in this Chapter these observations will be further discussed with regard to current models of the mode of action of chaperonins.

The synthesis of Rs cpn60 after *R. sphaeroides* was exposed to a variety of stresses, was investigated. These studies did not yield any major surprises. After a shift in growth temperature from 30ºC to 42ºC, there was a transient increase in Rs cpn60 synthesis. This increase in synthesis reached a peak 15 to 20 minutes after the temperature shift, followed by a rapid decline in synthesis to near control levels approximately 1 hour

after induction of the heat shock response. This response
was accompanied by an increase in the rate of synthesis of
a small number of proteins, while that of most other
proteins was repressed. When *E. coli* is exposed to an
identical shift in growth temperature, the synthesis of
the GroEL protein exhibits a broadly similar pattern of
synthesis (Neidhardt *et al.*, 1982; see Gross *et al.*,
1990). Other stresses, including exposure to 5% (v/v)
ethanol, 100 mM hydrogen peroxide, 500 mM sodium chloride,
600 µM cadmium sulphate and shift from chemoheterotrophic
to photoheterotrophic growth conditions, each produced
unique changes in the protein synthesis profiles.

Exposure to ethanol caused a relatively slow induction
of Rs cpn60 synthesis. The peak of the response occurred
more than 45 minutes after exposure to ethanol, when the
rate of synthesis of Rs cpn60 was elevated approximately
5-fold. This response is similar to that observed in a
number of organisms including *E. coli* (Neidhardt *et al.*,
1984), *B. subtilis* (Arnosti *et al.*, 1986) and *P.
aeruginosa* (Allan *et al.*, 1988). 500 mM sodium chloride
caused a similar induction of Rs cpn60, although there
appeared to be little repression of overall protein
synthesis after this treatment. High salt is known not to
induce a stress response in *E. coli* (Neidhardt *et al.*,
1984). Treatment with cadmium caused a substantial
repression in overall protein synthesis which was only
detected some 45 minutes after imposition of the stress,

while synthesis of Rs cpn60 was rapidly doubled and maintained at this level throughout the experiment. VanBogelen *et al.* (1987) reported that exposure of *E. coli* to cadmium did not induce the synthesis of the GroEL protein. However, it is possible that a low level of induction similar to that observed in *R. sphaeroides* might have escaped notice. Hydrogen peroxide is known to induce a stress response in which the synthesis of a small group of proteins is increased in both *E. coli* (VanBogelen *et al.*, 1987) and *M. voltae* (Hebert *et al.*, 1991). In *R. sphaeroides*, exposure to 100 mM hydrogen peroxide rapidly repressed the synthesis of all proteins. Thus it would seem that *R. sphaeroides* is particularly sensitive to oxidative stress. It is, however, possible that exposure to lower levels of hydrogen peroxide might elicit a more typical stress response.

A shift from chemoheterotrophic to photoheterotrophic growth conditions caused a complete cessation of protein synthesis which was maintained for a period of approximately 18 hours. This response is similar to that observed by Chory *et al.* (1984). When protein synthesis was resumed, no differential induction of Rs cpn60 was observed. Terlesky and Tabita (1991) determined the levels of cpn60 in *R. sphaeroides* strain HR, a derivative of strain ATCC 17023, grown under a variety of conditions:

When cultures were grown chemoheterotrophically at 30°C, the cpn60 comprised 3.5% of the soluble protein.

Under photoheterotrophic conditions, the corresponding percentage was 6.7%, and under photolithoautotrophic conditions, 9.3%. After growth at 40°C under photo-lithoautotrophic conditions, the cpn60 comprised an astonishing 28.5% of the soluble protein (Terlesky and Tabita, 1991). Although levels in photoheterotrophically grown cultures were approximately double those of chemoheterotrophically grown cultures, the growth shift experiments conducted in this work did not indicate that there was any strong induction of Rs cpn60 synthesis when photoheterotrophic growth commenced.

The material produced during the heat shock experiments was used to gain an impression of the range of proteins that normally interact with Rs cpn60, and of how this range might be influenced during heat shock. These experiments indicated that there might be a size constraint on proteins capable of interaction with Rs cpn60, and that the flux of proteins through Rs cpn60 is altered during heat shock. Reservations about this interpretation of the results have been discussed in Section 4.4.. The possibility that there is indeed a size restriction on the proteins that can interact with Rs cpn60 are discussed with regard to possible mechanisms of chaperonin action later in this Chapter.

## 6.1.2. The search for a possible cytosolic chaperonin in *Pisum sativum*

Although chaperonins appear to be ubiquitous in eubacteria and membrane-bound organelles, the identification of a cytosolic homologue has proved to be elusive. All the chaperonins so far identified display greater than 40% sequence identity at the amino acid level. A search of the data banks conducted by van der Vies (1989) did not identify any cytosolic proteins with this level of sequence identity. However, a protein of unknown function, the mouse TCP-1 protein, did show significant homology to the chaperonins.

Keith Willison of the Chester Beatty Laboratories kindly agreed to supply monoclonal antibodies that cross-react with this protein. These monoclonals were used in western blot analyses of protein extracts from a variety of sources. The 91a monoclonal was shown to cross-react with a polypeptide that was present in *P. sativum* total leaf extracts, but not in the chloroplasts of this plant, in mitochondria of *R. communis*, nor in *E. coli*. It was hoped that this monoclonal could be used in immunolocalization studies to confirm that the antigen had a cytosolic location. Immunogold localization experiments, however, failed to identify the subcellular distribution of this protein, and subsequent experiments demonstrated that 91a did not recognize the native form of this protein.

Attempts were then made to purify this protein. The

gross characteristics of the protein recognised by 9ia were found to be consistent with those of a chaperonin-type protein. The sedimentation of the native protein in sucrose density gradients was consistent with it being a member of a large multimeric complex which co-purified with the plastid chaperonin and Rubisco. This native protein also co-precipitated with the plastid chaperonin in ammonium sulphate cuts, and eluted from a Mono Q anion exchange at a slightly lower sodium chloride concentration. Native gel analysis of sucrose density gradient fractions revealed the presence of a number of large proteins that were considered to be likely candidates for the *P. sativum* cytosolic chaperonin. However, when these protein bands were excised from dried native gels, resolved in SDS polyacrylamide gels and subjected to western blot analysis using 9ia as a probe, no immunoreactive material was detected.

Subsequent attempts to purify the native putative TCP-1 homologue, using a variety of chromatographic techniques, were unsuccessful. The main reasons for this appeared to be significant losses of immunoreactive material during chromatography, and the fact that none of the columns used adequately separated the putative TCP-1 homologue from Rubisco. Western blot analysis of Mono Q fractions resolved in native gels revealed a number of immunoreactive bands. These observations indicated that the native multimeric complex tended to dissociate in the

presence of concentrations of sodium chloride typically used to elute this protein. Attempts to stabilize the native protein by increasing the hydrophobicity of elution buffers were unsuccessful. Etiolated peas were used to minimize the Rubisco problem. However, using this material as a source of protein proved to be problematic. Large amounts of biomass were required and the etiolated peas were susceptible to fungal contamination. Furthermore, partial purification of the putative TCP-1 homologue indicated that this protein was not very abundant in leaf extracts. Chaperonins are characteristically abundant proteins in eubacteria and membrane-bound organelles, and the apparently low abundance of the putative TCP-1 homologue is somewhat worrying. However, even though this protein appears to be of low abundance in pea leaf extracts, this is not sufficient reason to abandon the idea that the putative TCP-1 homologue might be a component of cytosolic chaperonin. Only further purification, sequence and functional analysis of this protein will demonstrate its role in pea leaves.

## 6.2. Possible mechanisms of chaperonin action

Studies on the possible mechanisms of chaperonin action were briefly discussed in the introduction (1.4.3.). As stated before, the mechanism by which cpn60s recognise nonnative proteins and modulate their folding, is only poorly understood. An idea of the uncertainty surrounding

the mode of action of the chaperonins is suggested by the
following observations:

Most studies have suggested that the cpn60s interact
with a molten globule state of the substrate protein (see
Martin *et al.*, 1991; Goloubinoff *et al.*, 1989a). However,
Badcoe *et al.* (1991) have presented evidence that GroEL
interacts with a more unfolded state of lactate
dehydrogenase and does not recognise the molten globule
state at all. GroEL has been shown to exhibit a weak
ATPase activity, but determinations of this activity vary
at least 40-fold between different laboratories (Gray and
Fersht, 1991; Viitanen *et al.*, 1990; Hendrix, 1979).
GroES, cpn10, inhibits this activity (Gray and Fersht,
1991; Martin *et al.*, 1991; Chandrasekhar *et al.*, 1986;
Viitanen *et al.*, 1990). When an unfolded protein,
rhodanese, is added to GroEL and GroES in the presence of
ATP there is a roughly 40-fold increase in the GroES-
supressed rate of ATP hydrolysis (Martin *et al.*, 1991). *In
vitro* experiments have shown that GroES and ATP are often
essential requirements for discharge of bound substrate
protein from GroEL (Martin *et al.*, 1991; Goloubinoff *et
al.*, 1989a). However, several proteins do not require
GroES for their discharge (Martin *et al.*, 1991; Buchner *et
al.*, 1991; Badcoe *et al.*, 1991; Höll-Neugebauer and
Rudolph, 1991), although discharge is somewhat slower in
these cases. The release of some proteins from GroEL has
been shown to take place in the absence of GroES and the

-263-

presence of nonhydrolyzable ATP analogues (Viitanen *et al.*, 1991; Badcoe *et al.*, 1991). These observations have been interpreted to mean that the energy required to dissociate the GroEL-substrate protein complex is provided by the ATP binding energy, and that ATP turnover is required to switch GroEL between high and low substrate affinity states.

