Protein phosphorylation in *Rhodomicrobium vannielii*

by

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RESULTS AND DISCUSSION

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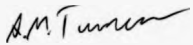
Declaration

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. N.H. Mann. All sources of information have been specifically acknowledged by way of reference.

None of the work contained in this thesis has been used in any previous application for a degree.



Andrew M. Turner

Dedication

To San, with love

"It is not without justice that I claim indulgence for this work, and I beg that no one will charge me with negligence, if he finds that I have passed over some illustration. For who could prove equal to the task of examining all the records which have come down to us....."

Frontius, Stratagems, I

Abbreviations

APBA	m-aminophenylboronicacid
ATP	adenosine triphosphate
Ci	Curie (3.7 x 10^{12} DPM)
cpm	counts per minute
DNase	deoxyribonuclease
DPM	disintegrations per minute
EDTA	ethylenediaminetetra-acetic acid, disodium salt
GTP	guanosine triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
mA	Amperes x 10^{-3}
min	minute
Mr	relative molecular weight
NP40	Nonidet P40
OD	optical density, absorbance
PMSF	phenylmethylsulphonylfluoride
P-Ser	O-phospho-L-serine
psi	pounds per square inch
P-Thr	O-phospho-L-threonine
P-Tyr	O-phospho-L-tyrosine
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'tetramethylenediamine
Tris	hydroxymethylaminomethane

α	alpha
β	beta
δ	gamma
δ	delta
σ	sigma
μ	mu

SUMMARY

Rm. vannielii was shown to contain phosphopolypeptides, the synthesis of which varied through the growth curve. Phosphoamino acid analysis demonstrated that the predominant phosphoamino acid was phosphoserine, though phosphotyrosine and phosphothreonine were also detected. The major phosphopolypeptide in the cell was shown to be the RuBisCO large subunit. Phosphorylation of this polypeptide was shown to be growth stage-dependent, commencing around mid log phase. The role of RuBisCO large subunit phosphorylation is as yet undetermined. A second phosphopolypeptide of Mr 12,700 was tentatively identified as a light harvesting chlorophyll binding protein. Analysis of phosphopolypeptides from swarmer and chain cells revealed that there were no detectable phosphopolypeptides in the undifferentiating swarmer cell. Analysis of phosphorylation during differentiation revealed that the accumulation of phosphopolypeptides did not become detectable until 1 hour into differentiation and the phosphorylation of most polypeptides appeared to reach a maximum rate by 2 hours into differentiation. In addition, two phosphopolypeptides specific to the end, and later stages of differentiation were observed.

Investigations in to protein kinase activities in cell-free extracts established that the conditions under which the experiments were carried out had to be extremely carefully controlled. On examining the effects of Mg^{2+} and Ca^{2+} on the pattern of phosphopolypeptides obtained from cell-free extracts it was found that both cations could influence the phosphopolypeptide patterns produced and that the effects were concentration-dependent in a way that supported the suggestion that there were multiple protein kinases active in the extracts. In particular, Ca^{2+} was found to prevent the phosphorylation of a Mr 47,000 phosphopolypeptide the phosphorylation of which was Mg^{2+}-dependent. The addition of chlorpromazine (a calmodulin antagonist) instead of Ca^{2+} produced the same effect leading to the suggestion that there may be a calmodulin-like molecule in Rm. vannielii that exerts its effect on protein kinases through an interaction with Mg^{2+} rather than Ca^{2+}. Other explanations are also put forward. As has been found in other studies on protein phosphorylation in prokaryotes, the resemblance between the proteins observed to be phosphorylated in vivo and in vitro was slight (see section 1.4). Examination of protein kinase activities through differentiation revealed that the dark-incubated swarmer cell contained potentially active protein kinases despite there being no detectable phosphorylation of polypeptides in vivo. This suggests that protein kinases are present in the inhibited swarmer cell, but inactive.

Examination of specific proteins for phosphopolypeptides by immunological techniques identified the β and β' subunits of a DNA-dependent RNA polymerase and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase as being phosphorylated. The large subunit of RuBisCO was identified on 1 dimensional polyacrylamide gels as the heavily-labelled Mr 54,500 phosphopolypeptide.

CHAPTER 1

INTRODUCTION

Protein phosphorylation/dephosphorylation is now recognized as a major regulator of cellular activity. Much of the evidence for this has been accumulated from studies in eukaryotes, but there is increasing evidence that prokaryotes are also subject to metabolic regulation in this fashion. This study is concerned with examining the role of protein phosphorylation in Rhodomicrobium vannielii. In this chapter I shall begin by discussing covalent protein modification in general. I will then review briefly the role of phosphorylation in eukaryotes, looking mainly at its function in the overall scheme of metabolic control before going on to review protein phosphorylation in prokaryotes. Finally, I will consider the physiology and cell cycle of Rm. vannielii.

1.1 Post-Translational Modification

The first essential for discussing post-translational modification is to clearly define the term. For our purposes we will define translation as the assembly of amino acids into proteins, the sequence being determined by the nucleotide sequence of messenger RNA molecules. Any subsequent alterations to the amino acid or to the polypeptide after polymerization can be classified as post-translational modifications.
Post-translational modification is a widely overlooked step in protein synthesis. In its broadest sense it is essential in most cases for the synthesis of an active protein localized in the correct cellular compartment (e.g. membrane, periplasmic space or

even extracellular). In this general sense post-translational modification includes three types of event (Uy & Wold, 1977). The first involves weak non-covalent interactions resulting in the folding of the polypeptide chain and the association of chains with each other and with noncovalently bound ligands. The second involves the transport of the synthesized polypeptide to its site of action and the third involves various covalent modifications. This third category of modification can be functional or regulatory. For our purposes it is the covalent post-translational modifications that are of interest. These are of two distinct types, peptide bond cleavage and amino acid derivatization.

1.1.1 Peptide bond cleavage

The significance of peptide bond cleavage becomes obvious when we consider that all bacterial proteins are synthesized with N-formylmethionine as the first amino acid, but very few retain the N-terminal formyl group or even the methionine residue. This cleavage is probably fairly nonspecific, as overproduction of a new protein often results in the production of a mixture of formylated and deformylated forms. A more specific cleavage occurs when proteins are translocated through or inserted into membranes. This results in the removal of an amino-terminal leader or signal sequence (see Wickner, 1979) on insertion into or transfer through the membrane. In eukaryotes many enzymes are known to be activated by proteolytic cleavage e.g. digestive enzymes (see Neurath & Walsh, 1976) and enzymes involved in blood coagulation (see Jackson & Nemerson, 1980). The same may be true of prokaryotes. A second aspect of proteolytic cleavage may be involved in the maintenance of the stoichiometry of protein

subunits. Luzikov (1986) hypothesized that the regulation of relative amounts of the subunits of a particular protein did not occur through coordinated synthesis of subunits, but through the elimination of unassembled, incorrectly assembled or incorrectly compartmentalized subunits by proteolysis.

1.1.2 Covalent protein modification

Covalent protein modification can be split into three categories (Wold, 1981), modifications involving the carboxyl-terminal, modifications involving the amino-terminal and modifications involving individual amino acid side chains. I shall briefly examine each in turn, examining the side chain modifications by the modifying group type. No attempt will be made to discuss all the individual modifications as Uy & Wold (1977) listed over 140 different amino acid derivatives, instead occasional examples will be listed to illustrate the potential roles played by these modifications.

1.1.2.1 Modifications of the carboxyl-terminal

There are three well characterized derivatives of the carboxyl-terminal group. Unsubstituted amides (α-$CONH_2$) are only known to be present on various amino acid residues of some short peptides (e.g. hormones (Lowry & Chadwick, 1970) and insect toxins (Suchanek et al., 1980)). Histone H1 is known to be ADP-ribosylated on the carboxyl-terminal lysine residue and tubulin forms a substituted amide derivative by the addition of a tyrosine residue in a peptide linkage to the carboxyl-terminal (see Wold, 1981). Modifications of the carboxyl group are quite uncommon, being restricted to only a few proteins.

1.1.2.2 Modifications of the amino-terminal

There are a considerably greater number of modifications observed to be made on the amino-terminal residue of a protein. The most common modification is an acylation with a variety of groups. Apart from N-formyl methionine, the formyl group is found attached to glycine in honey bee melittin (Kreil & Kreil-Kiss, 1967). The diaminopimelic acid of E. coli peptidoglycan forms an amide link with the amino group of the amino terminal lysine of the membrane lipoprotein. However, of all the N-acylations, N-terminal acetylation is by far the most common (Driessen et al., 1985). A large number of animal and plant proteins are N-terminal acetylated, but a few have also been identified in bacteria and fungi. Of the five bacterial proteins known to be N-terminal acetylated, four are ribosomal proteins. The majority of N-terminal acetylation occurs on alanine and serine residues, but other residues (methionine, glycine, aspartate, asparagine, isoleucine,valine and threonine) have also been observed. The N-terminal acetyltransferases are highly specific, suggesting that the acetylation reaction serves some important function. The actual nature of this function has not yet been determined.

The N-terminal amino group has also been observed to be alkylated, the best characterized of these reactions being the N-terminal methylations on ribosomal proteins. These occur on terminal methionine and alanine residues. An N-terminal dimethylproline residue has been identified on the cytochrome c557 of the protozoan Crithidia oncopelti (Pettigrew & Smith, 1977).

1.1.2.3 Modifications of the amino acid side chains

The largest category of covalent modifications are modifications to the amino acid side chains. Of these the most common modification is glycosylation, the introduction of covalently linked monosaccharides, oligosaccharides and polysaccharides onto proteins. Asparagine is the major acceptor of glycosyl groups and alone can form hundreds of glycosylasparagine derivatives. Other glycosylated amino acids include serine, threonine and cysteine, and the secondary amino acids hydroxylysine, hydroxyproline, β-hydroxyphenylalanine and β-hydroxytyrosine. It is thought that at least one of the roles of the sugar moieties is in biological communications involving cell-cell and cell-molecule interactions. An example would be the requirement by lectins of specific oligosaccharide sequences for tight binding. Glycosylation is also known to occur on a very small number of prokaryotic proteins. Examples are the glycoproteins found in the carboxysomes of Thiobacillus neapolitanus (Holthuijzen et al., 1986)

A second category of modification is methylation of which there are three forms. Firstly, there are the stable N-methylated derivatives of arginine, histidine, lysine and glutamine. Methylated arginine and lysine derivatives are found in both eukaryotes and prokaryotes, a methylated histidine derivative in muscle proteins and N^5-methylglutamine has been reported in a ribosomal protein of E. coli (Lhoest & Colson, 1977). Secondly, there are the proposed O-methyl ethers of serine and threonine and thirdly, there are the labile methyl esters of aspartate and glutamate. O-methylglutamate has been found to play an important role in bacterial chemotaxis (see Koshland, 1981).

A third category of modifications are halogenations. Histidine

is iodinated in thyroglobulin and tyrosine undergoes chlorination, bromination and iodination, the latter resulting in two derivatives that are key intermediates in the biosynthesis of the thyroxin family of hormones. ADP-ribosylation is another well characterized modification (Hayaishi & Ueda, 1982, Gaal & Pearson, 1986). It can occur in two forms, mono- and poly-ADP ribosylation on serine, glutamate, histidine and arginine. In eukaryotes, ADP-ribosylation seems to be primarily involved in the regulation of intra-nuclear events. In prokaryotes, mono-ADP ribosylation is known to reversibly regulate the activity of the nitrogenase iron proten in Rhodospirillum rubrum (Lowery et al., 1986). Other specific examples of modifications include the acetylation of histones on lysine (Gershey et al., 1968), the adenylylation of glutamine synthetase and the uridylylation of the P_{II} regulatory protein in the cascade control of glutamine synthetase in Escherichia. coli (Adler et al., 1975) and the fatty acid acylation of human apolipoprotein A-1 (Hoeg et al., 1986). There are many other specific amino acid modifications, most of which are uncommon. However, the best studied group of all are the phosphorylations. Phosphorylation can occur in several forms. An acyl-phosphate bond can be formed on aspartate and there are amidophosphate derivatives of lysine, histidine and arginine. However, by far the best studied, probably due to their relative stabilities, are the phosphomonoesters of serine, threoine and tyrosine. The latter are the subject of this thesis and will be discussed in detail later.

1.2 PROTEIN PHOSPHORYLATION

1.2.1 Introduction

Protein phosphorylation/dephosphorylation is now recognized as a major regulator of cellular activity. In this chapter I shall review briefly the role of phosphorylation in eukaryotes, looking mainly at its function in the overall scheme of metabolic control. I shall then review protein phosphorylation in prokaryotes. Firstly, however, the terms used and the reactions under consideration must be specified.

We shall discuss protein phosphorylation on the basis of the reactions below. The reason for this is the known existence of kinases not involved in regulation that form carboxyphosphates or histidyl phosphates often as intermediates in certain enzymic processes. Protein phosphorylation in our context, then, refers to the reaction

$$\text{protein} + n\text{NTP} \xrightleftharpoons[\text{kinase}]{\text{protein}} \text{protein-P}n + n\text{NDP}$$

whereas the reverse, phosphatase catalyzed reaction can be defined as

$$\text{protein-P}n + n\text{H}_2\text{O} \xrightleftharpoons[\text{phosphatase}]{\text{phosphoprotein}} \text{protein} + n\text{P}_i$$

i.e. phosphorylation is the transfer of a phosphate group from a nucleoside triphosphate to a protein and dephosphorylation is the removal of the phosphate group using water as an acceptor (Knowles, 1980). The nucleoside triphosphate is generally ATP,

though GTP has been documented (Rodnight & Lavin, 1964; Traught et al., 1973 & Nimmo & Cohen, 1974 as cited by Krebs & Beavo, 1979) and the accepting amino acid groups on the protein are the hydroxyl groups of serine, threonine or tyrosine.

1.2.2 The role of protein phosphorylation

As well as its role in the control of specific enzyme systems, protein phosphorylation is thought to have a central role in the regulation of diverse biological processes in eukaryotes in general, and mammalian tissues in particular. Not only is it involved in the control of many important specific cell functions, but more recently it has become clear that protein phosphorylation is the basis of a complex network of interlocking systems which control and coordinate multiple diverse biological functions acting through a small number of second messengers (Cohen, 1986). Regulatory agents of protein phosphorylation, i.e. those effectors that produce a change in the phosphorylation state of specific proteins either directly or through a second messenger, are many and varied. They include the second messengers cAMP, cGMP and calcium as well as steroid hormones, insulin, interferon, thyroid hormone, viruses, hemin and light (Greengard, 1978; Fig 1.1). The mechanisms by which these regulatory agents exert their effects may vary considerably, but in principle they can be summarized as follows.

(1) The agents could alter the amounts or properties of the protein kinase.

(2) The agents could alter the amounts or properties of the substrate protein.

(3) The agents could alter the amounts or properties of the phosphoprotein phosphatase.

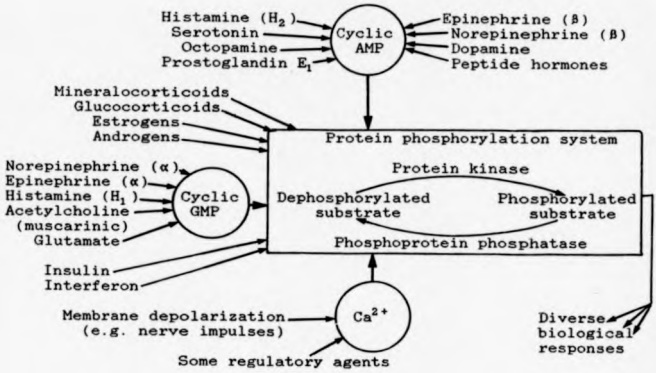


Fig 1.1 Schematic diagram of postulated role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents (after Greengard, 1978)

(4) The agents could alter the amounts or properties of an activator or inhibitor of the kinase or phosphatase. (Greengard, 1978).