The question of which features of the unfolded protein are recognised by the cpn60s remains largely unresolved. Landry and Gierasch (1991) have presented evidence that GroEL binds a peptide in an $\alpha$-helical conformation, and that modification of this peptide to reduce its intrinsic propensity to take up an $\alpha$-helical structure, lowered its affinity for GroEL. Although this work is the first to identify a structural motif which interacts with a chaperonin, the actual interactions are likely to be somewhat more complicated. Zahn and Plückthun (1992) have recently shown that a single-chain antibody containing no $\alpha$-helices was able to compete with $\beta$-lactamase for binding of GroEL, thus indicating that substrate recognition by GroEL cannot be limited to $\alpha$-helices.

The above gives an impression of the many controversial aspects of this field. Another question that has rarely been addressed in the literature, is that of the location of substrate proteins bound to cpn60s. There are two current views regarding the location of the substrate protein. One of these could be referred to as the cogwheel

hypothesis and the other as the Anfinsen cage hypothesis.

The cogwheel hypothesis was proposed by Georgopolous and Ang (1990). Although this model does not explicitly state the location of the substrate protein on the chaperonin, the general impression given is that substrate proteins are bound on the outer surface of GroEL, and that the role of the GroES protein is as a cogwheel which displaces the bound proteins. Georgopolous and Ang (1990) propose that the hydrolysis of ATP by GroEL provides the energy for this displacement (Figure 6.2.1.). This model would not be expected to impose any size limitation on substrate proteins.

The Anfinsen cage hypothesis was proposed by John Ellis (personal communication). This model explicitly states that substrate proteins are sequestered within the cpn60 multimer. Similar models have been proposed by Creighton (1991) and Nilsson and Anderson (1991). However, no evidence has been provided in support of this model. The observation that there may be a size limitation on proteins found in association with Rs cpn60, could indicate that only proteins that can fit, in their entirety, into the central cavity of Rs cpn60 can be substrates for this chaperonin. Evidence that such a cavity exists has been provided by Helen Saibil (personal communication). Three-dimensional reconstructions created from a series of tilted electron microscope images show that the Rs cpn60 multimer is composed of two halves, with

Figure 6.2.1.   "Cogwheel" model of chaperonin action



A model for the intracellular action of the GroEL and GroES chaperonins. The GroEL chaperonin can bind to many, but perhaps not all, unfolded polypeptides, some of which may still be nascent (4). The bound polypeptide is either not released or released very slowly. The envisioned role of the GroES protein is as a cogwheel displacing the bound polypeptides. The hydrolysis of ATP by GroEL provides the energy for such a displacement. The released polypeptide may undergo further intra- or intermolecular folding. Eventually, the GroE chaperonins dissociate, allowing GroEL to recycle.

After Georgopolous and Ang (1990).

each half having two pairs of "jaws" (data not shown). A
cartoon of the appearance of these "jaws" is shown in
Figure 6.2.2. The full three-dimensional reconstruction
looks very much like a cage (data not shown). Addition of
MgATP to partially purified Rs cpn60 causes a major
conformational change, in which the two outer striations
of the Rs cpn60 side view almost completely disappear
(Helen Saibil, personal communication). These images
suggest that the "jaws" might have opened up. Nilsson and
Anderson (1991) also invoke a major conformational change
under these conditions. They suggest that during this
process the interior reactive surface of the chaperonin is
converted into an unreactive relatively hydrophilic state.

How does the cpn10 protein fit into this scenario? While
in vitro studies have shown that a cpn10 protein is not
always required for discharge of substrate proteins, it is
known that cpn10 interacts with cpn60 in the presence of
MgATP. This interaction has been exploited in the
purification of cpn10 proteins from a number of sources
(Chandrasekhar et al., 1986; Terlesky and Tabita, 1991;
Lubben et al., 1990). Intracellular levels of ATP would
thus be expected to favour an association between cpn60
and cpn10 in vivo. It is possible that those proteins that
are discharged from cpn60 by ATP alone in vitro, are only
weakly bound to cpn60, while those that require the
presence of cpn10 are more strongly bound. The observation
of Gray and Fersht (1991) that the cooperativity of ATP

-267-

hydrolysis by GroEL is increased by GroES, could support this view. The points discussed above have been incorporated into the Anfinsen cage model shown in Figure 6.2.2.. In this model, GroES aids in the discharge of substrate proteins. This differs from the model of Creighton (1991), which proposes that GroES acts by simply preventing the dissociation of bound protein from GroEL until ATP hydrolysis is completed. Creighton (1991) also proposed that the substrate protein would tend to refold while the energy of ATP hydrolysis passes the substrate from one half of the tetradecamer to the other. This seems unlikely since the three-dimensional reconstruction of the Rs cpn60 tetratdecamer does not reveal a channel between the two halves of the molecule (data not shown).

The model proposed by Nilsson and Anderson (1991) differs from that presented in Figure 6.2.2. in that it does not include a size constraint. Nilsson and Anderson (1991) suggested that cpn60s bind target protein domains, and that in multidomain proteins the N- and C-terminal amino acid stretches would protrude from the holes at each end of the chaperonin. Again the three dimensional Rs cpn60 reconstruction indicates that this is unlikely to be the case.

The model presented in Figure 6.2.2. attempts to explain all the *in vitro* observations of the mode of action the chaperonins (see 1.4.) and present a scheme for the normal *in vivo* mode of action. An obvious problem with model is

Figure 6.2.2. "Anfinsen cage"-type model of chaperonin action

= intermediate folding state

-269-

that it only invokes a heptameric form of cpn60 for full
functionality. Such a form has been isolated from beef
liver mitochondria (Viitanen *et al.*, 1992a). This form was
shown to facilitate the formation of catalytically active
Rubisco from an unfolded state in the presence of
mammalian cpn10, K⁺ and MgATP (Viitanen *et al.*, 1992a).
Thus, it seems possible that the basic functional unit of
cpn60 is a single heptameric ring. However, the fact the
vast majority of the chaperonins examined are
tetradecamers indicates a functional significance of this
form. Helen Saibil (personal communication) has expressed
the view that cpn10 may bind to one side of the cpn60
tetradecamer thus causing the other side of the molecule
to be opened up with the resulting release of bound
substrate protein. This view, however, disregards the data
of Viitanen *et al.* (1992a).

### 6.3.  Future work

An obvious priority in any future work on the *R.
sphaeroides* cpn60 would be to obtain its gene sequence.
Some preliminary work was done in this direction, but this
work was abandoned for the reasons outlined in Chapter 3.
However, the superb two- and three-dimensional images of
the Rs cpn60 protein that have been obtained, have made
the need for the total gene sequence much more pressing.
Structural predictions from the gene sequence could
possibly be fitted to these images, and might provide

valuable information about the interior surface of the chaperonin in particular. Obviously X-ray crystallographic data would be most suitable for this purpose. Protein preparations have been supplied to Steve Wood of Birkbeck College but so far, as with other cpn60s, no satisfactory crystals have been obtained.

Another area warranting further investigation is the possible size constraint on chaperonin substrate proteins. One approach would be to select a protein of $M_r$ >100,000, denature it in a chaotrope and dilute it into a chaperonin-containing solution. It would then be possible to determine whether such a substrate forms a stable association with the chaperonin and what conditions are required for its discharge. Such experiments would perhaps be best conducted using the well-characterized GroEL/GroES system.

I am, however, somewhat sceptical about the overall validity of this approach. Firstly, the possibility that unfolded proteins might, upon dilution, form an "improper" association with a chaperonin cannot be entirely excluded. Secondly, evidence that such a protein is discharged from a chaperonin could be a side effect of gross conformational changes in the chaperonin, rather than a direct consequence of these changes. Thirdly, the final aim of all studies in cell biology must be to understand events *in vivo*. For these reasons, it would perhaps be better to attempt to determine the range of proteins that

associate with cpn60 *in vivo*. *In vivo* expression of large proteins from plasmid vectors could aid in determining whether the chaperonins would interact with such proteins. Even if such an association was demonstrated, it would be interesting to carry out competition studies, either *in vitro* or *in vivo*, to determine whether the chaperonins exhibit any preference for protein substrates in a particular size range.

The study of the putative TCP-1 homologue in *P. sativum* should probably, at least for the time being, be abandoned. It is clear that TCP-1 and its homologues can be more readily studied in archaebacteria (Phipps *et al.*, 1991; Trent *et al.*, 1991), yeast (Ursic and Culbertson, 1991) and mice (Lewis *et al.*, 1992). Studies conducted in reticulocyte lysates have begun to bear fruit regarding the chaperoning action of TCP-1 homologues (Yaffe *et al.*, 1992; Gao *et al.*, 1992). These studies have confirmed that TCP-1 is a cytosolic chaperonin and rapid advances can be expected in this field. Current studies are fairly similar to early studies in the chaperonin field. Many interesting questions still remain to be answered. For example: is there a cytosolic cpn10 protein? Is the protein folding capability of TCP-1-containing complexes regulated, at least in part, by their unique association with other polypeptides (see Lewis *et al.*, 1992)?

The introduction of the molecular chaperone concept has profoundly altered the way we think of protein folding in

the cell. It is extrordinary that while many of the molecular chaperones are abundant well characterized proteins their true functional significance is only now becomimg apparent. Undoubtedly this fascinating field will yield many important discoveries in the not too distant future.

# BIBLIOGRAPHY

Allan, B., Linseman, M., MacDonald, L. A., Lam, J. S. and
  Kropinski, A. M. (1988). Heat shock response of *Pseudomonas
  seruginosa*. J. Bacteriol. 170, 3668-3674.

Altman, A., Kumamoto, C. A. and Emr, S. D. (1991). Heat-shock
  proteins can substitute for SecB function during protein
  export in *Escherichia coli*. EMBO J. 10, 239-245.

Ananthan, J., Goldberg, A. L. and Voellmy, R. (1986). Abnormal
  proteins serve as eukaryotic stress signals and trigger the
  activation of the heat shock genes. Science 232, 522-524.

Andrews, T. J. and Ballment, B. (1983). The function of the
  small subunits of ribulose bisphosphate carboxylase-
  oxygenase. J. Biol. Chem. 258, 7514-7518.

Anfinsen, C. B. (1973). Principals that govern the folding of
  protein chains. Science 181, 223-230.

Anfinsen, C. B., Haber, E., Sela, M. and White, F. H. Jr.
  (1961). The kinetics of formation of native ribonuclease
  during oxidation of the reduced polypeptide chain.
  Proc. Natl. Acad. Sci. USA 47, 1309-1314.

Ang, D., Liberek, K., Skowyra, D., Zylicz, M. and Georgopolous,
  C. (1991). Biological role and regulation of the universally
  conserved heat shock proteins. J. Biol. Chem. 266, 24233-
  24236.

Anson, M. L. (1945). Protein denaturation and the properties of
  protein groups. Adv. Protein Chem. 2, 361-386.

Arnosti, D. N., Singer, V. L. and Chamberlin, M. J. (1986).
Characterization of heat shock in *Bacillus subtilis*. J.
Bacteriol. **168**, 1243-1249.

Ashburner, M. and Bonner, J. J. (1979). The induction of gene
activity in Drosophila by heat shock. Cell **17**, 241-254.

Auer, J., Lechner, K. amd Bock, A. (1989).Gene organization and
structure of two transcriptional units from *Methanococcus*
coding for ribosomal proteins and elongation factors. Can. J.
Microbiol. **35**, 200-204.

Badcoe, I. G., Smith, C. J., Wood, S., Halsall, D. J.,
Holbrook, J. J., Lund, P. and Clarke, A. R. (1991). Binding
of a chaperonin to the folding intermediates of lactate
dehydrogenase. Biochemistry **30**, 9195-9200.

Baker, D., Sohl, J. L. and Agard, D. A. (1992). A protein-
folding reaction under kinetic control. Nature **356**, 263-265.

Baker, K. P. and Schatz, G. (1991). Mitochondrial proteins
essential for viability mediate protein import into yeast
mitochondria. Nature **349**, 205-208.

Bardwell, J. C. A. and Craig, E. A. (1984). Major heat shock
gene of *Drosophila* and the *Escherichia coli* heat-inducible
*dnaK* gene are homologous. Proc. Natl. Acad. Sci. USA **81**, 848-
852.

Barraclough, R. and Ellis, R. J. (1980). Protein synthesis in
chloroplasts. IX. Assembly of newly synthesized large
subunits into ribulose bisphosphate carboxylase in isolated
intact pea chloroplasts. Biochim. Biophys. Acta **608**, 19-31.

Beckmann, R. P., Mizzen, L. A. and Welch, W. (1990).
  Interaction of Hsp 70 with newly synthesized proteins:
  implications for protein folding and assembly. Science 248,
  850-854.

Bennett, D. (1975). The T-locus of the mouse. Cell 6, 441-454.

Bernstein, H. D., Rapoport, T. A. and Walter, P. (1989).
  Cytosolic protein translocation factors. Is SRP still unique.
  Cell 58, 1017-1019.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline
  extraction procedure for screening recombinant plasmid DNA.
  Nucleic Acid Res. 7, 1513-1523.

Bloom, M. V., Milos, P. and Roy, H. (1983). Light-dependent
  assembly of ribulose-1,5-bisphosphate carboxylase.
  Proc. Natl. Acad. Sci. USA 80, 1013-1017.

Blumberg, H. and Silver, P. A. (1991). A homologue of the
  bacterial heat-shock gene *DnaJ* that alters protein sorting in
  yeast. Nature 349, 627-630.

Bochkareva, E. S., Lissin, N. M. and Girshovich, A. S. (1988).
  Transient association of newly synthesized unfolded proteins
  with the heat shock GroEL protein. Nature 336, 254-257.

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R.,
  Schmid, F. X. and Kiefhaber, T. (1991). GroE facilitates
  refolding of citrate synthase by suppressing aggregation.
  Biochemistry 30, 1586-1591.

Burglin, T. R., Mattaj, I. W., Newmeyer, D. D., Zeller, R. and
  De Roberts, E. M. (1987). Cloning of nucleoplasmin from
  *Xenopus laevis* oocytes and analysis of its developmental

expression. Genes Dev. 1, 97-107.

Burnette, W. N. (1981) "Western blotting": Electrophoretic
transfer of proteins from sodium dodecyl sulfate–
polyacrylamide gels to unmodified nitrocellulose and
radiographic detection with antibody and radioiodinated
protein A. Anal. Biochem. 112, 195-203.

Butt, T. R., Jonnalagadda, S., Monia, B. P., Sternberg, E. J.,
Marsh, J. A., Stadel, J. M., Ecker, D. J. and Crooke, S. T.
(1989). Ubiquitin fusion augments the yield of cloned gene
products in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 86,
2540-2544.

Cannon, S., Wang, P. and Roy, H. (1986). Inhibition of ribulose
bisphosphate carboxylase assembly by antibody to a binding
protein. J. Cell. Biol. 103, 1327-1335.

Carazo, J. M., Marco, S., Abella, G., Carrascosa, J. L.,
Secilla, J.-P. and Muyal, M. (1991). Electron microscope
study of GroEL chaperonin: different views of the aggregate
appear as a function of cell growth temperature. J. Struct.
Biol. 106, 211-220.

Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R. and
Georgopolous, C. (1986). Purification and properties of the
groES morphogenetic protein of *Escherichia coli*. J. Biol.
Chem. 261, 12414-12419.

Chapman, M. S., Suh, S. W., Curmi, P. M. G., Cascio, D.,
Smith, W. W. and Eisenberg, D. S. (1988). Tertiary structure
of plant RuBisCO: domains and their contacts. Science 241,
71-74.

Chappell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J. and Rothman, J. E. (1986). Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell 45, 3–13.

Chappell, T. G., Konforti, B. B., Schmid, S. L. and Rothman, J. E. (1987). The ATPase core of clathrin uncoating protein. J. Biol. Chem. 262, 746–751.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. and Horwich, A. L. (1989). Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature 337, 620–625.

Cheng, M. Y., Hartl, F.-U. and Horwich, A. L. (1990). The mitochondrial chaperonin hsp60 is required for its own assembly. Nature 348, 455–458.

Chirico, W. J., Waters, M. G. and Blobel, G. (1988). 70K heat shock related proteins stimulate protein translocation in microsomes. Nature 332, 805–810.

Chory, J., Donohue, T. J., Varga, A. R., Staehelin, L. A. and Kaplan, S. (1984). Induction of the photosynthetic membranes of *Rhodopseudomonas sphaeroides*: biochemical and morphological studies. J. Bacteriol. 159, 540–554.

Chung, C. T., Niemela, S. L. and Miller, R. H. (1989). One step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA 86, 2172–2175.

Collier, D. N., Bankaitis, V. A., Weiss, J. B. and Bassford, P. J. Jr. (1988). The antifolding activity of SecB promotes the export of the *E. coli* maltose-binding protein. Cell 53, 273-283.

Cotten, M., Sealy, L. and Chalkley, R. (1986). Massive phosphorylation distinguishes *Xenopus laevis* nucleoplasmin isolated from oocytes or unfertilized eggs. Biochemistry 25, 5063-5069.

Cozens, A. L. and Walker, J. E. (1987). The organization and sequence of the genes for ATP synthase subunits in the cyanobacterium *Synechococcus* 6301. Support for an endosymbiotic origin of chloroplasts. J. Mol. Biol. 194, 359-383.

Craig, E. A. and Gross, C. A. (1991). Is hsp70 the cellular thermometer? TIBS 16, 135-140.

Craig, E. A., Ingolia, T. D. and Manseau, L. J. (1983). Expression of *Drosophila* heat-shock cognate genes during heat shock and development. Dev. Biol. 99, 418-426.

Craig E. A., Kramer, J. and Kosic-Smithers, J. (1987). *SSC1*, a member of the 70-kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. Proc. Natl. Acad. Sci. USA 84, 4156-4160.

Creighton, T. E. (1984). Proteins. W. H. Freeman and Co. New York.

Creighton, T. E. (1991). Unfolding protein folding. Nature 352, 17-18.

Crooke, E., Brundage, L., Rice, M. and Wickner, W. (1988a).
ProOmpA spontaneously folds in a membrane competent state
which trigger factor stabilizes. EMBO J. 7, 1831-1835.

Crooke, E., Guthrie, B., Lecker, S., Lill, R. and Wickner, W.
(1988b). ProOmpA is stabilized for membrane translocation by
either purified *E. coli* trigger factor or canine signal
recognition particle. Cell 54, 1003-1011.

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G.,
Hegerl, R. and Baumeister, W. (1989). The multicatalytic
proteinase (prosome) is ubiquitous from eukaryotes to
archaebacteria. FEBS Lett. 251, 125-131.

Deshaies, R. J. Koch, B. D., Werner-Washburne, M., Craig, E. M.
and Scheckman, R. (1988). A subfamily of stress proteins
facilitates translocation of secretory and mitochondrial
precursor polypeptides. Nature 332, 800-805.

Dilworth, S. M., Black, S. J. and Laskey, R. A. (1987). Two
complexes that contain histones are required for nucleosome
assembly in vitro: Role of nucleoplasmin and N1 in Xenopus
egg extracts. Cell 51, 1009-1018.

Dingwall, C., Sharnick, S. V. and Laskey, R. A. (1982). A
polypeptide domain that specifies migration of nucleoplasmin
into the nucleus. Cell 30, 449-458.

Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E. and
Laskey, R. A. (1987). Nucleoplasmin cDNA sequence reveals
polyglutamic acid tracts and a cluster of sequences
homologous to putative nuclear localization signals. EMBO J.
6, 69-74.

Dingwall, C., Robbins, J., Dilworth, S. M., Roberts, B. and Richardson, W. D. (1988). The nucleoplasmin nuclear location sequence is larger and more complex than that of SV-40 large T antigen. J. Cell Biol. 107, 841-849.