The phosphorylation of the substrate protein could (assuming it to be an enzyme) alter its K_m for a substrate, alter its K_a for an activator or alter its K_i for an inhibitor. A fourth case is illustrated by the enzyme isocitrate dehydrogenase in E. coli where phosphorylation of the enzyme alters its V_{max}, reducing it virtually to zero (Garland & Nimmo, 1984). Cohen (1982) stressed that protein phosphorylation/dephosphorylation in the context of the neural and hormonal control of cellular activity should not merely be regarded as a mechanism for switching an enzyme on or off. Instead it should be seen as a means of switching the enzyme between two different forms capable of responding differently to various substrates and regulatory molecules. The subsequent changes in amounts of substrates, activators or inhibitors may then affect the rate at which an enzyme is phosphorylated or dephosphorylated (Cohen, 1983). In addition, it is also known that the second messengers Ca^{2+} and cAMP can modulate each others activity (Review see Pallen et al., 1986). These interactions serve to integrate extracellular and intracellular information to determine the precise activity of a metabolic pathway. The coordinated metabolic response of a cell to a simple regulatory signal arises from the presence in cells of a number of broad specificity protein kinases and phosphatases that will phosphorylate or dephosphorylate many proteins in response to that one signal (e.g. the mediation of many of the effects of calcium through the calmodulin-dependent 'multiprotein' kinase, Cohen, 1986), and from the amplification mechanisms of the signal transduction

system itself.

1.2.2.1 Protein phosphorylation and transcriptional control

There are few proven examples of transcriptional control by phosphorylation/dephosphorylation mechanisms. One of those that has been characterized, however, is the control of early gene expression in bacteriophage T7. It was first reported by Rahmsdorf et al. (1974) that T7 possessed a gene coding for a protein kinase responsible for the shutoff of RNA synthesis in the host. This occurs by the inhibition of initiation of host RNA polymerase (Ponta et al., 1974), and by enforced termination of transcription (Hercules et al., 1976) due to the phosphorylation of the host DNA-dependent RNA polymerase on the B' subunit (and to a lesser extent the B subunit (Zillig et al., 1975)). In addition, the kinase is thought to cause termination behind the M gene in the polycistronic unit of the early region (Ponta et al., 1974; Pfennig-Yeh et al., 1978) in 75% of transcripts (possibly by the binding of host RNA polymerase blocked in initiation to a weak promotor located behind the M gene (Pfennig-Yeh et al., 1978)). While this represents a fairly crude form of transcriptional control, there is a possibility that more subtle means of modulating transcriptional activity by phosphorylation exist. An example of this could be the phosphorylation of chromatin-associated non-histone proteins reported by Sinclair & Rickwood (1985) during the development of Dictyostelium discoideum. In this case major changes in protein and RNA synthesis accompany development in the absence of any major changes in the species of nuclear proteins, but in the presence of major changes in the state of phosphorylation of these nuclear proteins. Thus it is possible that protein phosphorylation/

dephosphorylation mechanisms may be important in regulating transcription in at least some organisms. Indeed, it is possible to envisage phosphorylation of the DNA-dependent RNA polymerase altering the template specificity of the polymerase and thus being able to control major changes in cellular activity. This may in fact occur in prokaryotes, as Enami & Ishihama (1984) reported that the β and β' subunits of an E. coli RNA polymerase were phosphorylated in vivo in uninfected cells.

1.2.2.2 Protein phosphorylation and translational control

As with transcription there is a defined role for protein phosphorylation/dephosphorylation only in the acute regulation of overall protein synthesis in reticulocytes. This process occurs at the initiation of translation rather than elongation or termination and was reviewed by Proud (1986). The regulation is mediated by the phosphorylation of eukaryotic initiation factor 2 (eIF-2). In brief, the phosphorylation of eIF-2 prevents its recycling and causes it to sequester another initiation factor, guanine nucleotide exchange factor (GEF, also known as eIF-2B, RF, ERF, ESP1 and Co-eIF-2C), bringing about almost total cessation of peptide chain initiation. How widespread this system is is not known, however there is evidence to suggest that inhibition of peptide chain initiation in HeLa cells in response to heat shock and serum deprivation is controlled in a similar fashion (Duncan & Hershey, 1984, 1985).

1.2.2.3 Protein phosphorylation and control of post-translational modification

Protein phosphorylation is itself a form of post-translational modification, and the direct modulation of enzyme activities is

by far its most common function. However there are also examples of protein phosphorylation/dephosphorylation mechanisms regulating other post-translational modifications. An example of this system is the activation of phosphorylase phosphatase. Protein kinase F_A phosphorylates the phosphorylase phosphatase resulting in its activation (Yang et al., 1980). The active phosphorylase phosphatase can then dephosphorylate rabbit muscle phosphorylase a. This type of cascade regulation is typified by the neural and hormonal control of glycogen metabolism reviewed by Cohen (1982). A well characterized example in prokaryotes is discused in section 1.4.4, the control of an acetylation/deacetylation post-translational control mechanism in Clostridium sphenoides by phosphorylation/dephosphorylation.

1.2.2.4 Protein phosphorylation and the alteration of physical properties

Protein phosphorylation does not only serve to alter the activity of enzymes, it can also be involved in the alteration of the physical properties of proteins. Perhaps the best example of this is the phosphorylation of the light harvesting chlorophyll a/b binding protein (LHCP) in the chloroplast (reviewed by Bennett, 1983 and Allen, 1983). In summary, the 'Z-scheme' model of photosynthesis involves the operation in series of two photochemical reactions in two photosystems (PS1 and PS2) linked by a series of electron carriers. As each photosystem has distinct absorbtion and action spectra, almost any particular wavelength of light will preferentially excite either PS1 or PS2, producing an imbalance in the rates of excitation and thus rendering the photosynthetic quantum yield wavelength-dependent (the optimum yield being at those wavelengths where both systems

are excited to the same extent). In practice this does not occur, the photosynthetic membranes can adjust so as to channel excitation energy into the unfavoured photosystem, resulting in equalization of the excitation rates between PS1 and PS2 regardless of the wavelength of the incident light. The redistribution of excitation energy between the photosystems involves changes in the distribution of LHCP, originaly thought to have a purely light harvesting role for PS2. It is now known that the photosynthetic components are not randomly distributed through the membrane, PS2 components appear to be concentrated in the appressed regions of the thylakoid stacks while the PS1 components are concentrated in the unappressed regions. The distribution of the LHCP, responsible for the harvesting of the excitation energy, is dependent on its phosphorylation state. On phosphorylation LHCP migrates from the appressed regions to the unappressed regions of the thylakoids, possibly due to repulsion between the negative charges on the phosphates, redistributing excitation energy in favour of PS1. The phosphorylation state of LHCP is coupled to the redox state of plastoquinone, an electron carrier between PS2 and PS1. Reduction of plastoquinone induces phosphorylation of LHCP, and as a result the system is self regulating. Thus if the wavelength of light favours PS2, plastoquinone is reduced and LHCP is phosphorylated, migrating to the unappressed (PS1 rich) regions of the thylakoids and increasing excitation transfer to PS1. Conversely if PS1 excitation is favoured plastoquinone becomes oxidized and phosphorylation of LHCP ceases. Net dephosphorylation of LHCP then causes excitation energy transfer back to PS2.

This system appears to hold for green algae and higher plants. What then of prokaryotic photosynthesis? Cyanobacteria have been

shown to regulate excitation energy between PS1 and PS2 and yet they lack LHCP, their light harvesting being done by phycobilins. Sanders et al. (1986) proposed that redox controlled protein phosphorylation of proteins in the phycobilisomes were responsible for excitation energy distribution, with electrostatic decoupling of the phycobilisome from PS2 acting instead of the migration of LHCP. Moreover, they also suggest that the 'lateral shuffling' of LHCP is only a side effect of phosphorylation and the primary effect in all photosynthetic systems is the decoupling of the light harvesting component from PS2.

Holmes et al. (1986) have extended the theory of electrostatic decoupling of light harvesting units from photosystems to the purple photosynthetic bacteria. These bacteria have only one type of photosystem and operate cyclic electron transport (Drews, 1985), so the type of adaptive mechanism to changing spectral quality described above (known as state 1-state 2 transitions) cannot apply. It is known, however, that phosphorylation of suspected light harvesting polypeptides occurs in Rhodobacter sphaeroides, and that this phosphorylation appears to be under redox control. Holmes et al. (1986) suggest that this phosphorylation may have a role in regulating cooperativity (the transferral of excitation energy from one photosynthetic unit to another when the reaction centre of the first is closed, i.e. already in a charge separated state). Such phosphorylation associated with cooperativity changes is also seen in Rs. rubrum (Loach et al., 1984). Increased cooperativity would occur in response to an increase in light intensity, and the net reduction of the ubiquinone pool. Holmes & Allen (1986) demonstrated the phosphorylation of the B880-$ subunit of the light harvesting

pigment-protein complex of Rhodospirillum rubrum under non-cooperative conditions, and phosphorylation of the B880-α subunit under cooperative conditions. They propose that protein phosphorylation plays a major role in the regulation of excitation energy transfer coupling in all photosynthetic systems by the system of electrostatic repulsion outlined above (Allen & Holmes, 1986). Moreover they suggest that the regulation of excitation energy distribution by alterations in the coupling of photosystems through phosphorylation/dephosphorylation is the main control system, and other factors (i.e. thylakoid stacking and lateral heterogeneity of photosystems) are "neither necessary nor sufficient for regulation of excitation energy distribution...".

1.2.3 Protein kinases

Protein kinases are often regulated by specific effector compounds or messengers, and have been classified into five categories on the basis of these interactions (Krebs & Beavo, 1979, Table 1.1). They have been studied in considerably more detail than the phosphoprotein phosphatases. This is probably a reflection of the facts that kinases are easier to purify than the phosphatases, and that kinases appear to have a major role in enzyme activation in eukaryotes, and thus have attracted more interest.

1.2.3.1 cAMP dependent protein kinases

cAMP is an important second messenger in cells. It is generally assumed that in higher organisms cAMP exerts its effects solely by activating protein kinases (Hoppe, 1985). The cAMP-dependent protein kinases (category 1 in table 1.1) can be divided into two

CATEGORY	DESIGNATION	RECOGNIZED EXAMPLE(S)
1	cAMP-dependent protein kinases	Type I and Type II
2	cGMP-dependent protein kinases	see section 1.2.3.2
3	Ca^{2+}-dependent protein kinases	phosphorylase kinase
4	dsRNA-dependent protein kinases	see section 1.2.3.4
5	non-specified protein kinases	many examples

Table 1.1 Classification of protein kinases based on regulation by specific agents (after Krebs & Beavo, 1979)

groups based on their elution from DEAE cellulose, type I and type II. The isoenzymes are both tetrameric complexes which are activated by the dissociation of the dimeric regulatory subunit (R2) on binding cAMP, releasing two monomeric catalytic subunits (C) (Flockhart & Corbin, 1982) with the stoichiometry

$$R2C4 + 4cAMP \rightleftharpoons R2(cAMP)4 + 2C$$

(Hoppe, 1985). The functional distinction between type I and type II cAMP-dependent protein kinases lies in their behaviour towards ATP. Type II kinases, in the presence of ATP, transfer a phosphoryl group from ATP to each monomeric regulatory subunit in an intra-molecular autophosphorylation reaction catalyzed by the catalytic subunits. Type I kinases do not carry out such a reaction, but instead the holoenzyme binds ATP with an unusually high affinity due to the substitution of an alanine residue for the serine residue phosphorylated in type II kinases. The role of these responses may be to stabilize the holoenzyme in the absence of cAMP, i.e. they inhibit kinase activity. After a period of uncertainty (Krebs & Beavo, 1979), it is now thought that cAMP activates the protein kinase by forming an intermediate ternary complex with nucleotide, regulatory subunits and catalytic subunits before the subunits dissociate (Hoppe, 1985) instead of binding to the regulatory subunit after dissociation (Ogez & Segel, 1976, as cited by Krebs & Beavo, 1979).

1.2.3.2 cGMP-dependent protein kinases

Much less is known about the cGMP-dependent protein kinases. Examples studied appear to have similar, but not identical, specificities to cAMP-dependent kinases. Among the best studied

examples are the enzymes obtained from beef lung (Lincon et al., 1978) and the silk worm (Inoue et al., 1976). The beef lung enzyme appears to be a dimer of identical subunits, contains no separate regulatory subunits and does not dissociate on cGMP binding. It contains two cGMP binding domains (binding site 1 and 2) per subunit which differ in their affinity for cGMP (Hofmann et al., 1985). Like the type II cAMP-dependent protein kinases, it is capable of autophosphorylation (deJonge & Rosen, 1977) incorporating 4 molecules of phosphate per subunit onto serine and threonine residues. This phosphorylation interferes with the positive cooperative binding of cGMP to binding site 1 without altering the affinities of either of the binding sites (Hofmann et al., 1985). It does, however, increase the affinity of the 'high'-affinity binding site 1 for cAMP (Landgraf et al., 1986), though the physiological significance of this is not known.

1.2.3.3 Ca^{2+}-dependent protein kinases

The best studied example of a Ca^{2+}-dependent protein kinase is phosphorylase kinase, the key to both the neural and hormonal mechanisms of stimulating gluconeogenesis (see Cohen, 1982). Phosphorylase kinase has a subunit structure of $(\alpha\beta\gamma\delta)_4$. The α-subunit and β-subunit are phosphorylated by cAMP-dependent protein kinase, the δ-subunit is the catalytic subunit and the γ-subunit, which is in fact calmodulin, is the calcium binding subunit. Studies on this enzyme have been instrumental in elaborating the role of protein phosphorylation in the control of cellular activity in response to neural and hormonal stimuli.

1.2.3.4 Double stranded RNA-dependent protein kinases

Double stranded RNA-dependent protein kinases constitute the class about which least is known. Such activities are found in cells treated with interferon (Roberts et al., 1976) and also in erythrocyte lysates (Sen et al., 1978) and are involved with the phosphorylation of at least two polypeptides. Sen et al. (1978) reported that double-stranded RNA was required to activate the kinase, but not for its subsequent continued activity and that the activation was shown to be independent of cAMP or cGMP.

1.2.3.5 Unclassified protein kinases

This category includes those kinases with no known effectors, as well as those that do not fit into any of the above classes. This includes most of the examples from prokaryotes known so far.

1.2.4 Phosphoprotein phosphatases

Compared with protein kinases, very little is known about phosphoprotein phosphatases, as is explained in 1.2.3. In eukaryotes there appear to be a few broad specificity phosphoprotein phosphatases participating in cellular regulation (see Cohen, 1983), termed protein phosphatase-1 (PP-1), -2A and -2C. At least two of these can be regulated by secondary messengers. For example PP-1 activity can be inhibited by cAMP through the action of Inhibitor-1, a protein effective when phosphorylated by cAMP-dependent protein kinase. Prokaryotic protein phosphatases on the other hand display much greater specificity, one example being the isocitrate dehydrogenase phosphatase of *E. coli* (see section 1.4.1.1).