Dingwall, C., Robbins, J. and Dilworth, S. M. (1989). Characterization of the nuclear location sequence of *Xenopus* nucleoplasmin. J. Cell Sci. Suppl. 11, 243-248.

Dingwall, C. and Laskey, R. A. (1990). Nucleoplasmin: the archetypical molecular chaperone. Sem. Cell Biol. 1, 11-17.

Dobson, C. M. (1992). Resting places on folding pathways. Curr. Biol. 2, 343-345.

Dodson, M., McMacken, R. and Echols, H. (1989). Specialized nucleoprotein structures at the origin of replication of bacteriophage λ. J. Biol. Chem. 264, 10719-10725.

Dudley, K., Potter, J., Lyon, M. F. and Willison, K. R. (1984). Analysis of male sterile mutations in the mouse using haploid stage expressed cDNA probes. Nucleic Acids Res. 12, 4281-4293.

Dunn, L. C. and Gluecksohn-Schoenheimer, S. (1950). Repeated mutations in one area of a mouse chromosome. Proc. Natl. Acad. Sci. USA. 36, 233-237.

Earnshaw, W. C. (1987). Anionic regions in nuclear proteins. J. Cell Biol. 105, 1479-1482.

Earnshaw, W. C., Honda, B. M., Laskey, R. A. and Thomas, J. O. (1980). Assembly of nucleosomes: the reaction involving X. laevis nucleoplasmin. Cell 21, 373-383.

Echols, H. (1990). Nucleoprotein structures initiating DNA replication, transcription, and site-specific recombination. J. Biol. Chem. 265, 14697-14700.

Eilers, M. and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature 322, 228-232.

Ellis, J. (1987). Proteins as molecular chaperones. Nature 328, 378-379.

Ellis, R. J. (1979). The most abundant protein in the world. TIBS 4, 241-244.

Ellis, R. J. and van der Vies, S. M. (1988). The RubisCo subunit binding protein. Photosynthesis Res. 16, 101-115.

Ellis, R. J. and Hemmingsen, S. M. (1989). Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. TIBS 14, 339-342.

Ellis, R. J. (1990). The molecular chaperone concept. Sem. Cell Biol. 1, 1-9.

Englard, S. and Seifter, S. (1990). Precipitation techniques. Methods in Enzymol. 182, 285-300.

Epstein, C. J., Goldberger, R. F. and Anfinsen, C. B. (1963). The genetic control of tertiary protein structure: studies with model systems. Cold Spring Harbor Symp. Quant. Biol. 28, 439-449.

Ewbank, J. J. and Creighton, T. E. (1992). Protein folding by stages. Curr. Biol. 2, 347-349.

Fayet, O., Louarn, J.-M., and Georgopoulis, C. (1986).
Supression of the *Escherichia coli dnaA46* mutation by
amplification of the *groES* and *groEL* genes. Mol. Gen. Genet.
202, 435-445.

Fayet, O., Ziegelhoffer, T. and Georgopoulos, C. (1989). The
*groES* and *groEL* heat shock gene products of *Escherichia coli*
are essential for bacterial growth at all temperatures. J.
Bacteriol. 171, 1379-1385.

Finley, D., Ozkaynak, E. and Varshavsky, A. (1987). The yeast
polyubiquitin gene is essential for resistance to high
temperatures, starvation, and other stresses. Cell 48, 1035-
1046.

Finley, D., Bartel, B. and Varshavsky, A. (1989). The tails of
ubiquitin precursors are ribosomal proteins whose fusion to
ubiquitin facilitates ribosome biogenesis. Nature 338, 394-
401.

Flynn, G. C., Chappell, T. G. and Rothman, J. E. (1989).
Peptide binding and release by proteins implicated as
catalysts of protein assembly. Science 245, 385-390.

Flynn, G. C., Pohl, J., Flocco, M. T. and Rothman, J. E.
(1991). Peptide-binding specificity of the molecular
chaperone BiP. Nature 353, 726-730.

Friedman, D. I., Olson, E. R., Georgopolous, C., Tilly, K.,
Herskowitz, I. and Banuett, F. (1984). Interactions of
bacteriophage and host macromolecules in the growth of
bacteriophage λ. Microbiol. Rev. 48, 299-325.

Gaitanaris, G. A., Papavassiliou, A. G., Rubock, P., Silverstein, S. J. and Gottesman, M. E. (1990). Renaturation of denatured λ repressor requires heat shock proteins. Cell 61, 1013-1020.

Gannon, P. M., Li, P. and Kumamoto, C. A. (1989). The mature portion of *Escherichia coli* maltose-binding protein (MBP) determines the dependence of MBP on SecB for export. J. Bacteriol. 171, 813-818.

Gao, Y., Thomas, J. O., Chow, R. L., Lee, G.-H. and Cowan, N. J. (1992). A cytoplasmic chaperonin that catalyzes β-actin folding. Cell 69, 1043-1050.

Gatenby, A. A. and Ellis, R. J. (1990). Chaperone function: the assembly of ribulose bisphosphate carboxylase-oxygenase. Ann. Rev. Cell Biol. 6, 125-149.

Georgopoulos, C. P., Hendrix, R. W., Kaiser, A. D. and Wood, W. B. (1972). Role of the host cell in bacteriophage morphogenesis: Effects of a bacterial mutation on T4 head assembly. Nature New Biol. 239, 38-41.

Georgopoulos, C. P., Hendrix, R. W., Casjens, S. R. and Kaiser, A. D. (1973). Host participation in bacteriophage lambda head assembly. J. Mol. Biol. 76, 45-60.

Georgopoulos, C. P. and Hohn, B. (1978). Identification of a host protein necessary for bacteriophage morphogenesis (the *groE* gene product). Proc. Natl. Acad. Sci. USA 75, 131-135.

Georgopoulos, C. and Ang, D. (1990). The *Escherichia coli groE* chaperonins. Sem. Cell Biol. 1, 19-25.

Georgopoulos, C., Ang, D., Liberek, K. and Zylicz, M. (1990). Properties of the *Escherichia coli* heat shock proteins and their role in bacteriophage λ growth. In: Stress proteins in biology and medicine (Eds Morimoto, R., Tissieres, A. and Georgopoulos, C.) pp. 191-221, Cold Spring Harbour Laboratory Press, New York.

Gething, M.-J. and Sambrook, J. (1992). Protein folding in the cell. Nature 355, 33-45.

Ghélis, C. and Yon, J. (1982). Protein folding. Academic Press, London.

Gibson, J. L. and Tabita, F. R. (1977). Different molecular forms of D-ribulose-1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. 252, 943-949.

Gibson, J. L. and Tabita, F. R. (1985). Structural differences in the catalytic subunits of form I and form II ribulose 1,5-bisphosphate carboxylase/oxygenase from *Rhodopseudomonas sphaeroides*. J. Bacteriol. 164, 1188-1193.

Glover, L. A. and Lindsay, J. G. (1992). Targeting proteins to mitochondria: a current overview. Biochem. J. 284, 609-620.

Gluecksohn-Waelsch, S. (1989). In praise of complexity. Genetics 122, 721-725.

Goff, S. A. and Goldberg, A. L. (1985) Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. Cell 41, 587-595.

Goldberger, R. F., Epstein, C. J. and Anfinsen, C. B. (1963). Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. 238, 628-635.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A. and Lorimer, G. H. (1989a). Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperone proteins and Mg-ATP. Nature 342, 884-889.

Goloubinoff, P., Gatenby, A. A. and Lorimer, G. H. (1989b). GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in *Escherichia coli*. Nature 337, 44-47.

Gottesman, S. (1984). Bacterial regulation: global regulatory networks. Ann. Rev. Genet. 18, 415-441.

Govezensky, D., Greener, T., Segal, G. and Zamir, A. (1991). Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. J. Bacteriol. 173, 6339-6346.

Gray, J. C. (1982). Use of proteolytic inhibitors during isolation of plastid proteins. In: Methods in chloroplast molecular biology. Eds. Edelman *et al*., 1093-1102.

Gray, T. E. and Fersht, A. R. (1991). Cooperativity in ATP hydrolysis by GroEL is increased by GroES. FEBS Lett. 292, 254-258.

Grimm, R., Speth, V., Gatenby, A. A. and Schafer, E. (1991). GroEL-related molecular chaperones are present in the cytosol of oat cells. FEBS Lett. 286, 155-158.

Gross, C. A., Straus, D. B., Erickson, J. W. and Yura, T. (1990). The function and regulation of heat shock proteins in *Escherichia coli*. In: Stress proteins in biology and medicine (Eds Morimoto, R., Tissieres, A. and Georgopoulos, C.) pp. 191-221, Cold Spring Harbour Laboratory Press, New York.

Guglielmi, G., Mazodier, P., Thompson, C. J. and Davies, J. (1991). A survey of the heat shock response in four *Strptomyces* species reveals two *groEL*-like genes and three GroEL-like proteins in *Streptomyces albus*. J. Bacteriol. 173, 7374-7381.

Gupta, R. S. (1990). Sequence and structural homology between a mouse T-complex protein TCP-1 and the 'chaperonin' family of bacterial (GroEL, 60-65 kDa heat shock antigen) and eukaryotic proteins. Biochem. International 20, 833-841.

Guthrie, B. and Wickner, W. (1990). Trigger factor depletion or overproduction causes defective cell division but does not block protein export. J. Bacteriol. 172, 5555-5562.

Haber, E. and Anfinsen, C. B. (1962). Side-chain interactions governing the pairing of half-cystine residues in ribonuclease. J. Biol. Chem. 237, 1839-1844.

Haider, M. Z., Knowles, B. H. and Ellar, D. J. (1986). Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protein by larval gut proteases. Eur. J. Biochem. 156, 531-540.

Hallberg, R.L. (1990). A mitochondrial chaperonin: genetic, biochemical, and molecular characteristics. Sem. Cell Biol. 1, 37-45.

Hammer, M. F., Schimenti, J. and Silver, L. M. (1989). Evolution of mouse chromosome 17 and the origin of inversions associated with *t* haplotypes. Proc. Natl. Acad. Sci. USA 86, 3261-3265.

Hardy, S. J. S. and Randall, L. L. (1991). A kinetic partitioning model of selective binding of nonnative proteins by the bacterial chaperone SecB. Science 251, 439-443.

Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J. P. and Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. Cell 63, 269-279.

Hebert, A. M., Kropinski, A. M. and Jarrell, K. F. (1991). Heat shock response of the archebacterium *Methanococcus voltae*. J. Bact. 173, 3224-3227.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W. and Ellis, R. J. (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature 333, 330-334.

Hemmingsen, S. M. and Ellis, R. J. (1986). Purification and properties of ribulosebisphosphate carboxylase large subunit binding protein. Plant Physiol. 80, 269-276.

Hendrix, R. W. (1979). Purification and properties of groE, a host protein involved in bacteriophage assembly. J. Mol. Biol. 129, 375-392.

Herendeen, S. L., VanBogelen, R. A. and Neidhardt, F. C. (1979). Levels of major proteins of *Escherichia coli* during growth at different temperatures. J. Bacteriol. 139, 185-194.

Herrmann, B. G., Barlow, D. P. and Lehrach, H. (1987). A large inverted duplication allows homologous recombination between chromosomes heterozygous for the proximal t complex inversion. Cell 48, 813-825.

Hershko, A. (1988). Ubiquitin-mediated protein degradation. J. Biol. Chem. 263, 15237-15240.

Hohn, T., Hohn, B., Engel, A., Wurtz, M. and Smith, P. R. (1979). Isolation and characterization of the host protein groE involved in bacteriophage lambda assembly. J. Mol. Biol. 129, 359-373.

Höll-Neugebauer, B. and Rudolph, R. (1991). Reconstitution of a heat shock effect in vitro: influence of GroE on the thermal aggregation of α-glucosidase from yeast. Biochemistry 50, 11609-11614.

Holmgren, A. and Brändén, C.-I. (1989). Crystal structure of chaperone protein PapD reveals an immunoglobulin fold. Nature 342, 248-251.

Holmgren, A., Kuehn, M. J., Brändén, C.-I. and Hultgren, S. J. (1992). Conserved immunoglobulin-like features in a family of periplasmic pilus chaperones in bacteria. EMBO J. 11, 1617-1622.

Houston, L. S., Cook, R. G. and Norris, S. J. (1990). Isolation and characterization of *Treponema pallidum* major 60-kilodalton protein resembling the *groEL* protein of

Escherichia coli. J. Bacteriol. 172, 2862-2870.

Hultgren, S. J., Lindberg, F., Magnusson, G., Kihlberg, J., Tennent, J. M. and Normark, S. (1989). The PapG adhesin of uropathogenic Escherichia coli contains separate regions for receptor binding and for the incorporation into the pilus. Proc. Natl. Acad. Sci. USA 86, 4357-4361.

Hultgren, S. J., Normark, S. and Abraham, S. N. (1991). Chaperone-assisted assembly and molecular architecture of adhesive pili. Ann. Rev. Microbiol. 45, 383-415.

Hutchinson, E. G., Tichelaar, W., Hofhaus, G., Weiss, H. and Leonard, K. R. (1989). Identification and electron microscopic analysis of a chaperonin oligomer from Neurospora crassa mitochondria. EMBO J. 8, 1485-1490.

Ikemura, H. and Inouye, M. (1988). In vitro processing of pro-subtilisin produced in Escherichia coli. J. Biol. Chem. 263, 12959-12963.

Ikemura, H., Takagi, H. and Inouye, M. (1987). Requirement of pro-sequence for the production of active subtilisin E in Escherichia coli. J. Biol. Chem. 262, 7859-7864.

Ingolia, T. D., Slater, M. R. and Craig, E. A. (1982). Saccharomyces cerevisiae contains a complex multigene family related to the major heat shock-inducible gene of Drosophila. Mol. Cell. Biol. 2, 1388-1398.

Ishii, N., Taguchi, H., Sumi, M. and Yoshida, M. (1992). Structure of holo-chaperonin studied with electron microscopy. Oligomeric cpn10 on top of two layers of cpn60 rings with two stripes each. FEBS Lett. 299, 169-174.

IUPAC-IUB. Joint Commission on Biochemical Nomenclature (JCBM) (1984). Nomenclature and symbolism for amino acids and peptides. Biochem. J. 219, 345-373.

Iwabe, N., Kuma, K.-I., Hasegawa, M., Osawa, S. and Miyata, T. (1989). Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. Proc. Natl. Acad. Sci. USA 86, 9355-9359.

Jenkins, A. J., March, J. B., Oliver, I. R. and Masters, M. (1986). A DNA fragment containing the *groE* genes can suppress mutations in the *Escherichia coli dnaA* gene. Mol. Gen. Genet. 202, 446-454.

Johnson, A. and Thorpe, R. (1982). Immunochemistry in practice. Blackwell Scientific Publications, Oxford.

Kamath-Loeb, A. and Gross, C. A. (1991). Translational regulation of $\sigma^{32}$ synthesis: requirement for an internal control element. J. Bacteriol. 173, 3904-3906.

Kang, P. J. and Craig, E. A. (1990). Identification and characterization of a new *Escherichia coli gene that is a dosage-dependent suppressor of a dnaK* deletion mutation. J. Bact. 172 2055-2064.

Kang, P. J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A. and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature 348, 137-143.

Kassenbrock, C. K., Garcia, P. D. Walter, P. and Kelly, R. B. (1988). Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature 333, 90-93.

Kleinschmidt, J. A., Fortkamp, E., Krohne, G., Zentgraf, H. and Franke, W. W. (1985). Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes. J. Biol. Chem. 260, 1166-1176.

Kleinschmidt, J. A., Dingwall, C., Maier, G. and Franke, W. W. (1986). Molecular characterization of a karyophilic, histone-binding protein: cDNA cloning, amino acid sequence and expression of nuclear protein N1/N2 of *Xenopus laevis*. EMBO J. 5, 3547-3552.

Kogoma, T. and Yura, T. (1992). Sensitization of *Escherichia coli* cells to oxidative stress by deletion of the *rpoH* gene, which encodes the heat shock sigma factor. J. Bacteriol. 174, 630-632.

Kozutsumi, Y., Segal, M., Normington, K., Gething, M.-J. and Sambrook, J. (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose regulated proteins. Nature 332, 462-464.

Krieg, U. C., Walter, P. and Johnson, A. E. (1986). Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle. Proc. Natl. Acad. Sci. USA 83, 8604-8608.

Kuehn, M. J., Normark, S. and Hultgren, S. J. (1991).
Immunoglobulin-like PapD chaperone caps and uncaps
interactive surfaces of nascently translocated pilus
subunits. Proc. Natl. Acad. Sci. USA 88, 10586-10590.

Kumamoto, C. A. (1989). *Escherichia coli* SecB protein
associates with exported protein precursors *in vivo*. Proc.
Natl. Acad. Sci. USA 86, 5320-5324.

Kumamoto, C. A. (1991). Molecular chaperones and protein
translocation across the *Escherichia coli* inner membrane.
Mol. Microbiol. 5, 19-22.

Kumamoto, C. A. and Beckwith, J. (1983). Mutations in a new
gene, *secB*, cause defective protein localization in
*Escherichia coli*. J. Bacteriol. 154, 253-260.

Kumamoto, C. A. and Beckwith, J. (1985). Evidence for
specificity at an early step in protein export in *Escherichia
coli*. J. Bacteriol. 163, 267-274.

Kumamoto, C. A. and Gannon, P. M. (1988). Effects of
*Escherichia coli secB* mutations on pre-maltose binding
protein conformation and export kinetics. J. Biol. Chem. 263,
11554-11558.

Kumamoto, C. A., Chen, L., Fandl, J. and Tai, P. C. (1989).
Purification of the *Escherichia coli secB* gene product and
demonstration of its activity in an *in vitro* protein
translocation system. J. Biol. Chem. 264, 2242-2249.

Kumamoto, C. A. and Nault, A. K. (1989). Characterization of
the *Escherichia coli* protein-export gene *secB*. Gene 75, 167-
175.

Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y. and Ito, K. (1989). Effects of mutations in heat-shock genes *groES* and *groEL* on protein export in *Escherichia coli*. EMBO J. 8, 3517-3521.

Laemmli, U. K., Beguin, F. and Gujer-Kellenberger, G. (1970). A factor preventing the head protein of bacteriophage T4 from random aggregation. J. Mol. Biol. 47, 69-85.

Laminet, A. A. and Plückthun, A. (1989). The precursor of β-lactamase: purification, properties and folding kinetics. EMBO J. 8, 1469-1477.

Laminet, A. A., Kumamoto, C. A. and Plückthun, A. (1991). Folding *in vitro* and transport *in vivo* of pre-β-lactamase are SecB independent. Mol. Microbiol. 5, 117-122.

Landry, S. J. and Gierasch, L. M. (1991). The chaperonin GroEL binds a polypeptide in an α-helical conformation. Biochemistry 30, 7359-7362.

Landry, S. J., Jordan, R., McMacken, R. and Gierasch, L. M. (1992). Different conformations of the same polypeptide bound to the chaperones DnaK and GroEL. Nature 355, 455-457.

Langer, T., Chi, L., Echols, H., Flanagan, J., Hayer, M. K. and Hartl, F. U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. Nature 356, 683-689.

Laskey, R. A., Honda, B. M., Mills, A. D. and Finch, J. T. (1978). Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature 275, 416-420.

Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C.,
Bassford, P. J. Jr, Kumamoto, C. A. and Wickner, W. (1989).
Three pure chaperone proteins of *Escherichia coli* — SecB,
trigger factor and GroEL — form soluble complexes with
precursor proteins *in vitro*. EMBO J. 8, 2703-2709.