1.3 Zero-order Ultrasensitivity

One aspect of covalent protein modification is worthy of further study, and that is the phenomenon of sensitivity amplification in regulatory covalent modification systems (for a review of amplification in regulatory systems in general see Koshland et al., 1982). In many cases it is necessary to amplify a signal to produce a response that is proportionally much larger than the initial stimulus. There are three recognized sources of ultrasensitivity (defined as an output response that is more sensitive to change than the hyperbolic Michaelis-Menten equation would allow) (Goldbeter & Koshland, 1982). The first is the positive cooperativity of allosteric proteins. Here a small percentage change in an allosteric effector can bring about a larger percentage change in the product generated by an allosteric enzyme by subunit interactions. The second is the multistep effect. This occurs when one effector acts at more than one point in the pathway. The simplest case of this would be where an effector activates an enzyme in the forward pathway and inhibits an enzyme in the reverse pathway. An actual example would be the involvement of cAMP at five different steps in the control of the glycogen cascade (Goldbeter & Koshland, 1982). The third source of ultrasensitivity is zero-order ultrasensitivity. This system of sensitivity amplification was first proposed by Goldbeter & Koshland (1981). It is based on the kinetic characteristics of two, opposing, energy-driven reactions at least one of which must operate outside the first order region (hence zero-order ultrasensitivity). As such this system is especially applicable to covalent modification systems. The way in which this system functions is illustrated in Figs 1.2 and 1.3. These illustrate the velocity curves for two converter

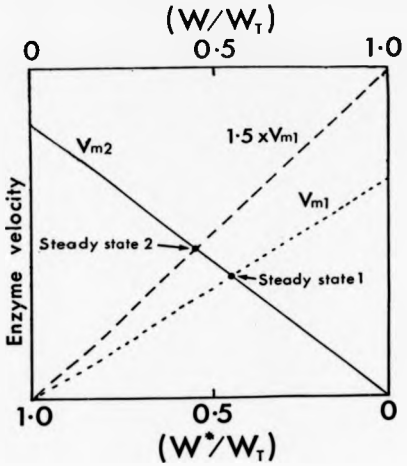


Fig 1.2 The effect on steady-state proportions of the modified (W^*) and unmodified (W) forms of a protein in response to a 1.5-fold increase in the maximum velocity of converter enzyme E_1 when the converter enzymes are both operating in the first-order region.

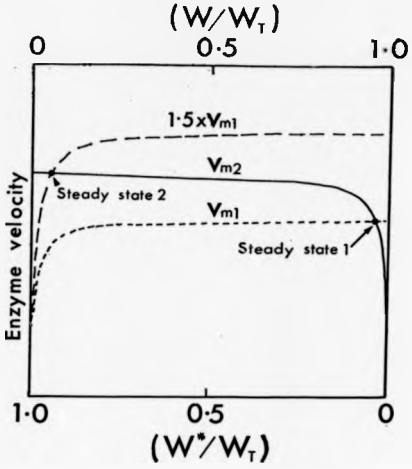


Fig 1.3 The effect on steady-state proportions of the modified (W^*) and unmodified (W) forms of a protein in response to a 1.5-fold increase in the maximum velocity of converter enzyme E_1 when the converter enzymes are both operating in the zero-order region.

enzymes E1 and E2 involved in the interconversion of a substrate protein between its unmodified (W) and modified (W*) forms (one of which will be the active form, the other the inactive form) according to the reaction:

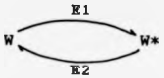


Fig 1.2 illustrates the velocity curves for these converter enzymes assuming both are in the first order region, i.e. not saturated by their substrates. The point at which the two plots intersect represents the steady state concentration of the modified (bottom axis) or unmodified (top axis) form of the substrate. The figure shows that a 50% increase in the maximum velocity of E1 only produces a small increase in the steady state proportion of W*. If, however, we assume that the converter enzymes are saturated by their substrate the velocity curves will show zero-order kinetics. In this case we can see (Fig 1.3) that a 50% increase in the maximum velocity of E1 can bring about a very large change in the steady state proportion of W*. If the activities of the converter enzymes E1 and E2 are regulated (perhaps allosterically) by an effector, it can be seen that a small change in effector concentration will produce a disproportionately large change in the state of modification of W. In this manner a high degree of sensitivity is maintained at the expense of having to maintain opposing energy driven covalent modification reactions. As mentioned above, this system was first proposed by Goldbeter & Koshland (1981), without any evidence for its actual occurence in vivo. The first observation of the reality of such a system came from LaPorte & Koshland (1983) when they demonstrated that the reversible phosphorylation of the

isocitrate dehydrogenase enzyme, a key enzyme in the distribution of isocitrate between the glyoxalate bypass and the TCA cycle (Kornberg & Madsen, 1957), demonstrated zero-order ultrasensitivity; a small change in kinase or phosphatase activity producing a large change in the steady state proportion of isocitrate dehydrogenase phosphorylated, *in vitro*. From this they calculated (from the concentrations of components used) that the effect *in vivo* would probably be even more marked. Subsequently zero-order ultrasensitivity has also been demonstrated in the regulation of glycogen phosphorylase (Meinke et al., 1986) illustrating that isocitrate dehydrogenase is not unique in showing zero-order ultrasensitivity amplification, indeed, Laporte & Koshland (1983) suggest that it may in fact be ubiquitous in nature. Further mathematical analysis of amplification in covalent modification systems showed that the multistep effect and the zero-order effect could act synergistically to enhance the sensitivity gained for either effect in isolation (Goldbeter & Koshland, 1984). It is this combination of sensitivity and fast response times that make covalent regulation systems so effective.

Protein phosphorylation is a central control mechanism for many important processes in eukaryotes, and occupies a key role in the coordination of these processes. In prokaryotes protein phosphorylation is a relatively recent discovery, and its role is still poorly defined. In the next section I shall review prokaryotic protein phosphorylation.

1.4 Prokaryotic Protein Phosphorylation

Protein phosphorylation in prokaryotes is a comparatively recent observation and consequently there are few examples of well defined systems. Much of the work so far has been concerned with the phenomenology of protein phosphorylation in various organisms, and so I shall discuss prokaryotic protein phosphorylation in the context of the organisms, begining with the best studied example, *E. coli*.

1.4.1 Protein phosphorylation in *Escherichia coli*

The earliest evidence for protein phosphorylation in prokaryotes came from Kuo & Greengard (1969). They reported the presence of an enzyme in cell extracts of *E. coli* that could catalyze the phosphorylation of exogenous histones and basic proteins. This report was followed by others (Gordon, 1971; Kurek et al., 1972; Powers & Ginsburg, 1973 as cited by Cozzone, 1984). All these reports, however, allowed no definite conclusions to be drawn due to their failure to completely characterize the phosphorylated moiety of the proteins. Bacteria are known to contain kinases that are stimulated by basic proteins (e.g. the histones used in the assays) and which transfer phosphate to polyphosphate (Li & Brown, 1973) or to proteins forming an acylphosphate bond (Agabian et al., 1972). Bacteria also contain enzymes that form phosphorylated intermediates (most of which are carboxylphosphates or histidylphosphates) as a part of the catalytic mechanism (Walsh & Spector, 1971; Walinder, 1968). In many cases reports of kinase activities were attributed to kinases other than protein kinases.

The first conclusive report of protein kinase activity having a physiological role *in vivo* in prokaryotes came from studies of *E.*

coli infected with bacteriophage T7 (Zillig et al., 1975). It was demonstrated that after infection with T7 bacteriophage the β' and to a lesser extent the β subunits of a DNA-dependent RNA polymerase were phosphorylated by a phage protein kinase. Subsequent work (Ponta et al., 1974; Pfennig-Yeh et al., 1978) demonstrated that the kinases were involved in the control of early gene expression (see section 1.2.2.1). It had been known prior to this that ribosomes of E. coli could be phosphorylated by rabbit skeletal muscle protein kinase (Traugh & Traut, 1972), but this was the first example of an asigned role to the observed kinase activity in vivo. The fact that the kinase activity observed was conferred by a phage-coded protein kinase and that E. coli contained much less phosphoserine and phosphothreonine than eukaryotes led Rubin & Rosen as recently as 1975 to conclude that there was no protein kinase activity in uninfected prokaryotic cells.

In recent years the whole aspect of bacterial protein phosphorylation has been re-investigated with much attention being paid to the characterization of the phosphorylated moiety of the proteins. Manai & Cozzone (1979a) first demonstrated the presence of phosphoproteins in E. coli in vivo after cells were grown in the presence of ^{32}P-orthophosphate. They found four polypeptides to be phosphorylated in the soluble fraction (Mr 12,000, 13,000, 35,000 and 49,000) and one (Mr 68,000) in the ribosomal fraction. Phosphorylation occured on serine and threonine residues. Manai & Cozzone (1979b) expanded these observations to include crude ribosomal preparations. They observed the four major bands described above and several fainter ones. All five bands were observed in crude ribosomal preparations. Phosphoserine was the predominant phosphoamino acid

in the soluble fraction and phosphothreonine was predominant in the ribosomal fraction. These were the first conclusive observations of protein phosphorylation in E.coli. The first identification of the endogenous substrate for a protein kinase came from Garnak & Reeves (1979) when they observed that the addition of acetate to a stationary phase culture of E. coli grown in the presence of ^{32}P-orthophosphate resulted in the incorporation of radiolabelled phosphate into the enzyme isocitrate dehydrogenase concomitant with a loss of isocitrate dehydrogenase activity. The study of isocitrate dehydrogenase regulation by reversible phosphorylation has proved to be a major field of study, and so it shall be considered separately in detail in a subsequent section (1.4.1.1). Manai & Cozzone (1982a) went on to analyze the protein kinase activity of E. coli cell free extracts and their ability to phosphorylate endogenous proteins. They suggested that previous failures to demonstrate protein phosphorylation in prokaryotes were due to the use of exogenous proteins as substrates for the kinase reactions. The standard procedure in eukaryotes was to look for the phosphorylation of added histone, casein or phosvitin by the extract at the expense of adenosine triphosphate. It has since been demonstrated that bacterial kinases do not phosphorylate these proteins (Manai & Cozzone, 1979a; Wang & Koshland, 1978); so to detect bacterial protein kinases in vitro, phosphorylation of endogenous proteins at the expense of ATP must be demonstrated. Manai & Cozzone (1982a) demonstrated the presence of kinase activities phosphorylating polypeptides of Mr 55,000, 51,000 and 45,000 with minor components of Mr 37,000 and 35,000 and other very faint bands. Preparations of crude ribosomes revealed the presence of a Mr 51,000 component and minor

components of Mr 55,000 and 37,000 all of which are lost on salt washing of the ribosomes suggesting that they are not part of the actual structure. The only phosphoamino acid observed was phosphoserine and it was demonstrated that the proteins were phosphorylated directly at the expense of ATP, and that there was no cAMP-dependent kinase activity. The fact that different subcellular fractions demonstrated different protein kinase activities was used as evidence for the presence of multiple kinases with different intracellular locations in E. coli, rather than a small number of enzymes with broad substrate specificity. This conclusion was probably premature as the proteins phosphorylated in the ribosome fractions appear to be a subset of those phosphorylated in the cytoplasm, so the differences could be attributed to differences in the substrates available rather than kinase specificities. One important observation was that the polypeptides phosphorylated in vitro showed considerable discrepancies in Mr from those phosphorylated in vivo. In addition discrepancies were observed in the behaviour of the major phosphorylated ribosomal components on salt washing (unlike the major Mr 51,000 component in vitro, the Mr 68,000 component in vivo is not released on salt washing) and the nature of the amino acids modified (phosphoserine, phosphothreonine and phosphotyrosine are all observed in vivo). The reason for these discrepancies is not known. Manai & Cozzone (1983) carried out a study of the phosphoamino acids present in various fractions of extracts prepared from E. coli cells grown in the presence of ^{32}P-orthophosphate. They demonstrated the presence of phosphoserine, phosphothreonine and phosphotyrosine, but the proportions of each differed considerably between the fractions (cytoplasmic, ribosomal and salt washed ribosomes). In addition

it was shown that the Mr 65,000 phosphopolypeptide, the major phosphopolypeptide detectable in the ribosomes *in vivo*, was phosphorylated exclusively on tyrosine residues by exploiting the property of resistance of phosphotyrosine to alkaline hydrolysis. The second phosphoprotein to be identified in *E. coli* was the DnaK protein (Zylicz et al., 1983). The DnaK protein has been shown to be essential for bacteriophage lambda DNA replication, bacterial growth and for bacterial DNA and RNA metabolism. Zylicz et al. (1983) reported that it possesed a weak DNA-independent ATPase activity that could be modulated *in vitro* by lambda O and P replication proteins and that it was phosphorylated *in vitro* and *in vivo*, apparently as a result of an autophosphorylation reaction. Analysis of the phosphoamino acids present in the phosphoprotein revealed phosphotyrosine, and it was proposed that the phosphorylation of the DnaK protein may have a role in the regulation of its activity (though the nature of this activity was, at that time, unknown) or some other intracellular enzyme activity. Subsequently, Wada et al. (1986) demonstrated that the DnaK and DnaJ proteins were involved in the phosphorylation of glutaminyl-tRNA synthetase and threonyl-tRNA synthetase. Further studies of protein phosphorylation in *E. coli* were carried out by Enami & Ishihama (1984). They examined the phosphoprotein patterns at various points in the growth cycle, specifically during log phase, stationary phase and during the transition between these two. They observed five predominant species of Mr 84,000, 74,000, 66,000, 56,000 and 45,000 after a short period of ^{32}P-orthophosphate labelling, with the Mr 56,000 species having the highest rate of incorporation of radioactive label. Over a longer period other species were also labelled with Mr values of 90,000, 76,000, 73,000 and 61,000. Qualitative differences

between the different growth phases could be seen, with the Mr 76,000 polypeptide being predominant in the log phase culture but the Mr 90,000 or 45,000 polypeptide being predominant in the stationary phase culture. Prolonged exposure revealed phosphorylated polypeptides corresponding to the stained bands of the β and β' subunits of a DNA-dependent RNA polymerase, and this labelling of the RNA polymerase was later confirmed by immunoprecipitation. Phosphorylation of the σ subunit was also seen. Analysis of kinase activities in cell extracts in vitro revealed the phosphorylation of a number of polypeptides that appeared (by Mr values) to be a subset of those observed in vivo, and different kinase activities in the log phase and the stationary phase extracts. Further studies resulted in the purification of a Mr 120,000 protein kinase capable of phosphorylating a Mr 90,000 protein in vitro. This was tentatively identified as the enzyme isocitrate dehydrogenase kinase. A second kinase detected was a Mr 100,000 protein with the capacity to undergo autophosphorylation. It was suggested that this may be an intermediate of an enzyme involved in DNA metabolism. One of the most significant factors in these observations is the differences in the Mr values of observed phosphopolypeptides between Enami & Ishihama (1984) and Manai & Cozzone (1979b and 1982) (table 1.2). One possible explanation is the use of different strains of E. coli by the two groups, CP78 by Manai & Cozzone and W3350 by Enami & Ishihama. A second alternative would be that the pattern of phosphopolypeptides observed was dependent on the growth conditions to which the cells are exposed. There is a known precedent for the latter, as cells grown on acetate phosphorylate the enzyme isocitrate dehydrogenase and it is not unreasonable to suppose that other

CP78	:	W3350
17,000	:	
	:	20,000
35,000	:	
38,000	:	
39,000	:	
44,000	:	45,000
54,000	:	
54,100	:	56,000
	:	61,000
65,000	:	66,000
72,000	:	73,000
74,000	:	74,000
	:	76,000
83,000	:	84,000
94,000	:	90,000

Table 1.2 A comparison of the Mr values of phosphopolypeptides identified in the E. coli strains CP78 (after Cozzone, 1984) and W3350 (from Enami & Ishihama, 1984). Phosphopolypeptides of similar Mr values are paired.

medium components could produce similar changes.