Levinthal, C. (1968). Are there pathways for protein folding?
J. Chim. Phys. 65, 44-45.

Lewis, M. J. and Pelham, H. R. B. (1985). Involvement of ATP in
the nuclear and nucleolar functions of the 70 kd heat shock
protein. EMBO J. 12, 3137-3143.

Lewis, V. A., Hynes, G. M., Zheng, D., Saibil, H. and Willison,
K. (1992). T-complex polypeptide-1 is a subunit of a
heteromeric particle in the eukaryotic cytosol. Nature 358,
249-252.

Liberek, K., Georgopolous, C. and Zylicz, M. (1988). Role of
the *Escherichia coli* DnaK and DnaJ heat shock proteins in the
initiation of bacteriophage λ DNA replication. Proc. Natl.
Acad. Sci. USA 85, 6632-6636.

Lill, R., Crooke, E., Guthrie, B. and Wickner, W. (1988). The
"trigger factor cycle" includes ribosomes, presecretory
proteins, and the plasma membrane. Cell 54, 1013-1018.

Lindberg, F., Tennet, J. M., Hultgren, S. J., Lund, B. and
Normark, S. (1989). PapD a periplasmic transport protein in
P-pilus biogenesis. J. Bacteriol. 171, 6052-6058.

Lindquist, S. (1986). The heat-shock response. Ann. Rev.
Biochem. 55, 1151-1191.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. Ann. Rev. Genet. 22, 631-677.

Lipman, D. J. and Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. Science 227, 1435-1441.

Lissin, N. M., Venyaminov, S. Y. and Girschovich, A. S. (1990). (Mg-ATP)-dependent self-assembly of molecular chaperone GroEL. Nature 348, 339-342.

Liu, G., Topping, T. B. and Randall, L. L. (1989). Physiological role during export for the retardation of folding by the leader peptide of maltose-binding protein. Proc. Natl. Acad. Sci. USA 86, 9213-9217.

Liu, S.-K., and Tessman, I. (1990). *groE* genes affect SOS repair in *Escherichia coli*. J. Bacteriol. 172, 6135-6138.

Louie, D. D., Kaplan, N. O. and McLean, J. D. (1972). Allosteric effects of 2--adenylic acid on the *Pseudomonas* pyridine nucleotide transhydrogenase. J. Mol. Biol. 70, 651-664.

Lubben, T. H., Gatenby, A. A., Donaldson, G. K., Lorimer, G. H. and Viitanen, P. V. (1990). Identification of a groES-like chaperonin in mitochondria that facilitates protein folding. Proc. Natl. Acad. Sci. USA 87, 7683-7687.

Lund, B., Lindberg, F., Marklund, B.-I. and Normark, S. (1987). The PapG protein is the α-D-galactopyranosyl-(1→4)-β-D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA 84, 5898-5902.

Lyon, M. F., Zenthon, J., Evans, E. P., Burtenshaw, M. D. and
   Willison, K. R. (1988). Extent of the mouse t complex and its
   inversions shown by *in situ* hybridization. Immunogenetics 24,
   125-127.

McCallum, K. L., Butler, B. J. and Innis, W. E. (1989). Stress
   or heat shock protein synthesis and cellular filamentation in
   psychrophilic and psychrotrophic bacteria. Arch. Microbiol.
   152, 148-153.

McMullin, T. W. and Hallberg, R. L. (1987). A normal
   mitochondrial protein is selectively synthesized and
   accumulated during heat shock in *Tetrahymena thermophila*.
   Mol. Cell. Biol. 7, 4414-4423.

McMullin, T. W. and Hallberg, R. L. (1988). A highly
   evolutionarily conserved mitochondrial protein is
   structurally related to the protein encoded by the
   *Escherichia coli groEL* gene. Mol. Cell. Biol. 8, 371-380.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular
   cloning. A laboratory manual. Cold Spring Harbour Laboratory,
   New York.

Manning-Krieg, U. C., Scherer, P. E. and Schatz, G. (1991).
   Sequential action of mitochondrial chaperones in protein
   import into the matrix. EMBO J. 10, 3273-3280.

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.
   L. and Hartl, F.-U. (1991). Chaperonin-mediated protein
   folding at the surface of groEL through a 'molten globule'-
   like intermediate. Nature 352, 36-42.

Mascagni, P., Tonolo, M., Ball, H., Lim, M., Ellis, R. J. and
    Coates, A. (1991) Chemical synthesis of 10 kDa chaperonin.
    Biological activity suggests chaperonins do not require other
    molecular chaperones. FEBS Lett. 286, 201-203.

Merck Index (1984). An encyclopedia of chemicals, drugs and
    biologicals. Merck & Co., Inc. Rahway, New Jersey.

Meury, J. and Kohiyama, M. (1991). Role of heat shock protein
    DnaK in osmotic adaptation of *Escherichia coli*. J. Bacteriol.
    173, 4404-4410.

Miki, T., Orita, T., Furuno, M. and Horiuchi, T. (1988).
    Control of cell division by sex factor F in *Escherichia coli*.
    III. Participation of the *groES* (*mopB*) gene of the host
    bacteria. J. Mol. Biol. 201, 327-338.

Miller, M. J., Xuong, N.-H. and Geiduschek, E. P. (1979). A
    response of protein synthesis to temperature shift in the
    yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA
    76, 5222-5225.

Miller, M. J., Xuong, N.-H. and Geiduschek, E. P. (1982).
    Quantitative analysis of the heat shock response of
    *Saccharomyces cerevisiae*. J. Bacteriol. 151, 311-327.

Mills, A. D., Laskey, R. A., Black, P. and De Roberts, E. M.
    (1980). An acidic protein which assembles nucleosomes *in
    vitro* is the most abundant protein in *Xenopus* oocyte nuclei.
    J. Mol. Biol. 139, 561-568.

Mitraki, A. and King, J. (1989). Protein folding intermediates
    and inclusion body formation. Biotech. 7, 690-697.

Miziorko, H. M. and Lorimer, G. H. (1983). Ribulose-1,5-bisphosphate carboxylase-oxygenase. Ann. Rev. Biochem. 52, 507-535.

Moore, S. and Stein, W. H. (1973). Chemical structures of pancreatic ribonuclease and deoxyribonuclease. Science 181, 458-464.

Moreau, N., Angelier, N., Bonnanfant-Jais, M.-L., Gounon, P. and Kubisz, P. (1986). Association of nucleoplasmin with transcription products as revealed by immunolocalization in the amphibian oocyte. J. Cell. Biol. 103, 683-690.

Morimoto, R. I., Tissière, A. and Georgopoulos, C. (1990). The stress response, function of the proteins, and perspectives. In: Stress proteins in biology and medicine (Eds Morimoto, R., Tissieres, A. and Georgopoulos, C.) pp. 191-221, Cold Spring Harbour Laboratory Press, New York.

Mues, G. I., Munn, T. Z. and Raese, J. D. (1986). A human gene family with sequence homology to *Drosophila melanogaster* *Hsp70* heat shock genes. J. Biol. Chem. 261, 874-877.

Munro, S. and Pelham, H. R. B. (1985). What turns on heat shock genes? Nature 317, 477-478.

Munro, S. and Pelham, H. R. B. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46, 291-300.

Musgrove, J. E., Johnson, R. A. and Ellis, R. J. (1987). Dissociation of the ribulosebisphosphate-carboxylase large-subunit binding protein into dissimilar subunits. J. Biochem. 163, 529-534.

Neidhardt, F. C., Vanbogelen, R. A. and Lau, E. T. (1982). In:
Heat shock from bacteria to man (Eds. Schlesinger, M. J.,
Ashburner, M. and Tissieres, A.) pp. 139-145, Cold Spring
Harbor, New York.

Neidhardt, F. C., VanBogelen, R. A. and Vaughn, V. (1984). The
genetics and regulation of heat-shock proteins. Ann. Rev.
Genet. 18, 295-329.

Nilsson, B. and Anderson, S. (1991). Proper and improper
folding of proteins in the cellular enviroment. Ann. Rev.
Microbiol. 45, 607-635.

Nivinskas, R. and Black, L. W. (1988). Cloning, sequence, and
expression of the temperature-dependent phage T4 capsid
assembly gene 31. Gene 73, 251-257.

Norgren, M., Normark, S., Lark, D., O'Hanley, P., Schoolnik,
G., Falkow, S., Svanborg-Edén, C., Båga, M. and Uhlin, B. E.
(1984). Mutations in E. coli cistrons affecting adhesion to
human cells do not abolish Pap pili fiber formation. EMBO J.
3, 1159-1165.

Norgren, M., Båga, M., Tennent, J. M. and Normark, S. (1987).
Nucleotide sequence, regulation and functional analysis of
the papC gene required for cell surface localization of Pap
pili of uropathogenic Escherichia coli. Mol. Microbiol. 1,
169-178.

Nozaki, M., Iwakura, Y. and Matsushiro, A. (1986). Studies of developmental abnormalities at the molecular level of mouse embryos homozygous for the t¹² lethal mutation. Dev. Biol. 113, 17-28.

Ohta, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, Z., Jordan, F. and Inouye, M. (1991). Pro-peptide as an intermolecular chaperone: renaturation of denatured subtilisin E with a synthetic pro-peptide. Mol. Microbiol. 5, 1507-1510.

Ostermann, J., Horwich, A. L., Neupert, W. and Hartl, F.-U. (1989). Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. Nature 341, 125-130.

Ozkaynak, E., Finley, D., Solomon, M. J. and Varshavsky, A. (1987). The yeast ubiquitin genes: a family of natural gene fusions. EMBO J. 6, 1429-1439.

Paech, C. and Dybing, C. D. (1986). Purification and degradation of ribulose bisphosphate carboxylase from soybean leaves. Plant Physiol. 81, 97-102.

Paek, K.-H. and Walker, G. C. (1987). *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. 169, 283-290.

Park, S., Liu, G., Topping, T. B., Cover, W. H. and Randall, L. L. (1988). Modulation of folding pathways of exported proteins by the leader sequence. Science 239, 1033-1035.

Parsell, D. A. and Sauer, R. T. (1989). Induction of a heat
shock-like response by unfolded protein in *Escherichia coli*:
dependence on protein level not protein degradation. Genes
Dev. 3, 1226-1232.

Patel, B. K. R., Banerjee, D. K. and Butcher, P. D. (1991).
Characterization of the heat shock response in *Mycobacterium
bovis* BCG. J. Bacteriol. 173, 7982-7987.

Pelham, H. R. B. (1984). Hsp70 accelerates the recovery of
nucleolar morphology after heat shock. EMBO J. 3, 3095-3100.

Pelham, H. R. B. (1986). Speculations on the functions of the
major heat shock and glucose-regulated proteins. Cell 46,
959-961.

Pelham, H. (1988). Coming in from the cold. Nature 332, 776-
777.

Pelham, H. R. B. (1989). Heat shock and sorting of luminal ER
proteins. EMBO J. 8, 3171-3176.

Pelham, H. R. B., Hardwick, K. G. and Lewis, M. J. (1988).
Sorting of soluble ER proteins in yeast. EMBO J. 7, 1757-
1762.

Phillips, G. J. and Silhavy, T. J. (1990). Heat-shock proteins
DnaK and GroEL facilitate export of LacZ hybrid proteins in
*E. coli*. Nature 344, 882-884.

Phipps, B. M., Hoffmann, A., Stetter, K. O. and Baumeister, W.
(1991). A novel ATPase complex selectively accumulated upon
heat shock is a major cellular component of thermophilic
archaebacteria. EMBO J. 10, 1711-1722.

Poritz, M. A., Bernstein, H. D., Strub, K., Zopf, D., Wilhelm, H. and Walter, P. (1990). An *E. coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. Science 250, 1111-1117.

Porter, D. (1984). Protein synthesis during differentiation of *Rhodomicrobium vannielii* swarmer cells. Ph. D. thesis, University of Warwick, Coventry.

Prasad, T. K. and Hallberg, R. L. (1989). Identification and metabolic characterization of the *Zea mays* mitochondrial homologue of the *Escherichia coli* groEL protein. Plant Mol. Biol. 12, 609-618.

Pushkin, A. V., Tsuprun, V. L., Solovjeva, N. A., Shubin, V. V., Evstigneeva, Z. G. and Kretovich, W. L. (1982). High molecular weight pea leaf protein similar to the groE protein of *Escherichia coli*. Biochim. Biophys. Acta 704, 379-384.

Randall, L. L. and Hardy, S. J. S. (1986). Correlation of competence for export with lack of tertiary structure of the mature species: a study *in vivo* of maltose-binding protein in *E. coli*. Cell 46, 921-928.

Randall, L. L., Topping, T. B. and Hardy, S. J. S. (1990). No specific recognition of leader peptide by SecB, a chaperone involved in protein export. Science 248, 860-863.

Rapoport, T. A. (1990). Protein transport across the ER membrane. TIBS 15, 355-358.

Rapoport, T. A. (1991). A bacterium catches up. Nature 349, 107-108.

Reading, D. S., Hallberg, R. L. and Myers, A. M. (1989).
Characterisation of the yeast *HSP60* gene coding for a
mitochondrial assembly factor. Nature 337, 655-659.

Rechsteiner, M. (1987). Ubiquitin-mediated pathways for
intracellular proteolysis. Ann. Rev. Cell Biol. 3, 1-30.

Ribes, V., Römisch, K., Giner, A., Dobberstein, B. and
Tollervey, D. (1990). *E. coli* 4.5S RNA is part of a
ribonucleoprotein particle that has properties related to
signal recognition particle. Cell 63, 591-600.

Richardson, W. D., Mills, A. D., Dilworth, S. M., Laskey, R. A.
and Dingwall, C. (1988). Nuclear protein migration involves
two steps: rapid binding at the nuclear envelope followed by
slower translocation through nuclear pores. Cell 52, 655-664.

Römisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingron,
M. and Dobberstein, B. (1989). Homology of 54K protein of
signal-recognition particle, docking protein and two *E. coli*
proteins with putative GTP-binding domains. Nature 340, 478-
482.

Rothman, J. E. (1989). Polypeptide chain binding proteins:
catalysts of protein folding and related processes in cells.
Cell 59, 591-601.

Russell, G. C. (1984). Inverted repeat sequences in the genome
of *R. vannielii*. Ph. D. thesis, University of Warwick,
Coventry.

Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J. and Silver, P. (1989). A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. J. Cell Biol. 109, 2665-2675.

Saibil, H., Dong, Z., Wood, S. and auf der Mauer, A. (1991). Binding of chaperonins. Nature 353, 25-26.

Saier, M. H. Jr., Werner, P. K. and Müller, M. (1989). Insertion of proteins into bacterial membranes: mechanism, characteristics, and comparisons with the eukaryotic process. Microbiol. Rev. 53, 333-366.

Sanz, P. and Meyer, D. I. (1988). Signal recognition particle (SRP) stabilizes the translocation-competent conformation of pre-secretory proteins. EMBO J. 7, 3553-3557.

Scherer, P. E., Kreig, U. C., Hwang, S. T., Vestweber, D. and Schatz, G. (1990). A precursor protein partly translocated into yeast mitochondria is bound to a 70 kd mitochondrial stress protein. EMBO J. 9, 4315-4322.

Schlesinger, M. J. (1990). Heat shock proteins. J. Biol. Chem. 265, 12111-12114.

Schmidt-Zachmann, M. S., Hügle-Dörr, B. and Franke, W. W. (1987). A constitutive nuclear protein identified as a member of the nucleoplasmin family. EMBO J. 7, 1881-1890.

Schneider, G., Lindqvist, Y., Brandén, C.-I. and Lorimer, G.
(1986). Three-dimensional structure of ribulose-1,5-
bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum*
at 2.9 Å resolution. EMBO J. **5**, 3409-3415.

Sealy, L., Cotten, M. and Chalkley, R. (1986). *Xenopus*
nucleoplasmin: egg vs. oocyte. Biochemistry **25**, 3064-3072.

Shalniene, V. and Nivinskas, R. (1987). Temperature sensitivity
of the multiplication of bacteriophage T4 amber mutants on
nonpermissive host: characterization of the phenomenon.
Virology **160**, 426-432.

Shinnick, T. M. (1987). The 65-kilodalton antigen of
*Mycobacterium tuberculosis*. J. Bacteriol. **169**, 1080-1088.

Silen, J. L. and Agard, D. A. (1989). The α-lytic protease pro-
region does not require a physical linkage to activate the
protease domain *in vivo*. Nature **341**, 462-464.

Silen, J. L., Frank, D., Fujishige, A., Bone, R. and Agard, D.
A. (1989). Analysis of prepro-α-lytic protease expression in
*Escherichia coli* reveals that the pro region is required for
activity. J. Bacteriol. **171**, 1320-1325.

Silver, L. M. (1985). Mouse *t* haplotypes. Ann. Rev. Genet. **19**,
179-208.

Silver, L. M. and Artzt, K. (1981). Recombination suppression
of mouse *t*-haplotypes due to chromatin mismatching. Nature
**290**, 68-70.

Silver, L. M. and White, M. (1982). A gene product of the mouse
    *t* complex with chemical properties of a cell surface-
    associated component of the extracellular matrix. Dev. Biol.
    91, 423-430.

Silver, L. M., Artzt, K. and Bennett, D. (1979). A major
    testicular cell protein specified by a mouse T/t complex
    gene. Cell 17, 275-284.

Silver, L. M., Uman, J., Danska, J. and Garrels, J. I. (1983).
    A diversified set of testicular cell proteins specified by
    genes within the mouse *t* complex. Cell 35, 35-45.

Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990). The E.
    coli *dnaK* gene product, the hsp70 homolog, can reactivate
    heat-inactivated RNA polymerase in an ATP hydrolysis-
    dependent manner. Cell 62, 939-944.

Somerville, C. R. and Somerville, S. C. (1984). Cloning and
    expression of the *Rhodospirillum rubrum* ribulosebisphosphate
    carboxylase gene in *E. coli*. Mol. Gen. Genet. 193, 214-219.

Spence, J. and Georgopoulos, C. (1989). Purification and
    properties of the *Escherichia coli* heat shock protein, HtpG.
    J. Biol. Chem. 264, 4398-4403.

Squires, C. and Squires, C. L. (1992). The Clp proteins:
    proteolysis regulators or molecular chaperones. J. Bacteriol.
    174, 1081-1085.

Sternberg, N. (1973). Properties of a mutant of *Escherichia
    coli* defective in bacteriophage λ head formation (*groE*). II.
    The propagation of phage λ. J. Mol. Biol. 76, 25-44.

Straus, D. B., Walter, W. A. and Gross, C. A. (1987). The heat
shock response of *E. coli* is regulated by changes in the
concentration of $\sigma^{32}$. Nature 329, 348-351.

Straus, D. B., Walter, W. A. and Gross, C. A. (1988).
*Escherichia coli* heat shock gene mutants are defective in
proteolysis. Genes Dev. 2, 1851-1858.

Straus, D. B., Walter, W. A. and Gross, C. A. (1989). The
activity of $\sigma^{32}$ is reduced under conditions of excess heat
shock protein production in *Escherichia coli*. Genes Dev. 3,
2003-2010.

Straus, D. B., Walter, W. A. and Gross, C. A. (1990). DnaK,
DnaJ, and GrpE heat shock proteins negatively regulate heat
shock gene expression by controlling the synthesis and
stability of $\sigma^{32}$. Genes Dev. 4, 2202-2209.

Struck, J. C. R., Toschka, H. Y., Specht, T. and Erdmann, V. A.
(1988). Common structural features between eukaryotic 7SL
RNAs, eubacterial 4.5S RNA and scRNA and archaebacterial 7S
RNA. Nucl. Acids Res. 16, 7740.