Desmarquets et al. (1984) catalogued the phosphopolypeptides produced by the CP78 strain by their molecular weights and isoelectric points on two-dimensional O'Farrell gels (O'Farrell, 1975). Their analysis revealed 12 phosphopolypeptides, 10 cytoplasmic, 1 ribosomal and 1 both cytoplasmic and ribosomal, with Mr values showing increased correspondence to those observed in the K12 strain used by Enami & Ishihama (1984) and decreased correspondence with the earlier work of Manai & Cozzone (1979b). The differences in the pattern of phosphorylated polypeptides obtained from the CP78 strain between the work of Manai & Cozzone (1979b) and Desmarquets et al. (1984) is possibly due to the use of slightly differing growth conditions. In particular, the work of Desmarquets et al. used a highly defined medium with cells grown on acetate as the sole carbon source. Under these conditions the enzyme isocitrate dehydrogenase is phosphorylated and Desmarquets et al. claim that the polypeptide of Mr 44,000 pHi 5.3 is the isocitrate dehydrogenase subunit. Recently Cortay et al. (1986b) have carried out detailed two-dimensional analysis of the phosphopolypeptides present in the CP78 strain of E. coli. They reported that an estimated 10% of all the polypeptides in the organism were phosphorylated. Dadsii & Cozzone (1985) proved the cAMP-independence of this phosphorylation by utilizing adenylate cyclase deficient mutants.

Quentmeir & Antranikian (1986) studied protein phosphorylation in E. coli cells grown anaerobically with nitrate as an electron acceptor. They used a K12 strain DSM498 and identified 10 phosphopolypeptides with Mr's again quite different from those obtained by Enami & Ishihama (1984) and Desmarquets et al. (1984) (Table 1.3). These differences were again attributed to the very

Protein	Mr	Isoelectric point	Alkaline phosphatase	Phospho-diesterase
a	52,000	5.6	+	
b	52,000	5.7		
c	52,000	5.8		
d	52,000	6.0	+	
e	45,000	5.6		
f	41,000	5.6		
g	41,000	5.8	+	
h	38,000	5.8	+	
i	45,000	6.0	+	
j	46,000	6.2		
k	44,000	6.4		
l	43,000	6.4		
m	35,000	6.3	+	
n	37,000	6.4		
o	32,000	6.4		+
p	32,000	6.6		(+)
q	31,000	6.2	+	
r	29,000	6.2	+	+
s	26,000	6.3	+	
t	21,000	5.6	+	

Table 1.3 Mr values and isoelectric points of ^{32}P-labelled proteins from E. coli grown anaerobically with nitrate as an electron acceptor (after Quentmeier & Antranikian, 1986)

different growth conditions used. Cortay et al. (1986a) reported that the phosphorylation of the Mr 54,500 polypeptide found in the cytoplasmic and ribosomal fractions of E. coli strain CP78 by Desmarquets et al. (1984) occurred on tyrosine residues. They also reported finding a polypeptide with identical Mr and pHi in E. coli strains CP79 and AB1111, and Salmonella typhimurium, and advanced the theory that this may be a common polypeptide among bacteria.

There has, then, been considerable work on protein phosphorylation carried out in Escherichia coli. The majority of this work has been cataloguing the patterns of phosphopolypeptides produced by different strains under different growth conditions, and very few of the phosphopolypeptides found have actually been identified. The reason for this is probably that although many phosphorylated polypeptides can be seen in the cell (Enami & Ishihama (1984) claimed to be able to see 40 different species, and Cortay et al. (1986) estimate that 10% of the proteins in the cell could be phosphorylated), this is still only a small proportion of the total number of species. Neihardt et al. (1983) resolved 1100 different proteins from bacterial extracts on O'Farrell gels. This combination of a large number of bands being still only a fraction of the total number of proteins means that it will be very difficult to identify proteins by their position on polyacrylamide gels alone. On this basis the only real ways the substrates of the kinases have been identified are:

1) By examination of known, easily isolated proteins or cell components for phosphorylation (e.g. DNA-dependent RNA polymerase

or proteins present in ribosomal fractions). These have often been well characterized in the past and are thus easily identified. This method has been frequently used.
2) By groups working on a particular protein or enzyme system identifying phosphorylation, and possibly its function, in their system. Examples of this are the discovery of the phosphorylation of the DnaK protein (Zylicz et al., 1983) and isocitrate dehydrogenase (Garnak & Reeves, 1979a). Much of the work done on protein phosphorylation in E. coli has in fact been carried out on this enzyme and its kinase/phosphatase. This work will be considered in the following section.

1.4.1.1 NADP$^+$-Isocitrate dehydrogenase phosphorylation in E coli

The control of E. coli NADP$^+$-isocitrate dehydrogenase activity by phosphorylation/dephosphorylation (see Nimmo, 1984) is the only phosphorylation system in prokaryotes in which the understanding of the molecular details is comparable to that of many eukaryotic systems. NADP$^+$-isocitrate dehydrogenase (ICDH) catalyzes a key step in the tricarboxylic acid (TCA) cycle converting isocitrate to α-ketoglutarate. Under normal growth conditions there is no reason to believe that ICDH plays a significant role in regulating flux through the TCA cycle. During growth on acetate, however, the glyoxalate bypass comes in to operation (Kornberg & Madsen, 1957) (Fig 1.4). The glyoxalate bypass allows the cell to utilize two-carbon compounds as carbon sources and is required as a result of the need for net biosynthesis of carbon skeletons. The glyoxalate bypass results in the synthesis of 2 four-carbon compounds from 1 molecule of oxalacetate and 2 molecules of acetate. 1 succinate is produced

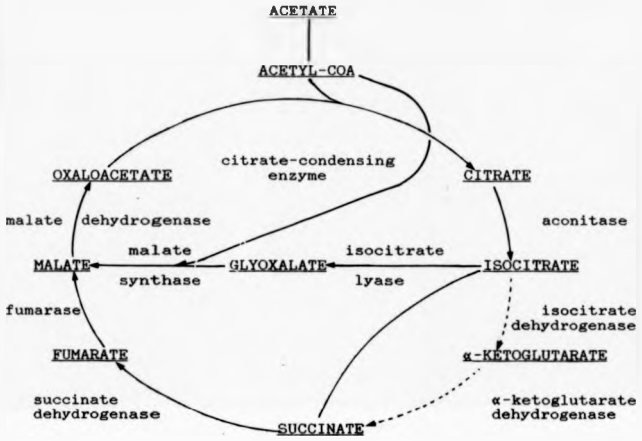


Fig 1.4 The TCA cycle and glyoxalate bypass. Dashed lines indicate reactions not occurring in the glyoxalate bypass.

from the splitting of isocitrate (produced via the TCA cycle from oxalacetate and acetate) into succinate and glyoxalate by isocitrate lyase, and 1 malate is produced from the combination of the glyoxalate produced with the second molecule of acetate by malate synthase. During growth on acetate, then, this mechanism results in competition between isocitrate lyase and ICDH for isocitrate. Holms & Bennett (1971) first reported the substantial loss of activity of ICDH on growth on acetate. They further demonstrated (Bennett & Holms, 1975) that the inactivation was rapidly and readily reversed by the addition of various metabolites (e.g. pyruvate, malate, glucose) which are products of the glyoxalate bypass or are metabolized more readily than acetate to yield such products. These compounds have been shown to inhibit the glyoxalate bypass at isocitrate lyase (Kornberg, 1966). On restoration to an acetate medium the activity of ICDH again fell. The changes observed were rapid and occurred in the absence of protein synthesis and this, along with the absence of any detectable low molecular weight effectors, led Bennett & Holms (1975) to propose reversible covalent modification as one possible mechanism for the regulation.

The first evidence implicating phosphorylation in the control mechanism came from Garnak & Reeves (1979a). They observed that concomitant with a loss of isocitrate dehydrogenase activity on growth on acetate there was incorporation of ^{32}P orthophosphate in to the enzyme. This was the first identification of a substrate for a prokaryotic protein kinase. Subsequent work (Garnak & Reeves, 1979b) confirmed this observation and estimated the Mr of the enzyme subunit at 51,000. Immunological techniques were used to confirm that the phosphorylated and non phosphorylated forms of the enzyme were identical and partial

acid hydrolysis permitted the identification of phosphoserine.

The purification of the ICDH kinase and phosphatase was achieved by LaPorte & Koshland (1982). The kinase was purified by ammonium sulfate fractionation, ion exchange chromatography and affinity chromatography on ICDH immobilised on sepharose 4B. When they tried to purify the phosphatase they found that the steps required were identical and that the ratio of the kinase activity to phosphatase activity remained constant. On trying to separate the two activities they found that the they were always superimposable and therefore suggested that the they existed on the same polypeptide chain (though other explanations were not excluded). It was also discovered that the phosphatase activity had an absolute requirement for ATP or ADP, with ATP being the superior substrate (thus eliminating the possibility that the phosphatase activity is merely a back reaction of the kinase activity). There are precedents for the location of opposing activities on the same protein. For example, the enzymes responsible for the adenylation-deadenylation of glutamine synthase in E. coli are located on the same polypeptide chain (Caban & Ginsburg, 1976; Rhee et al., 1978). LaPorte & Koshland (1982) suggested that this is not an evolutionary accident, and may have evolved to allow simultaneous regulation of both activities from a single site on the protein. They also speculate that the phosphatase site may have arisen as a result of an imperfect gene duplication of, and fusion with, the kinase site. The cAMP-independence of the E. coli ICDH kinase was demonstrated (Malloy & Reeves, 1983) by the use of adenylate cyclase deficient mutants. LaPorte & Koshland (1983) established the stoichiometry of the phosphorylation reaction at 1 mole of phosphate per mole of ICDH monomer, and reported that the phenomenon known as

zero-order ultrasensitivity could be demonstrated in the reversible phosphorylation of ICDH (see section 1.3). Garland & Nimmo (1984) compared the active and inactive forms of ICDH, especially as regarding their susceptibility to proteases in the presence and absence of $NADP^+$. They found that $NADP^+$ could protect active (unphosphorylated) ICDH from attack by several proteases. In contrast, inactive (phosphorylated) ICDH was much less susceptible to protease digestion, and was unable to bind $NADP^+$. They concluded from this and other data that $NADP^+$ binding and phosphorylation induced similar conformational states and proposed that the phosphorylation site occured close to, or at, the $NADP^+$ binding sites. This hypothesis allowed them to suggest explanations to several of the properties of the phosphorylation system. Firstly, the observation that phosphorylation of ICDH reduced the maximum catalytic activity of the enzyme almost to zero could be explained by charge repulsion between a negatively charged phosphate group in the $NADP^+$ binding site and the $NADP^+$ coenzyme. Secondly the phosphate group could induce a conformational change in the same way as the negatively charged coenzyme does on binding. This would explain the effects of $NADP^+$ and phosphorylation on susceptibility to proteolysis. Thirdly, this would also explain the failure to reactivate inactive ICDH by proteolysis. This hypothesis could also explain the observation of Nimmo & Nimmo (1984) that ICDH kinase is inhibited by $NADP^+$ and NADH. The binding of the coenzyme to ICDH could block the phosphorylation site. Further circumstantial evidence for this theory was presented by Borthwick et al.(1984b) when they determined the amino acid sequence around the site of phosphorylation in ICDH. They discovered that the sequence was dissimilar to sites phosphorylated by cAMP-dependent protein

kinases, but contained a sequence identical to one found close to the NADP-binding site of chicken liver dihydrofolate reductase.

Borthwick et al. (1984a) purified the active and inactive forms of ICDH from E. coli ML308. They found that they could separate a partially active and an inactive form by chromatography on procion-red-Sepharose and elution with $NADP^+$. The partially active form was found to consist of a mixture of active and inactive forms, and all three forms were demonstrated to be identical by the criterion of one-dimensional peptide mapping. The inactive form was shown to be identical to the active form apart from the addition of one phosphate group per subunit. The inability of the inactive form to bind to procion-red-Sepharose further supports Garland & Nimmo's (1984) hypothesis that the inactive form is inactive because it is unable to bind its cofactor. Borthwick et al. (1984a) drew attention to the fact that they were unable to detect any monophosphorylated species of the ICDH dimer. Their theoretical explanation for this is that the partially active form of ICDH actually consists of unphosphorylated dimers, and monophosphorylated dimers that disproportionate into unphosphorylated and diphosphorylated dimers on electrophoresis and point to a precedent for this in the disproportionation of partially phosphorylated rabbit muscle glycogen phosphorylase (Fischer et al., 1968, as cited by Borthwick et al., 1984a). Nimmo et al. (1984) purified the kinase and phosphatase from the ML308 strain and confirmed the observation of LaForte & Koshland (1982) that a single protein bore both activities. In addition they demonstrated that ICDH could be interconverted in vitro between the active and inactive form by the kinase/phosphatase, and that the phosphatase was tenfold less active than the kinase in the assay system they were

using. Nimmo & Nimmo (1984) examined the regulatory properties of the kinase/phosphatase in the ML308 strain. They found that the kinase activity could be inhibited by DL-isocitrate, ADP, AMP, NADPH, phosphoenolpyruvate (PEP) and some other effectors. The phosphatase activity was shown to be inhibited by NADPH and stimulated by AMP, oxaloacetate, pyruvate, PEP, 2-oxoglutarate and DL-isocitrate. It can be seen that many of the metabolites that inhibit the kinase stimulate the phosphatase, the most significant from a regulatory standpoint probably being AMP, ADP, isocitrate, PEP and NADPH. The suggestion was that on growth on acetate phosphorylation of ICDH rendered it rate limiting in the TCA cycle. As a result isocitrate accumulates and this increases the flux through isocitrate lyase (El-Mansi et al., 1985), which has a K_m for isocitrate at least 1000-fold lower than ICDH, thus allowing the glyoxalate bypass to function. Addition of another carbon source would cause a further rise in the levels of isocitrate, stimulating the ICDH phosphatase and allowing more flux through the TCA cycle. In addition carbon sources that do not require the operation of the glyoxalate bypass induce the dephosphorylation of the ICDH. This dephosphorylation appears to be stimulated by pyruvate (El-Mansi et al., 1986). Nimmo & Nimmo (1984) thus suggest that the function of the phosphorylation and dephosphorylation of ICDH is to optimise the division of carbon flux between the glyoxalate bypass and the TCA cycle. LaPorte et al. (1985) discuss the ICDH regulatory system in terms of its ability to detect and adapt to widely differing conditions within the cell. They describe how when the levels of ICDH differ markedly between strains, the phosphorylation system compensates for this by maintaining constant ICDH activity. They describe two methods for regulation of the extent of phosphorylation of ICDH.

The first is the modulation of the kinase and phosphatase activities by allosteric effectors that are sensitive to the total levels of ICDH, e.g. isocitrate (as described above). The second mechanism is dependent on the inherent kinetic parameters of the modifying enzymes. During log phase growth on acetate, ICDH is extensively phosphorylated. As a result the kinase is operating in the first order region and the phosphatase in the zero order region (being saturated with its substrate). consequently the velocity of the phosphatase is independent of its substrate concentration over a wide range. This results in a nearly constant steady state concentration of ICDH, while the concentration of phospho-ICDH varies. Thus any change in the overall level of both forms of ICDH is not reflected in the amount of ICDH activity.

LaPorte et al. (1985) located the gene for the ICDH kinase/phosphatase (aceK) in the glyoxalate bypass operon downstream from the genes coding for isocitrate lyase and malate synthase. They also found that ICDH kinase/phosphatase-deficient mutants were capable of reverting to growth on acetate by second site mutations that did not map to any of the genes for the enzymes involved in this branch point. This , they thought, could be indicative of another, unidentified, mechanism of control. The gene coding for ICDH kinase/phosphatase was cloned by LaPorte & Chung (1985). They discovered that both activities were encoded on an 1,800 base pair sequence, which being sufficient for only one Mr 66,000 polypeptide is strong evidence for both activities being located on the same polypeptide chain (rather than two very similar but non-identical subunits of the holoenzyme). Their working model for the ICDH kinase/phosphatase speculates that the two opposing reactions occur at independent sites, and that the

phosphatase domain was produced by an imperfect partial gene duplication of the primordial kinase resulting in a fused product with the duplicate site allowing the access of water. This theory suggests certain problems for the primaeval organism. Isocitrate lyase has a much lower affinity for isocitrate than does ICDH. As a result, some form of regulatory system for ICDH is required to permit growth on acetate. If the kinase and phosphatase evolved sequentially (as the above theory suggests) the kinase must have evolved first as there would have been no selective pressure for a phosphatase without the prior existence of the kinase. The eqilibrium constant of the kinase favours phosphorylation (Nimmo et al., 1984) and as a result flux through the TCA cycle would be almost totally diverted through the glyoxalate bypass. The result would be a shortfall in energy supply. Holms (1986) considered this and hypothesized an alternative cycle, the glyoxalate oxidation cycle (Fig 1.5). This cycle oxidizes glyoxalate by way of three enzymes found in the modern organism, malate synthase, malic enzyme and pyruvate dehydrogenase in a cyclic process capable of generating the same reducing power per cycle as flux through the TCA cycle. Thus the proposition is that evolution of the modern organism could have involved a stage in which the ICDH kinase shut off most of the flux through the TCA cycle. They go further and suggest the possibility that the glyoxalate-oxidizing cycle antedates the TCA cycle. Evidence for a role for the glyoxalate oxidation cycle in the modern organism comes from mutants isolated (LaPorte et al., 1985) that are capable of growing on acetate despite having only 2.5% of the ICDH activity necessary to sustain growth via the glyoxalate bypass and the TCA cycle. This suggests some way of generating reducing power from acetate independent of the TCA cycle.

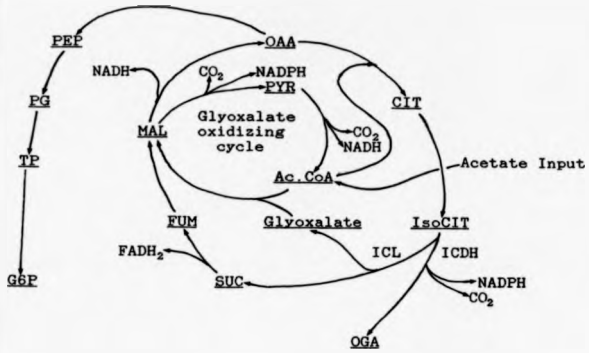


Fig 1.5 The Glyoxalate oxidizing cycle proposed by Holms (1986). Ac.CoA, acetylcoenzyme-A; CIT, citrate; FUM, fumarate; G6P, glucose 6-phosphate; ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; IsoCIT, isocitrate; MAL, malate; OAA, oxaloacetate; OGA, 2-oxoglutarate; PEP, phosphoenolpyruvate; PG, phosphoglycerate; PYR, pyruvate; SUC, succinate; TP, triose phosphate. After Holms (1986).

1.4.2 Protein phosphorylation in Salmonella typhimurium

Salmonella typhimurium was the first prokaryotic organism in which protein phosphorylation was definitively identified in the absence of phage infection (Wang & Koshland, 1978). At least four polypeptides were shown to be phosphorylated in vivo with apparent molecular weight on SDS polyacrylamide gels Mr 88,000, 53,000, 48,000 and 45,000. The same four polypeptides could be labelled in vitro, along with an additional polypeptide of Mr 63,000 in a magnesium and ATP-dependent reaction. Phosphoamino acid analysis of the Mr 53,000 and 45,000 polypeptides revealed phosphoserine and phosphothreonine respectively. Subsequent work (Wang & Koshland, 1981) expanded the number of known phosphopolypeptides to 10 and identified at least four protein kinases and more than one phosphatase. Extracts were fractionated and fractions were tested for the ability to phosphorylate known polypeptides in vitro. None of the phosphorylation reactions observed were cAMP-dependent. The Mr 45,000 polypeptide was later sized at 46,000, and identified as isocitrate dehydrogenase (Wang & Koshland, 1982). It was discovered that as well as the activity, the synthesis of the ICDH kinase and possibly the phosphatase were regulated by environmental conditions.

1.4.3 Protein phosphorylation in Streptococci

The first evidence for protein phosphorylation in Streptococci came from studies on the mechanism of inducer expulsion in Streptococcus pyogenes (Reizer et al., 1983). Inducer expulsion is a system whereby bacteria can selectively accumulate a preferable carbon-energy source whilst expelling the less favourable source in order to avoid futile energy consumption and

inducible enzyme synthesis. Inducer expulsion is distinct from inducer exclusion, as the former involves expulsion of the less favourable source from the intracellular pool while the latter reduces the intracellular concentration only by inhibition of further uptake. It was found that phosphorylation of a Mr 10,000 polypeptide occured concomitant with glucose-elicited inducer expulsion of methyl-ß-D-thiogalactopyranoside (TMG, a non metabolizable ß-galactoside analogue). Deutscher & Saier (1983) identified the phosphopolypeptide as the HPr phosphate carrier protein of the phosphotransferase system (PTS). The PTS of S. pyogenes can be described by the reaction scheme shown in Fig 1.6. Phosphate is transferred from phosphoenolpyruvate (PEP) on to a histidyl residue of enzyme I of the system. Enzyme I transfers the phosphoryl group on to a histidyl residue of HPr which then transfers its phosphate group via the sugar-specific enzyme II to a sugar. It was shown that HPr, as well as being phosphorylated on a histidyl residue could be phosphorylated on a seryl residue in an ATP-dependent process (Deutscher & Saier, 1983). This phosphate could not be transfered to a sugar, and the phosphorylation/dephosphorylation reaction was controlled by a Mr 20,000 protein kinase and a Mr 70,000 protein phosphatase. The kinase activity was shown to be stimulated by glycolytic intermediates, and it was postulated that the inhibition of sugar uptake by intracellular carbohydrate phosphates (Dills et al., 1980) could be a consequence of the phosphorylation on serine of HPr. Reizer et al. (1984) examined the properties of the HPr kinase. It was found that PEP-dependent phosphorylation of HPr on histidine strongly inhibited the ATP-dependent phosphorylation on the seryl residue by the kinase. The kinase was demonstrated to be dependent on divalent cations (Mg^{2+} and Mn^{2+} conferring

$$\text{PEP} + \text{enzyme I} \xrightleftharpoons{Mg^{2+}} \text{P-enzyme I} + \text{pyruvate} \qquad (1)$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I} \qquad (2)$$

$$\text{P-HPr} + \text{sugar} \xrightarrow[\text{enzyme III, } Mg^{2+}]{\text{enzyme II}} \text{sugar-P} + \text{HPr} \qquad (3)$$

Fig 1.6 The phosphotransferase system of Streptococcus faecalis. Enzyme II is a sugar-specific membrane component, enzyme III is enzyme II specific.

greatest activity) and to have a requirement for one of several intermediates of glycolysis or the pentose phosphate pathway. Waygood et al. (1986) showed that the HPr of Streptococcus salivarius was phosphorylated in vitro at the expense of ATP. Both acid stable and acid labile groups were phosphorylated. This is probably due to a phosphoryl exchange reaction between phosphoenolpyruvate and ATP (Matoo et al., 1984). No role was identified for this phosphorylation. Reizer et al. (1984) proposed that the ATP-dependent phosphorylation of HPr was involved in the regulation of carbohydrate transport and metabolism. However, the exact nature of this role will be hard to establish until the effect of ATP-dependent phosphorylation on the function of HPr in the phosphotransferase system has been determined.

1.4.4 Protein phosphorylation in Clostridia

The demonstration of protein phosphorylation in Clostridium sphenoides was the first example of protein phosphorylation in a strictly anaerobic organism (Antranikian et al., 1985a). 15 polypeptides were phosphorylated with apparent molecular weights ranging from Mr 10,000 to Mr 82,000 (including a Mr 53,000 and Mr 56,000). Antranikian et al. (1985b) identified one of the phosphoproteins as citrate lyase ligase. Citrate lyase is the enzyme responsible for the breakdown of citrate (Fig 1.7) into oxaloacetate and acetate. In some organisms (including Clostridium sphenoides and Rhodocyclus gelatinosus) a regulatory system is required for the citrate lyase as it competes with another enzyme, citrate synthase, for citrate. Citrate synthase is required for the biosynthesis of L-glutamate in the absence of substrate amounts of citrate. The main regulatory system present

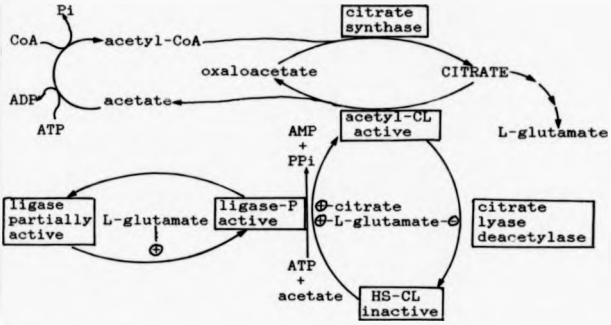


Fig 1.7 The regulation of citrate metabolism in Clostridium sphenoides by phosphorylation and acetylation, based on Antranikian et al. (1985). HS-CL, citrate lyase (sulfhydryl form); acetyl-CL, citrate lyase (acetyl form).

is an acetyletion/deacetylation system. On exhaustion of citrate a futile cycle is avoided by the deacetylation of citrate lyase, inactivating it. There is, however, a second level of control. The cycle of acetylation/deacetylation is halted by the deactivation of citrate lyase ligase. Antranikian et al. (1985a) demonstrated that the regulation of citrate lyase ligase activity was mediated by a phosphorylation/dephosphorylation reaction. Citrate lyase ligase exists in two forms, a partialy active form and a fully active form. The fully active form was found to be phosphorylated and the partially active dephosphorylated. Though the kinase and phosphatase for this reaction were not isolated, it was suggested that the stimulation of citrate lyase ligase activity by L-glutamate could occur by the direct modulation of the activities of the kinase or phosphatase. Thus it appears that protein phosphorylation/dephosphorylation could be at the root of an important prokaryotic regulatory cascade.

A second species of Clostridium known to phosphorylate proteins is the thermophile and obligate anaerobe Clostridium thermohydrosulfuricum (Londesborough, 1986). It was found that there were several polypeptides phosphorylated in vivo and in vitro, with Mr values ranging from Mr 13,000 to Mr 100,000. All but one of the polypeptides labelled in vitro were labelled in vivo, along with a number of additional polypeptides. Phosphorylation of one polypeptide (Mr 30,000) could be supressed by the heat treated soluble fraction of the cell extract. This suggests the presence of a heat stable protein kinase inhibitor, such as is often found in animals (Krebs & Beavo, 1979). On testing for modulation of the kinase activities in vitro it was found that phosphorylation of a Mr 53,000 polypeptide could be strongly enhanced by micromolar concentrations of fructose

1,6-bisphosphate and glucose 1,6-bisphosphate. Hexose monophosphates, however, inhibited phosphorylation suggesting the possibility of a physiological role. Possibly more interestingly, it was shown that micromolar concentrations of brain calmodulin could suppress the phosphorylation of three polypeptides (including the Mr 30,000 polypeptide). Londesborough (1986) hypothesized that C. thermohydrosulfuricum contained a calmodulin-like protein involved in the regulation of phosphorylation that was sufficiently similar to brain calmodulin for the latter to be weakly recognized in the bacterial system. Calmodulin-like proteins are not unknown in prokaryotes, having been shown to exist in Escherichia coli (Iwasa et al., 1981), Myxococcus xanthus (Inouye et al., 1983), the cyanobacteria Oscillatoria limnetica (Kerson at al., 1984) and in Bacillus subtilis (Fry et al., 1986), so this explanation can not be discounted. The fact that two of the three polypeptides inhibited in phosphorylation are phosphorylated on tyrosine residues (the third is as yet undetermined) suggests that the calmodulin could be exerting its effect through the modulation of a single tyrosine kinase or phosphatase activity.

1.4.5 Protein phosphorylation in Myxococcus xanthus

Komano et al. (1982) first demonstrated protein phosphorylation in Myxococcus xanthus. They showed that 6 bands on SDS-polyacrylamide gels were labelled after in vivo exposure to ^{32}P-orthophosphate. Five of these bands were confirmed as proteins by their susceptibility to pronase. No phosphoamino acid determination was attempted and no distinction was made between acyl- and ester-linked phosphate bonds, though two of the phosphopolypeptides could be phosphorylated in vitro at the

expense of ATP. Interestingly, these same two phosphopolypeptides were shown to be produced only on the induction of spore formation and Komano et al. (1982) suggested that protein phosphorylation may be required for spore formation. This was the first suggestion that protein phosphorylation could be implicated in microbial differentiation.

1.4.6 Protein phosphorylation in Sulfolobus acidocaldarius

acidocaldarius, a sulphur-dependent archaebacterium, was shown to contain phosphorylated proteins by Skorko (1984). Four phosphopolypeptides of Mr 71,000, 52,000, 42,000 and 31,500 could be detected, none of which were ribosomal or DNA-dependent RNA polymerase components. The amount of kinase activity in the organism was growth phase dependent, being low in log phase and increasing to a higher constant level in stationary phase. In vitro labelling experiments gave rise to only two phosphopolypeptides of Mr 46,000 and 42,000, the reaction being optimal in the presence of divalent cations. Phosphoamino acid analysis of the in vivo labelled polypeptides suggested the presence of phosphoserine and phosphothreonine.

1.4.7 Protein phosphorylation in Caulobacter crescentus

Hodgson et al. (1985) examined the phosphorylation of proteins in Caulobacter crescentus after infection with the DNA phage φCd1. About 40 ^{32}P phosphate-labelled polypeptides were detected on SDS-polyacrylamide gels. Two major polypeptides (Mr 94,000 and 50,000) were labelled before infection with the phage, but these were later shown to be acyl-phosphate linkages (possibly phosphorylated by the phosphoacyl kinase reported by Agabian et al. (1972). However, two minor polypeptides (Mr 90,000 and

67,000) were shown to be alkaline phosphatase-susceptible (implying an ester-linked phosphate bond). After phage infection, the β' subunit of the host DNA-dependent RNA polymerase and seven ribosomal polypeptides were phosphorylated. The RNA polymerase was phosphorylated on a threonine residue, and it was suggested that the role of the phosphorylation may be to affect host transcriptional processes in a manner similar to that seen in the infection of E. coli by T7 bacteriophage (Ponta et al. 1974). Other phosphoplypeptides seen post infection included DNA-binding and membrane proteins.

1.4.8 Protein phosphorylation in Halobacterium halobium

The extreme halophile Halobacterium halobium was shown to phosphorylate proteins in vivo by Spudich & Stoeckenius (1980). A number of polypeptides were labelled in the dark, but on exposure to light two polypeptides (Mr 100,000 and 80,000) were extremely rapidly dephosphorylated (dephosphorylation is extensive 30 seconds after exposure to light). After 15 minutes in the dark label appeared to be incorporated into bacteriorhodopsin though the significance of this is not known. Induction of dephosphorylation of the Mr 100,000 and Mr 80,000 polypeptides could be prevented by retinal synthesis inhibitors, and re-addition of exogenous retinal restored sensitivity. The retinal pigments have several roles in Halobacterium, being involved in phototaxis, photoenergy transduction and functioning as a light driven sodium ion pump. The exact function of the phosphorylation/dephosphorylation system has still to be established.