Suwanto, A. and Kaplan, S. (1989a). Physical and genetic map of
*Rhodobacter sphaeroides* 2.4.1 genome: genome size, fragment
identification, and gene localization. J. Bacteriol. 171,
5840-5849.

Suwanto, A. and Kaplan, S. (1989b). Physical and genetic map of
*Rhodobacter sphaeroides* 2.4.1 genome: presence of two unique
circular chromosomes. J. Bacteriol. 171, 5850-5859.

Tabita, F. R. (1988). Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. 52, 155-189.

Taguchi, H., Konishi, J., Ishii, N. and Yoshida, M. (1991). A chaperonin from a thermophilic bacterium, *Thermus thermophilus*, that controls refoldings of several thermophilic enzymes. J. Biol. Chem. 266, 22411-22418.

Takano, T. and Kakefuda, T. (1972). Involvement of a bacterial factor in morphogenesis of bacteriophage capsid. Nature New Biol. 239, 34-37.

Terlesky, C. T. and Tabita, F. R. (1991). Purification and characterization of the chaperonin 10 and chaperonin 60 proteins from *Rhodobacter sphaeroides*. Biochemistry 30, 8181-8186.

Thole, J. E. R., Hindersson, P., de Bruyn, J., Cremers, F., van der Zee, J., de Cock, H., Tommassen, J., van EDEN, W. and van Embden, J. D. A. (1988). Antigenic relatedness of a strongly immunogenic 65 kDa mycobacterial protein antigen with a similarly sized ubiquitous bacterial common antigen. Microbial Pathogenesis 4, 71-83.

Tilly, K., McKittrick, N., Zylicz, M. and Georgopolous, C. (1983). The *dnaK* protein modulates the heat-shock response of *Escherichia coli*. Cell 34, 641-646.

Tilly, K., Murialdo, H., and Georgopoulos, C. (1981). Identification of a second *Escherichia coli groE* gene whose product is necessary for bacteriophage morphogenesis. Proc. Natl. Acad. Sci. USA 78, 1629-1633.

Tilly, K., Spence, J. and Georgopoulos, C. (1989). Modulation of stability of the *Escherichia coli* regulatory factor $\sigma^{32}$. J. Bacteriol. 171, 1585-1589.

Torres-Ruiz, J. A. and McFadden, B. A. (1988). A homologue of ribulose bisphosphate carboxylase/oxygenase-binding protein in *Chromatium vinosum*. Arch. Biochem. Biophys. 261, 196-204.

Trent, J. D., Nimmesgern, E., Wall, J. S., Hartl, F.-U. and Horwich, A. L. (1991). A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. Nature 354, 490-493.

Tsuprun, V. L., Boekema, E. J., Samsonidze, T. G. and Pushkin, A. V. (1991). Electron microscopy of the complexes of ribulose-1,5-bisphosphate carboxylase (Rubisco) and Rubisco subunit-binding protein from pea leaves. FEBS Lett. 289, 205-209.

Ungewickell, E. (1985). The 70-kd mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO J. 4, 3385-3391.

Ursic, D. and Culbertson, M. R. (1991). The yeast homolog to mouse *Tcp-1* affects microtubule mediated processes. Mol. Cell. Biol. 11, 2629-2640.

Ursic, D. and Ganetzky, B. (1988). A *Drosophila melanogaster* gene encodes a protein homologous to the mouse *t* complex polypeptide 1. Gene 68, 267-274.

van der Vies, S. M. (1989). Isolation and expression of a plastid α chaperonin cDNA sequence from *Triticum aestivum*. Ph. D. thesis, University of Warwick, Coventry.

Van Dyk, T. K., Gatenby, A. A. and LaRossa, R. A. (1989). Demonstration by genetic supression of interaction of GroE products with many proteins. Nature 342, 451-453.

VanBogelen, R. A., Kelley, P. M. and Neidhardt, F. C. (1987). Differential induction of heat shock, SOS and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. 169, 26-32.

Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P. and Lorimer, G. H. (1990). Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K+ dependent. Biochemistry 29, 5665-5671.

Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H. and Gatenby, A. A. (1991). Complex interactions between the chaperonin 60 molecular chaperone and dihydrofolate reductase. Biochemistry 30, 9716-9723.

Viitanen, P. V., Lorimer, G. H., Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O. and Cowan, N. J. (1992a). Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. J. Biol. Chem. 267, 695-698.

Viitanen, P. V., Gatenby, A. A. and Lorimer, G. H. (1992b). Purified chaperonin 60 (groEL) interacts with the nonnative states of a multitude of *Escherichia coli* proteins. Protein Science 1, 363-369.

Vodkin, M. H. and Williams, J. C. (1988). A heat shock operon in *Coxiella burnetii* produces a major antigen homologous to a protein in both Mycobacteria and *Escherichia coli*. J. Bacteriol. 170, 1227-1234.

Wada, M. and Itikawa, H. (1984). Participation of *Escherichia coli* K-12 *groE* gene products in the synthesis of cellular DNA and RNA. J. Bacteriol. 157, 694-696.

Watanabe, M. and Blobel, G. (1989). Cytosolic factor purified from *Escherichia coli* is necessary and sufficient for the export of a preprotein and is a homotetramer of SecB. Proc. Natl. Acad. Sci. USA 86, 2728-2732.

Weiss, J. B., Ray, P.H. and Bassford, P. J. Jr. (1988). Purified SecB protein of *Escherichia coli* retards folding and promotes membrane translocation of the maltose-binding protein *in vitro*. Proc. Natl. Acad. Sci. USA 85, 8978-8982.

Weiss, J. B., MacGregor, C. H., Collier, D. N., Fikes, J. D., Ray, P. H. and Bassford, P. J. Jr. (1989). Factors influencing the *in vitro* translocation *Escherichia coli* maltose-binding protein. J. Biol. Chem. 264, 3021-3027.

Weiss, J. B. and Bassford, P. J. Jr. (1990). The folding properties of the *Escherichia coli* maltose-binding protein influence its interaction with SecB in vitro. J. Bacteriol. 172, 3023-3029.

Welch, W. J. and Suhan, J. P. (1986). Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. 103, 2035-2052.

Wells, B. (1985). Low temperature box and tissue handling device for embedding biological tissue for immunostaining in electron microscopy. Micron and Microscopica Acta 16, 49-53.

Wermuth, B. and Kaplan, N. O. (1976). Pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa*: Purification by affinity chromatography and physiochemical properties. Arch. Biochem. Biophys.

Werner-Washburne, M., Stone, D. E. and Craig, E. A. (1987). Complex interactions among members of an essential subfamily of *hsp70* genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7, 2568-2577.

West, S. M. and Price, N. C. (1988). The unfolding and refolding of glutamate dehydrogenases from bovine liver, baker's yeast and *Clostridium symbosium*. Biochem. J. 251, 135-139.

West, S. M. and Price, N. C. (1989). The unfolding and refolding of cytoplasmic aspartate amoinotransferase from pig heart. Biochem. J. 261, 189-196.

West, S. M., Kelly, S. M. and Price, N. C. (1990). The unfolding and attempted refolding of citrate synthase from pig heart. Biochim. Biophys. Acta 1037, 332-336.

Wetlaufer, D. B. and Ristow, S. (1973). Acquisition of three-dimensional structure of proteins. Ann. Rev. Biochem. 42, 135-158.

Whittenbury, R. and Dow, C. S. (1977). Morphogenesis and differentiation in *Rhodomicrobium vannielii* and other budding and prosthecate bacteria. Bacteriol. Rev. 41, 754-808.

Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992). Hsp90 chaperones protein folding *in vitro*. Nature 358, 169-170.

Willison, K. R., Dudley, K. and Potter, J. (1986) Molecular cloning and sequence analysis of a haploid expressed gene encoding *t* complex polypeptide 1. Cell 44, 727-738.

Willison, K., Kelly, A., Dudley, K., Goodfellow, P., Spurr, N., Groves, V., Gorman, P., Sheer, D., and Trowsdale, J. (1987). The human homologue of the mouse t-complex gene, *TCP-1*, is located on chromosome 6 but is not near the HLA region. EMBO J. 6, 1967-1974.

Willison, K., Lewis, V., Zuckerman, K. S., Cordell, J., Dean, C., Miller, K., Lyon, M. F. and Marsh, M. (1989). The *t* complex polypeptide 1 (TCP-1) is associated with the cytoplasmic aspect of Golgi membranes. Cell 57, 621-632.

Woese, C. R. (1987). Bacterial evolution. Microbiol. Rev. 221-271.

Woodland, H. R. and Adamson, E. D. (1977). The synthesis and storage of histones during oogenesis of *Xenopus laevis*. Dev. Biol. 57, 118-135.

Yaffe, M. B., Farr, G. W., Miklos, D., Horwich, A. L. Sternlicht, M. L. and Sternlicht, H. (1992). TCP-1 complex is a molecular chaperone in tubulin biogenesis. Nature 358, 245-248.

Young, D. B. (1990). Chaperonins and the immune response. Sem. Cell Biol. 1, 27-35.

Young, D., Lathigra, R., Hendrix, R., Sweetser, D. and Young, R. A. (1988). Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA 85, 4267-4270.

Yuen, S., Hunkapiller, M. W., Wilson, K. J. and Yuen, P. M. (1986). SDS-PAGE electroblotting. User bulletin 25. Applied Biosystems protein sequencer.

Zahn, R. and Plückthun, A. (1992). The molecular chaperone GroEL can act as a temperature-reversible "unfoldase" of an active enzyme. Nature *in press*.

Zhu, X., Ohta, Y., Jordan, F. and Inouye, M. (1989). Pro-sequence of subtilisin can guide the refolding of subtilisin in an intermolecular process. Nature 339, 483-484.

Zweig, M. and Cummings, D. J. (1973). Cleavage of head and tail proteins during bacteriophage T5 assembly: selective host involvement in the cleavage of a tail protein. J. Mol. Biol. 80, 505-518.