1.4.9 Protein phosphorylation in Synechococcus 6301

Synechococcus 6301 (Anacystis nidulans) was shown to contain a number of phosphopolypeptides in the thylakoids and soluble fraction both in vivo and in vitro (Sanders et al., 1986). The ressemblance between polypeptides phosphorylated in vivo and in vitro was slight. The phosphorylation of some low Mr polypeptides (Mr < 20,000) was shown to be light-dependent. It was speculated that the phosphorylation/dephosphorylation of an Mr 15,000 polypeptide shown to be phosphorylated in vivo and in vitro was involved in the regulation of coupling between the light-harvesting phycobiliproteins and the antenna chlorophyll proteins of photosystem 2.

1.4.10 Protein phosphorylation in Rhodobacter sphaeroides

Nine polypeptides, including Mr 54,000, 13,500 and 12,000 polypeptides, were shown to be phosphorylated in vivo (Holmes et al., 1986). Labelling in vitro at the expense of ^{32}P-δ-ATP resulted in a completely different set of phosphopolypeptides, including several of low apparent Mr (Mr 6000-12000) where polypeptides from the light harvesting complexes are usually seen. Phosphorylation of the low Mr polypeptides appeared to be under redox control, with oxidizing conditions favouring phosphorylation and reducing conditions favouring dephosphorylation.

1.4.11 Protein phosphorylation in Rhodospirillum rubrum

Loach et al. (1984) were the first to demonstrate protein phosphorylation in the photosynthetic bacterium Rhodospirillum rubrum. They reported that there was a correlation between an increase in cooperativity between photosynthetic units (see

section 1.2.2.4) and the amount of phosphate incorporated into the α-polypeptide of the light harvesting complex, and suggested that this may be due to the phosphorylation of the light harvesting complexes (associated with the reaction centres). A definitive demonstration of protein kinase activities in cell free extracts of Rs. rubrum was presented by Holuigue et al. (1985) when they showed that six polypeptides could be phosphorylated at the expense of ATP in cell free extracts. Phosphorylation occured on serine, threonine and tyrosine (Vallejos et al., 1985) residues and the phosphopolypeptides were distributed between membrane and cytoplasmic fractions. One of the polypeptides phosphorylated in vitro (Mr 13,000) overlapped purified apoprotein of the light harvesting chlorophyll-protein complex, supporting the suggestion of Loach et al. (1984) that the light harvesting complex could be phosphorylated. Holmes & Allen (1986) examined the phosphorylation state of two specific polypeptides (Mr 10,500 and 13,000), thought to correspond to the B880-β and B880-α subunits of the light harvesting pigment protein complex, under cooperative and non-cooperative conditions. They observed that the Mr 10,500 polypeptide (B880-β) was specifically phosphorylated under non-cooperative conditions, and the Mr 13,000 polypeptide (B880-α) was specifically phosphorylated under cooperative conditions. From the polypeptide sequence of B880-α, assuming phosphorylation on a serine or threonine residue, it was concluded the site of phosphorylation must be on the periplasmic side of the membrane. It was suggested that phosphorylation of the B880-β subunit could decrease cooperativity by mutual electrostatic repulsion between adjacent photosynthetic units, but the reason for an increase in cooperativity in response to phosphorylation on the B880-α

subunit was not clear.

Protein phosphorylation in prokaryotes, whilst by no means as well studied as in eukaryotes, is clearly a rapidly expanding field of study. Phosphorylation has been observed in eubacteria and archaebacteria and in organisms growing under many different growth conditions (aerobes, anaerobes, photosynthetics, halophiles etc.). This suggests that phosphorylation may prove to be a universal form of regulation amongst all life forms. In the work described above, some authors have made no attempt to distinguish between acyl- and ester-linked phosphate bonds. This is in many ways entirely justifiable, as there is no evidence to suggest that only ester-linked phosphorylation has any regulatory significance, though it does seem so far to be a general case (Cozzone, 1984). However the inclusion of acyl-linked phosphopolypeptides in the detection of phosphopolypeptides does handicap potential visualization of regulatory phenomena due to the role of acyl-phosphoprotein likages as intermediates in certain enzymatic mechanisms. For this reason we chose to confine our studies to ester-linked phosphopolypeptides. Many authors also fail to consider the possibility that some of the phosphate labelled bands they visualize on gel autoradiographs are the result of ADP-ribosylation. This too, in some ways, may be justifiable. ADP-ribosylation appears to occur less extensively in prokaryotes than does phosphorylation. Indeed Gaal & Pearson (1986) in their recent review of ADP-ribosylation state "There is little evidence to suggest that ADP-ribosyl transferases exist in uninfected bacterial cells". This is, however, misleading. Skorko & Kur (1981) presented evidence for the ADP-ribosylation of proteins in uninfected Escherichia coli cells and it has been

shown that the nitrogenase iron protein of Rhodospirillum rubrum is ADP-ribosylated in vivo (Pope et al., 1985), and can be reversibly regulated by ADP-ribosylation in vitro (Lowery et al., 1986). Thus ADP-ribosylation should not be ignored. One of the problems is the stability of the ADP-ribosyl group. I have seen no evidence to suggest that it is susceptible to the standard trichloroacetic acid heat treatment used to eliminate acyl-phosphates, carboxy-phosphates e.t.c. Though the ADP-ribosyl group of the nitrogenase iron protein of Rhodospirillum rubrum has been shown to be temperature sensitive (Dowling et al., 1982), data were only presented for lability at pH's of greater than 7.0 and so the acidic stability remains an unknown quantity. The problem is compounded by the differential stability of ADP-ribosyl groups. Treatment with neutral hydroxylamine usually degrades (ADP-ribosyl)-protein linkages, but 10-30% of linkages are resistant to this treatment and are only labile in hot alkali (Burzio, 1982), as are phosphoserine and phosphothreonine. In addition a small proportion of ADP-ribose appears to be attached through linkages that are hydroxylamine and alkali stable (Hayaishi & Ueda, 1982). Thus there are no easy techniques for distinguishing between phosphorylation and ADP-ribosylation, enzymatic methods that may place constraints upon experimental procedure are the only possibility.

It is interesting to consider the speculation of Cortay et al. (1986) that the Mr 54,500 polypeptide they observe as being phosphorylated on tyrosine in E. coli may be a widespread prokaryotic phosphopolypeptide. Allowing for the variations obtained between polyacrylamide gels several organisms appear to possess a phosphopolypeptide of Mr 52,000-57,000. Confirmation of this theory will require the identification of this polypeptide

and its role within the cell.

Another point of note is the frequent discrepancy between phosphorylated polypeptide patterns observed from *in vivo* labelling experiments and *in vitro* labelling experiments. Only in a few cases has there been any real correspondence between *in vivo* and *in vitro* results. There are several possible explanations for this. Firstly, all the non-corresponding results obtained *in vitro* could be artifacts, produced by non-specific phosphorylation of proteins normally compartmentalized away from the kinases. This is probably unlikely, as prokaryotic kinases appear to be highly specific. A second alternative is that they are polypeptides not normally phosphorylated (or phosphorylated to a very low level) *in vivo* due to the growth conditions used. The conditions used for *in vitro* kinase assays are never remotely physiological, and this could lead to the activation and inactivation of a different set of kinases to those seen *in vivo*. This can be cleary envisaged by considering the *Escherichia coli* isocitrate dehydrogenase kinase/phosphatase. The activities of this enzyme are tightly controlled by a number of effectors including isocitrate, ADP, phosphoenolpyruvate and NADPH (Nimmo & Nimmo, 1984). The presence or absence of these effectors in the assay buffer will profoundly effect the phosphorylation state of isocitrate dehdrogenase. The phosphorylation of the light harvesting chlorophyll a/b binding protein in chloroplasts is under redox control (Bennett, 1983 and Allen, 1983), and with the proposal of a unified theory for the regulation of photosystem coupling through phosphorylation it is possible that photosynthetic prokaryotes may also regulate the kinases phosphorylating the light harvesting subunits by some form of redox control. A third possibility is that the proteins seen to

be phosphorylated in vitro are proteins that are phosphorylated in vivo. the differences in apparent Mr arising from the further processing (e.g. proteolytic cleavage) of the protein in vivo. This would only apply in cases where phosphorylation takes place early post-translationally (before further modification). Other possibilities include, during the extract fractionation procedure, the loss of regulatory polypeptides, the separation of the kinase or phosphatase and their substrate (if the substrate is both phosphorylated and dephosphorylated simultaneously in vivo the removal of one enzyme will have a marked effect on the phosphopolypeptide pattern) and the degradation of particularly unstable kinases, phosphatases or substrates. Taking all these factors into consideration, it is perhaps surprising that anybody ever obtains an in vitro phosphopolypeptide pattern resembling those seen in vivo. In conclusion, I believe that the phosphorylation observed in vitro is significant, though not representative of the condition in which the cells are grown.

1.5 Rhodomicrobium vannielii

1.5.1 The Rhodospirillaceae

Rhodomicrobium vannielii is a member of the Rhodospirillaceae, a family in which, it has recently been suggested (Imhoff et al., 1984), there is a requirement for some reorganization. Internal consistency within this thesis will be maintained by adhering to the system proposed by Imhoff et al. (1984). To prevent confusion the changes involved are outlined below.

The main changes involve the creation of two new genera, Rhodobacter and Rhodopila. Rhodobacter includes all the old Rhodopseudomonas species that contained vesicular

intracytoplasmic membranes, i.e. Rhodobacter capsulatus (type species), Rhodobacter sphaeroides, Rhodobacter sulfidophilus, Rhodobacter adriaticus and Rhodobacter veldkampii. The exeption to this is Rhodopila (formerly Rhodopseudomonas) globiformis, which displayed sufficient disparity from the Rhodopseudomonas and Rhodobacter genera (based on biochemical, 16S rRNA analysis and DNA-rRNA hybridization studies) to warrant the creation of a separate genus. The remaining change is the inclusion of (formerly) Rhodopseudomonas gelatinosus and (formerly) Rhodospirillum tenue into the genus Rhodocyclus. The genus Rhodopseudomonas is retained for the rod-shaped, motile Rhodospirillaceae showing polar growth, asymmetrical division and possessing intracytoplasmic membranes that are adjacent and parallel to the cytoplasmic membrane. The genus Rhodomicrobium is retained on the basis of its characteristic morphological properties. The genus Rhodospirillum is recognized as being heterogenous, but currently insufficient data are available for separation and creation of new genera.

1.5.1.1 Physiological versatility

The Rhodospirillaceae display a high degree of metabolic versatility and adaptation to their natural environment. The environment is usually characterized by two features, the presence of light and the microbial breakdown of organic matter under conditions of oxygen limitation or anaerobiosis (Pfennig, 1978), a characteristic example of such habitats being the anaerobic muds of shallow ponds.

The mode of growth for which the Rhodospirillaceae are best adapted is photoheterotrophy. Under these conditions the organic compound is used as a source of carbon and as an electron donor,

and light is used as an energy source. Nitrate and sulfate replace oxygen as the terminal electron acceptors. A wide variety of carbon sources can be utilized, the best such sources are acetate, pyruvate, and the dicarboxylic acids, but some species can grow well on fatty acids, alcohols, sugars, one-carbon compounds (Sojka, 1978) and aromatics (Dutton & Evans, 1978). This ability to utilize a variety of organic substrates (both in the light and the dark) was attributed (Sojka, 1978) to the presence of a set of inducible catabolic pathways and the derivation of biosynthetic intermediates through the TCA cycle. The utilization of acetate remains unexplained in some Rhodospirillaceae. It has been known for many years that Rs. rubrum can grow on acetate as a sole carbon source, but despite considerable investigation no glyoxalate bypass mechanism has ever been found in this organism. Other members of the family appear to possess one or other or both of the two essential enzymes for the glyoxalate bypass, isocitrate lyase and malate synthase (Sojka, 1978). In the presence of CO_2 the acetate is incorporated into polysaccharide (rather than poly-ß-hydroxybutyrate as it is in the absence of CO_2). A possible pathway for this incorporation is the reductive carboxylic acid cycle (Fuller, 1978). This cycle is the main system used by the Chlorobiaceae for the fixation of CO_2 which is achieved by fixing it with acetate (via acetyl-CoA) to produce pyruvate, and operating a system analagous to the TCA cycle operating in reverse (Fig 1.8). The system is dependent on reduced ferredoxin for certain crucial carboxylations. It is known that Rs. rubrum and Rp. palustris can carry out similar reductive carboxylations (Fuller, 1978).

Many members of the Rhodospirillaceae are capable of

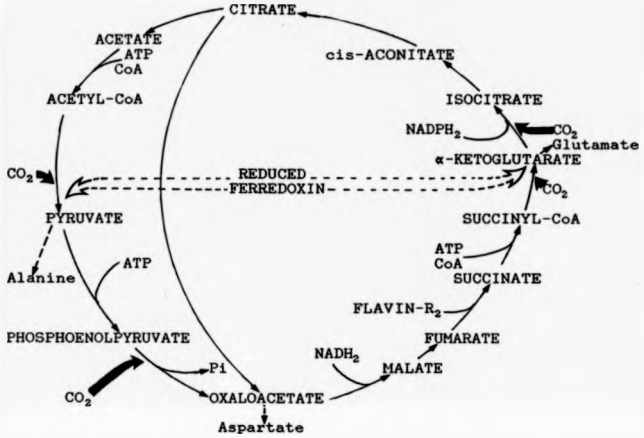


Fig 1.8 The reductive carboxylic acid cycle or Arnon cycle, after Fuller (1978).

photoautotrophic growth, growing using CO_2 as the sole carbon source, molecular hydrogen as the source of electrons and light as a source of energy. Despite their classification as the purple non-sulphur bacteria, it has been shown that some Rhodospirillaceae are capable of, indeed depend on, dissimilatory sulphur metabolism for a supply of electrons under photoautotrophic conditions (Neutzling et al., 1985). The key enzyme in photoautotrophic growth is ribulose bisphosphate carboxylase/oxygenase (RubisCO). RubisCO fixes CO_2 onto ribulose 1,5-bisphosphate as the first step in the Calvin cycle (reductive pentose phosphate cycle) (see Tabita et al., 1984). There are other CO_2 fixing reactions that use reduced Calvin cycle compounds as substrates, an example being the carboxylation of phosphoenol pyruvate to oxalacetate by Rp. palustris (reviewed by Fuller, 1978).

In addition to photosynthetic modes of growth many Rhodospirillaceae (mostly members of the genus Rhodopseudomonas) are capable of growing chemoheterotrophically in the dark utilizing the TCA cycle for substrate oxidation and oxygen as the terminal electron acceptor. Some Rhodospirillum species are also capable of this mode of growth, though due to their low oxygen tolerance only at low growth rates under microaeropholic conditions (Pfennig, 1978). Certain species are also capable of anaerobic growth in the dark with sugars as sole carbon and energy sources (Uffen, 1978) when provided with a fermentable carbon source (e.g. Rs. rubrum, Rb. sphaeroides). Finally, Rb. capsulatus, whilst being able to assimilate carbon under all of the above conditions, has been shown to possess a fifth growth mode. It is capable of chemoautotrophic growth in the dark under aerobic conditions using CO_2 as a carbon source, oxygen as the

terminal electron acceptor and H_2 as an electron donor (Madigan & Gest, 1979). Rb. capsulatus is, then, a remarkable organism with unusually great metabolic capacity that clearly demonstrates how this ancient group of organisms have been able to remain competitive throughout evolutionary history by retaining an extremely flexible metabolism that permits growth under practically any condition in the natural aquatic environment (Uffen, 1978).

The metabolic flexibility of the Rhodospirillaceae also manifests itself in two other areas, the metabolism of nitrogen and sulphur. The fixation of nitrogen was first observed in Rs. rubrum in 1949 (see Yoch, 1978). Since then it has been established in all properly tested strains of the Rhodospirillaceae (Stewart, 1973) with the exeption of Rhodocyclus purpureus (Madigan et al., 1984). Optimal fixation only occurs in the light, but significant amounts of fixation can be observed in the dark with the organisms obtaining the ATP and reductant necessary for nitrogenase activity through the fermentation of carbohydrate reserves (Yoch, 1978). The Rhodospirillaceae are also capable of the utilization of NH_4^+ (Brown & Herbert, 1977). As mentioned briefly above, at least some members of the Rhodospirillaceae are capable of dissimilatory sulfur metabolism as well as the assimilatory metabolism they depend on for the synthesis of sulfur containing cellular components (e.g. CoA, methionine). Both types of metabolism are discussed in detail by Truper, (1978).

Thus the metabolic flexibility of the Rhodospirillaceae confers on them the ability to grow under a wide range of growth conditions allowing them to exploit a variety of differnt habitats. However, the flexibility conferred by their metabolisms

is enhanced by some of the growth strategies these organisms have adopted (Kelly, 1985). These include the utilization of wavelenghths of light not used by other phototrophs, the ability to vary the composition of their light harvesting machinery to compensate for alterations in the light quality and the variation in cell-type expression associated with dispersal and survival. The most extreme example of variation in cell-type expression in the Rhodospirillaceae occurs in Rhodomicrobium vannielii, the organism of this study.

1.5.2 Rhodomicrobium vannielii

Rhodomicrobium vannielii is the sole member of the genus Rhodomicrobium, characterized apart from the genus Rhodopseudomonas on the basis of its morphological properties. Physiologically it resembles many other Rhodospirillaceae, capable of nitrogen fixation, ammonia assimilation, growth on a variety of carbon sources (though not sugars) under different growth regimes. It has been proposed, however, that Rm. vannielii is perhaps on the whole somewhat less flexible in its metabolism than other members of the Rhodospirillaceae. Indeed, across all the Rhodospirillaceae there appears to be a correlation between decreased metabolic flexibility and increased morphological complexity, possibly suggesting some sort of "trade off" between flexibility and persistance in the family (Kelly, 1985). Of all the aspects of Rm. vannielii, the area that has attracted most interest has been the cell cycle. This is because the extreme morphological variation displayed through the cell cycle of this organism renders it extremely amenable to study. Before proceeding further with this discussion it will be necessary to define some of the terms that will be in use. The important

distinction is the one that must be made between morphogenesis and differentiation. Morphogenesis applies to the changes seen in the external morphology and internal architecture during the cell cycle, whereas differentiation is applied to those events arising from a change in the cell cycle that results in the formation of a functionally distinct cell type. Development is defined as a process involving both morphogenesis and differentiation under intercellular influence such as is seen in the development of heterocysts in filamentous cyanobacteria. These definitions were proposed by Whittenbury & Dow (1977).

1.5.2.1 The cell cycle of Rhodomicrobium vannielii

Rm. vannielii displays a polymorphic cell cycle, i.e. it is capable of more than one vegetative cell cycle depending on the nutrient conditions (Whittenbury & Dow, 1977). In fact Rm. vannielii is capable of two distinct cell cycles, two modes of cell division and the production of three cell types (Fig 1.9). As a budding bacterium it displays obligate polar growth and asymmetric cell division, the obligate nature of these events being the significant factor as it appears that the phenomena themselves may be much more widespread than had previously been anticipated (Kelly & Dow, 1984). The intracytoplasmic membrane systems are "horseshoe" shaped, with a gap at the point of growth preventing any complications at cell division. A consequence of polar growth is the concept of cell ageing. As all new cell material is inserted at the growing pole, the daughter cell produced is structurally younger than the mother cell. Indeed, a single mother cell produces no more than four daughter cells whatever the nutrient conditions, possibly indicating cellular mortality (Whittenbury & Dow, 1977). This differential age of the

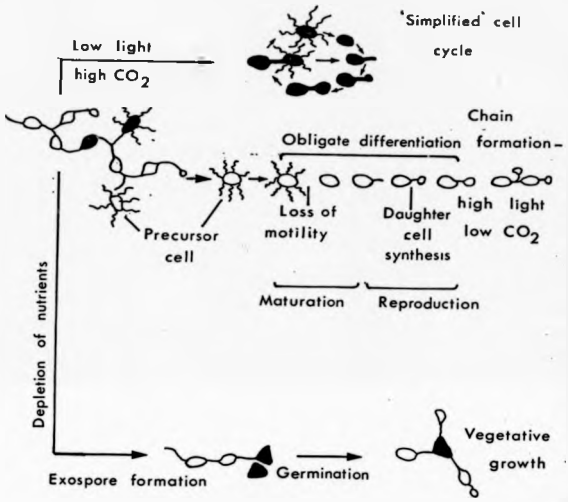


Fig 1.9 Cell expression and the swarmer cell cycle in Rhodomicrobium vannielii. indicating the effect of various environmental stimulii. After Dow et al. (1983).

progeny of a cell division as a result of polar growth is the basis of the "mother-daughter" cell distinction. Cells undergoing intercalary growth give rise to progeny that are indistinguishable from each other whereas cells undergoing asymmetric polar growth yield non-identical progeny (Dow et al., 1983).

The typical culture composition of a mid log phase photoheterotrophically grown batch culture of Rm. vannielii is mixed. The majority of cells exist in multicellular arrays, consisting of many individual cells linked by frequently branching filaments. In addition many motile, unattached "swarmer" cells will be seen and it is from these cells (termed "growth precursor" cells by Dow et al., 1983) that discussion of the cell cycle is best begun.

From the starting point of the swarmer cell there are two phases that can be identified, maturation and reproduction (Whittenbury & Dow, 1977). The maturation phase is instigated by an increase in light intensity and lasts between 130 and 150 minutes after a latent phase in which viability can be maintained for several days (Porter, 1985). During maturation the flagellae are lost and thus motility lost, a major landmark event for following the progress of swarmer cell differentiation. The onset of the reproductive phase is marked by the commencement of filament synthesis from one, or occasionally both, poles of the cell along with the initiation of DNA synthesis. The next stage, after 220-250 minutes from the initiation of the cycle is bud synthesis from the distal end of one filament followed by completion and separation of the daughter cell from the mother cell. The separation can occur by one of two means, binary fission in the case of swarmer cell production and plug formation

during the formation of vegetative cells. The type of daughter cell formed is environmentally controlled, low light intensities and high CO_2 concentrations leading to swarmer cell formation, the lack of such limitations resulting in the formation of vegetative chain cells. Nutrient depletion gives rise to the formation of angular (tetrahedral) exospores which on nutrient enrichment germinate to produce vegetative daughter cells under the same constraints as normal vegetative growth. i.e. one vegetative cell or exospore can give rise to no more than four daughter cells. In normal batch culture a small proportion (1-2%) of cells display a "simplified cell cycle" (Dow & France, 1980) in which the swarmer cell is the only product of reproduction. The amenability of Rm. vannielii to cell cycle studies stems from this variation in cell type expression. In practice, attention has been focused on the morphogenesis and differentiation of the swarmer cell. This is due to the existence of easily identified "landmark" events, making the current stage of the cell cycle easy to determine, the suitability of swarmer cell differentiation to monitoring via cell volume distribution analysis, and the ease with which high cell density, homogeneous, populations of swarmer cells can be selectively obtained with minimal physiological disturbance by filtration (Whittenbury & Dow, 1977). The swarmer cell has attracted considerable attention in its own right, with its role as a "shut down" or "growth precursor" cell being subject to investigation (Dow et al., 1983).

We have seen how protein phosphorylation plays a central role in the regulation of eukaryotic cells, and how protein phosphorylation is emerging as an important control mechanism in

prokarvotes. Rhodomicrobium vannielii provides an excellent vehicle for the further study of prokaryotic protein phosphorylation, providing the opportunity to examine its physiological versatility in terms of protein phosphorylation, and the chance to extend these studies to examine protein phosphorylation in the cell cycle. The initiation of such an investigation is the aim of this study.

CHAPTER 2

MATERIALS AND METHODS

The methods described here are those that were used as routine. Techniques specific to particular experiments and derivations of the following methods are described in the appropriate results section.

2.1 Reagents

All reagents, unless otherwise indicated here, were BDH AnalR grade. Acrylamide was BDH Electran grade. Tris (Trizma base), Sodium pyruvate, DNase I, RNase A, RNase T_1, lysozyme, Brij 58, O-phospho-L-serine, O-phospho-L-tyrosine, O-phospho-DL-threonine, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, snake venom phosphodiesterase, protease K, chlorpromazine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), m-aminophenylboronicacid, adenosine triphosphate (vanadate-free), phenylmethylsulphonylfluoride, protein A and bovine alkaline phosphatase were obtained from Sigma. Glycine, bisacrylamide, SDS and TEMED were BioRad electrophoresis purity reagents. Bacto-tryptone and bacto-agar were obtained from Difco, and yeast extract was obtained from Oxoid.

2.2 Bacterial Strain

Rhodomicrobium vannielii strain Rm5 (Whittenbury & Dow, 1977) was used throughout this study.

2.3 Media and Buffer Sterilization

Buffers and media were sterilized by one of two methods. All components were sterilized by autoclaving at 15 psi for 15 minutes unless otherwise noted. Volatile and labile components were filter sterilized through Millipore GS or FlowPore D disposable filter units.

2.4 Growth and Maintenance of Rm. vannielii

Rm. vannielii stocks were grown in liquid PM medium (Whittenbury & Dow, 1977) containing:

Ammonium chloride.....0.50 gl^{-1}

Magnesium sulphate (.$7H_2O$).....0.40 gl^{-1}

Sodium chloride.....0.40 gl^{-1}

Calcium chloride.....0.05 gl^{-1}

Sodium pyruvate.....1.50 gl^{-1}

Sodium hydrogen malate.....1.50 gl^{-1}

The pH of this medium was adjusted to 6.8 with potassium hydroxide before autoclaving. Solid medium was obtained by the addition of Difco Bacto-agar to 1.5% (w/v). Prior to inoculation 5 $ml l^{-1}$ sterile phosphate buffer (1 M) was added aseptically.

Phosphate Buffer pH 6.8

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)....7.8 gl^{-1}

Disodium hydrogen orthophosphate (Na_2HPO_4).....7.1 gl^{-1}

These stocks were subcultured every four weeks using a 1% inoculum from frozen stocks maintained in LB medium plus 50% (v/v) glycerol at -20°C.

Luria-Bertani (LB) medium:

Bacto-Tryptone.....10 gl^{-1}

Sodium Chloride.....10 gl^{-1}

Yeast Extract.....5 gl^{-1}

D-glucose.....1 gl^{-1}

Experimental cultures were required to contain the minimum ammounts of phosphate possible. The usual phosphate concentration is 5 mM, but for phosphorylation experiments a lower concentration is desirable. As a result a lower concentration of 312.5 μM was used (see section 3.1.1). This corresponds to an addition of 3.125 ml 0.1 M phosphate buffer per litre of culture, and this was used as standard for most experiments. For certain experiments in which very high concentrations of radioactive label were required and the ability to produce swarmer cells with the capacity to undergo synchronous differentiation was not, the only added exogenous phosphate was that supplied by the 2% (v/v) inoculum. In these cultures buffering capacity was supplied by supplementing the cultures with 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid).

Cells were grown by one of several methods.

1) Cells were grown in 100 or 500 ml serum bottles containing magnetic stirrer bars and sealed with sterile rubber Suba seals (William Freeman & Co., Barnsley, W. Yorkshire). Prior to inoculation, and after the addition of phosphate, bottles were flushed with oxygen-free nitrogen for 15 minutes. Bottles were incubated in a warm room at 30°C on a magnetic stirrer at an incident light intensity of 35 $\mu Em^{-2}s^{-1}$ provided by tungsten bulbs (as measured by Kelly, 1985).

2) Cells were grown in 100 or 250 ml B19 conical flasks sealed and gassed as above. Incubation was carried out in a shaking

water bath at 30°C with light provided as above.
3) Large scale cultures were prepared by using 5 or 20 litre flat bottomed vessels (244/1350, Baird and Tatlock) sealed with Quickfit tops capable of receiving Suba seals. Vessels were pre-gassed for 30 minutes and incubation carried out in a 30°C warm room on a magnetic stirrer with light provided as 1).

All experimental cultures received a standard inoculum of 2% (v/v) and were stirred or shaken continuously. Growth was followed by measuring the optical density of cultures at 540 nm in a Pye-Unicam SP500 or an LKB Ultrospec 4050 spectrophotometer. Culture purity was checked by phase contrast microscopy. If there was any doubt regarding the purity, samples were streaked out to single colonies on PM plates and individual colonies were checked. Plates were incubated under anaerobic conditions using the anaerobic bag technique (Westmacott & Primrose, 1975). Plates were placed on a tray and enclosed in a nylon bag (Portex Ltd., Hythe, Kent) which was then heat-sealed and flushed with oxygen-free nitrogen for 15 minutes. A beaker containing 15 ml saturated pyrogallol solution, 15 ml 10% (w/v) sodium hydroxide / 15% (w/v) potassium bicarbonate solution was also included in the bag to remove the last traces of oxygen. The tray was incubated in a 30°C warm room under the light conditions described above.

2.5 Preparation of Homogeneous Populations of Swarmer Cells

Populations of *Rm. vanniellii* swarmer cells for synchrony experiments were prepared by a derivation of the method of Whittenbury & Dow (1977). The column length, bore, and amount of glass wool used varied depending on the culture volume to be synchronised as follows:

Culture volume (ml)	Column dimensions (length/bore, cm)	glass wool (g)
100	24x2.8	5.5
500	37x4.8	18.0
20,000	4x(60x7.0)	45

The columns were covered with aluminium foil and sterilized by dry heat at 160°C overnight. Impurities were removed from the column by washing through with 4 column volumes of sterile distilled water. Simultaneously the column and the foil covered collection vessel (containing a magnetic stirrer bar) were pre-gassed with oxygen free nitrogen. The gassing of the column and collection vessel continued as the mid/late exponential phase (A_{540} 0.8-1.0) heterogeneous culture was poured into the column. The collection vessel was then Suba-sealed, and gassing continued via hypodermic needles for a further 1-5 minutes (depending on culture volume). Differentiation was initiated by the removal of the foil covering and incubation of the culture in a 30°C warm room at an incident light intnsity of 35 $\mu Em^{-2}s^{-1}$. Differentiation was followed by phase contrast microscopy and Coulter counter cell volume distribution analysis. Samples were removed via a hypodermic needle and syringe after first injecting an equal volume of oxygen-free nitrogen to prevent the influx of air on sampling.

2.6 Preparation of Multicellular Arrays From Batch Cultures

Multicellular arrays were prepared from batch cultures by the differential centrifugation method of Kelly (1985). Culture volumes (50 ml) were centrifuged in polycarbonate Oakridge tubes in a MSE Minor swingout bench centrifuge. The samples were centrifuged 5 times for 2 minutes each at 1,200 g. After each

spin the supernatant (containing mostly swarmer cells) was removed from above the loose pellet (containing mostly multicellular arrays), and the pellet was gently resuspended in 20-30 ml 10 mM Tris-HCl pH 7.4. The homogeneity of the preparation was monitored by phase contrast microscopy.

2.7 Coulter Counter Analysis of Cell Volume Distribution

The quality of swarmer cell preparations and the progress of synchronization experiments were monitored by examining cell size distribution on a Coulter Counter Model ZBI and Coulter Channelyzer C1000. This was interfaced with a BBC model B microcomputer, 5.25 inch floppy disc drive and Tandy TRS-80 plotter for profile processing, storage and hardcopy production.

Samples of cultures (20-250 µl) were added to 20 ml Isoton (Coulter Electronics Ltd.) and profiles obtained using the 30 µm orifice probe, a window width of 100, a current of 0.5 mA, a base channel threshold of 15, count range 4000 and an amplification factor of 0.5.

2.8 Incorporation of Radioisotopes in vivo

Radioisotopes utilized were acid-free carrier-free ^{32}P-orthophosphate (Amersham or NEN) in aqueous solution with a radioactive concentration of 10 mCiml^{-1}, and L-^{35}S-methionine (specific activity >800 Cimmol^{-1}) in 20 mM potassium acetate solution containing 0.1% (v/v) 2-mercaptoethanol. ^{32}P orthophosphate was routinely added to a final radioactive concentration of 10-50 µCiml^{-1} (or exeptionally 100 µCiml^{-1}) to cultures in early exponential phase for continuous labelling experiments, or to cultures or swarmer cell populations pre-use for pulse-labelling experiments. For phosphoamino acid

determinations a final specific activity of 1 mCiml^{-1} was used. ^{35}S-methionine labelling was carried out at a final radioactive concentration of 1 μCiml^{-1}.

2.9 Cell Harvesting and Preparation of Whole Cell Extracts

Cells were harvested by centrifugation (40,000 g at 4°C for 30 minutes). When ^{32}P-labelled cells were to be analyzed on SDS-PAGE, sodium fluoride was added to cultures to a final concentration of 100 mM immediately prior to harvesting, and the cells were washed twice with 20 mM Tris-HCl pH 7.4, 50 mM NaF and then twice with ice-cold acetone. The pellet was then dried and protein solubilized by heating in 100 μl sample buffer (Laemmli, 1970). A similar method has subsequently been published (Bhaduri & Demchick, 1983). They reported that extracts made by this method gave protein patterns comparable to extracts made by other methods. Samples were then incubated with 40 μg RNase A overnight at 20°C.

2.10 Protein Determinations

Protein determinations were carried out by using the Bio-Rad protein assay system (Bio-Rad Laboratories Ltd).

2.11 Preparation of Cell-Free Extracts

Three methods were used for the preparation of cell-free extracts.

a) Preparation of cell-free extracts for in vitro kinase assays

- Based on the method used by Wang & Koshland (1978)

Cell were harvested and washed twice in 10 mM Tris-HCl pH 7.4 before being resuspended in a minimal volume of double-strength

kinase buffer (100 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol). Cells were broken in one of two ways. Radioactive samples or samples of small volume (less than 2 ml) were broken by sonicating 5 times for 20 seconds at a peak to peak amplitude of 24 microns, with a 60 second cooling period in a methanol-ice bath between each pulse. Larger volumes of non-radioactive samples were broken by two passages through a French pressure cell (pre-cooled to 4°C) at 1000 psi, a method that was found to give far superior breakage. Extracts were treated with DNase I (20 $\mu g ml^{-1}$) and RNase A (20 $\mu g ml^{-1}$) for 30 minutes prior to the removal of particulate material (if required) by centrifugation at 38,000 g for 1 hour. The samples were diluted to a protein concentration of 1 $mg ml^{-1}$ and separated into 200 µl aliquots. Those aliquots not being used immediately were stored at -70°C.

b) Preparation of cell-free extracts for in vitro kinase assays :- Developed from the method used by Iwakura et al. (1974).

Cells were harvested as above and resuspended in 40 mM Tris-HCl pH 8.1, 25% (w/v) sucrose and treated with 1 mM EDTA and 500 $\mu g ml^{-1}$ lysozyme at 0°C for 10 minutes. Brij 58, a non ionic detergent was added to 0.5% (w/v) and after 5 minutes magnesium chloride to 10 mM and potassium chloride to 200 mM were added, and the cells sonicated as described above. Subsequent to sonication extracts were treated as al above.

c) Preparation of cell-free extracts for non-denaturing gel electrophoresis.

Cells were harvested and washed as above (including pre-treatment and washing with NaF in the case of ^{32}P-labelled

samples) and resuspended in 60 mM Tris-HCl 30 mM H_3PO_4 pH 6.8 (pH adjusted with NaOH). The cells were disrupted by sonication (as above) and the sonicate incubated with DNase I (20 $\mu g ml^{-1}$), and RNase A (20 $\mu g ml^{-1}$). The sonicate was cleared by centrifugation at 12,000 g for 5 minutes in an Eppendorf 5412 microcentrifuge.

2.12 In Vitro Kinase Assays

In vitro kinase assays were carried out by incubating 40 µl of cell free extract (prepared by adding 50 µl of cell-free extracts prepared as above (sections 2.11a and 2.11b) to 50 µl of a solution containing all the additions required in the assay) with 10 µl 0.5 mM ATP containing 10 µCi adenosine-5'-(δ-^{32}P) triphosphate (Amersham, specific activity >5000 $Ci mmol^{-1}$, supplied as an aqueous solution of activity 10 $mCi ml^{-1}$ containing 5 mM 2-mercaptoethanol) at room temperature for 30 minutes. Reactions were stopped by the addition of an equal volume of 2x concentrated Laemmli (1970) sample buffer and boiling for 5 minutes. Samples were then analyzed by SDS polyacrylamide gel electrophoresis.

2.13 Polyacrylamide Gel Electrophoresis of Proteins

2.13.1 One dimensional 10-30% exponential gradient SDS polyacrylamide gels

This technique was routinely used for the analysis of polypeptides and phosphopolypeptides in cell extracts and from in vitro kinase assays. The method was that of Kelly (1985). Exponential 10-30% gels were cast using the apparatus described by Porter (1984) in 25 x 20 cm glass plates using 2 mm thickness spacers. These gels were found to be highly reproducible and to

give good resolution. Gels were run at 20 mA constant current at 4°C overnight (16 hours) until the dye front had migrated off the bottom of the gel in perspex tanks.

The stock solutions used were as follows:

60% (w/v) high bisacrylamide acrylamide

acrylamide (BDH Electran grade).....60.0 g

bisacrylamide (BioRad).....1.6 g

Acrylamide and bisacrylamide were dissolved in 40 ml of warm distilled water, and the volume was then made up to 100 ml.

60% (w/v) low bisacrylamide acrylamide

acrylamide.....60.0 g

bisacrylamide.....0.3 g

Made up as above.

10% (w/v) stacking gel acrylamide

acrylamide.....10.0 g

bisacrylamide.....0.5 g

Lower gel buffer

Tris base.....36.6 g

The pH was adjusted to 8.8 with HCl and the volume made up to 100 ml.

Stacking gel buffer

Tris base.....5.98 g

The pH was adjusted to 6.8 with HCl and the volume made up to 100 ml.

10x running buffer stock

Tris base (25 mM final).....60.4 g

Glycine (192 mM final).....288.0 g

Sodium dodecyl sulphate (0.1% (w/v) final).....20.0 g

Made up with distilled water to 2 l

Final concentrations given are after dilution. For the 10-30% exponential gradient polyacrylamide gel two solutions of acrylamide were required, one of 30%, the other of 10%. These were prepared from the above stocks as follows:

30% (w/v) acrylamide

Low bisacrylamide stock.....10.0 ml

75% (w/v) glycerol.....7.3 ml

lower gel buffer.....2.5 ml

10% (w/v) SDS.....0.2 ml

The final total volume was 20 ml. This mixture was then degassed on a water pump vacuum line for 30 minutes. Immediately before pouring, 4.8 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 48 µl fresh 10% (w/v) ammonium persulphate were added.

10% (w/v) acrylamide

High bisacrylamide stock.....8.30 ml

distilled water.....34.90 ml

lower gel buffer.....6.25 ml

10% (w/v) SDS.....0.50 ml

The final total volume was 50 ml. This mixture was degassed as above and 12 µl TEMED, 120 µl ammonium persulphate added immediately before pouring.

The 30% acrylamide gel solution was poured into a 25 ml glass scintillation vial containing a magnetic stirrer bar. The top of this scintillation vial could be sealed with a Suba seal that was penetrated by two hypodermic needles, one 21Gx1.5, one 19Gx2 (Sabre, Sabre International Products Ltd.). A length of silicone rubber tubing was attached to the 21G needle, this tubing being passed through a peristaltic pump. After penetration of the suba seal both ends were removed from the 19G needle and thin plastic tubing slid over each end of the protruding cannula, a short length (~2 cm) to reach just above the stirrer bar in the scintillation vial and a long length that was taped to the back plate of the assembled casting plates. The ammonium persulphate and TEMED were added to the 10% acrylamide (contained in a conical flask), the silicone rubber tube was immersed and the mixture pumped through until it just appeared at the tip of the 21xG needle. The ammonium persulphate and TEMED were then added to the 30% acrylamide in the scintillation vial and the suba seal pushed into place, thus forcing a few drops of 30% acrylamide through the 19G needle to run down the backplate of the casting apparatus. The stirrer speed was increased to 600 rpm and the pump turned on to pump the 10% acrylamide through at a rate of 3 $mlmin^{-1}$. This system with a constant volume in the mixing vial being diluted with a larger volume of the lower percentage gel mix results in an exponential gradient. The gel was poured until it was 4 cm from the top of the front (bevelled) gel plate. The surface was overlayed with a small volume of butan-2-ol, and the gel left to set for three hours. After polymerization the surface of the gel was very thoroughly washed to remove all traces of the butan-2-ol, and a stacking gel layed on top, containing:

Stacking gel mixture

stacking gel acrylamide.....3.0 ml

distilled water.....4.4 ml

stacking gel buffer.....2.4 ml

10% (w/v) SDS.....0.1 ml

Final total volume 10 ml. This mixture was degassed as usual, and 5 µl TEMED, 100 µl 10% (w/v) ammonium persulphate added immediately before pouring. Immediately after pouring a 9 or 12 well teflon gel comb was inserted into the stacking gel. The stacker was allowed to set for 30 minutes, the comb was removed and the gel transferred to the tank for loading. The running buffer was prepared by diluting the running buffer concentrate tenfold with distilled water. Mr estimations were carried out by the preparation of a calibration curve utilising standards of known molecular ratio (plotting $\log_{10}$ Mr against distance migrated). The standards used were Pharmacia low molecular weight standards containing phosphorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), soybean trypsin inhibitor (Mr 20,000) and α-lactalbumin (Mr 14,400). Protein samples were prepared as described above, the composition of the sample buffers used were as follows:

Sample buffer stock

stacking gel buffer.....1.25 ml

distilled water.....5.25 ml

glycerol.....1.00 ml

10% (w/v) SDS.....2.00 ml

Just prior to use 50 µl of 2-mercaptoethanol was added to each 950 µl of sample buffer stock. After boiling 1% (v/v) 0.5% (w/v) bromophenol blue was added to each sample.

2x Sample buffer stock

stacking gel buffer.....2.50 ml

distilled water.....0.50 ml

glycerol.....2.00 ml

10% (w/v) SDS.....4.00 ml

Just prior to use 100 µl of 2-mercaptoethanol was added to each 900 µl of sample buffer stock. After boiling 1% (v/v) 0.5% (w/v) bromophenol blue was added to each sample.

2.13.2 One dimensional polyacrylamide gels (non-gradient)

For some applications, e.g. the resolution of high molecular weight polypeptides single percentage gels were found to give better resolution than the 10-30% gradient gels. The only stock required not included in the above list was:

40% (w/v) acrylamide, 0.5% (w/v) bisacrylamide

acrylamide.....40.0 g

bisacrylamide.....0.5 g

Acrylamide and bisacrylamide were dissolved in 40 ml of warm distilled water, and the volume was then made up to 100 ml.

Mixtures were made as below. The stocks used are listed on the left, the gel concentrations produced are listed across the top. Cross-indexing gives the appropriate volume (in ml) of each component required for a gel of that percentage.

Gel percentage components	7%	10%	15%
40% acrylamide stock	12.25	17.5	26.25
lower gel buffer	8.82	8.82	8.82
distilled water	48.21	42.98	34.23
10% (w/v) SDS	0.7	0.7	0.7

The mixture was degassed as usual, and polymerization initiated by the addition of 16.8 µl TEMED and 168 µl ammonium persulphate. Gel plates as described in 2.13.1 were used with the solution being pipetted between the plates. Overlaying with butan-2-ol and casting of the stacking gel proceeded as described above. Gels were run at 25 mA constant current at 4°C for 5-8 hours. The dye front was retained on the gel.

2.13.3 One dimensional non-denaturing polyacrylamide gels

These gels were used for the analysis of native proteins. One stock additional to those described above was required:

25% (w/v) acrylamide, 1.25% (w/v) bisacrylamide

acrylamide.....25.00 g

bisacrylamide.....1.25 g

Acrylamide and bisacrylamide were dissolved in 40 ml of warm distilled water, and the volume was then made up to 100 ml.

The non-denaturing gel system used was a 4-30% gradient. This was found to give excellent resolution in the molecular weight range required. Preparation of the gradient was basically as described in section 2.13.1 with differences detailed below. The solutions used were a 4% (w/v) and a 30% (w/v) acyrylamide solution, made up as follows:

30% (w/v) acrylamide solution

60% low bis acrylamide solution (2.13.1).....10.0 ml

lower gel buffer (2.13.1).....2.6 ml

30% (v/v) glycerol.....7.4 ml

The mixture was degassed as usual, and polymerization initiated with the addition of 5.2 µl TEMED and 52 µl 10% (w/v) ammonium persulphate.

4% (w/v) acrylamide solution

25% (w/v) acrylamide solution .....9.2 ml

lower gel buffer (2.13.1).....7.5 ml

distilled water.....40.8 ml

The mixture was degassed as usual, and polymerization initiated with the addition of 12.2 µl TEMED and 104.25 µl 10% (w/v) ammonium persulphate.

The gradient was poured using the apparatus described in 2.13.1 as described in that section. The only difference was that 5 ml of the 4% gel mixture was retained and applied to the top of the gel after the pouring of the gradient to ensure the top of the gel was genuinely 4%. The well comb was immediately inserted into this, and the gel left to set for three hours. The comb was then removed and the gel placed in the tank in a cold room for running overnight at 18 mA. The running buffer was prepared as follows:

Non-denaturing gel running buffer

glycine.....43.2 g

Trizma base.....9.0 g

The above were dissolved in 1.5 l of distilled water.

Mr estimations were obtained by comparing the relative migration of proteins with proteins of known molecular weight. Molecular weight markers used were Pharmacia high molecular weight markers consisting of thyroglobulin (Mr 669,000), ferritin (Mr 440,000), catalase (Mr 232,000), lactate dehydrogenase (Mr 140,000) and bovine serum albumin (Mr 67,000). These markers were dissolved in a buffer identical to that used by the samples. The effect of charge on the mobility of the proteins was minimized by running the samples until migration was halted by the increasing concentration of acrylamide in the gradient of the gel. Porter (1984) reported that this method would separate proteins on the basis of molecular ratio and that a calibration curve could be obtained.

2.13.4 Two-dimensional non-denaturing/denaturing gel electrophoresis

Two dimensional non-denaturing/denaturing gel electrophoresis was carried out as described by Porter (1984) with a few modifications. Samples were run in the first dimension on a 5-15% exponential gradient non-denaturing gel. Stocks required were as follows:

30% (w/v) acrylamide stock

acrylamide.....28.5 g

bisacrylamide.....1.5 g

Volume made up to 100 ml.

Stacking gel acrylamide stock

acrylamide.....10.0 g

bisacrylamide.....2.5 g

Volume made up to 100 ml.

Lower gel buffer stock

Tris base.....36.60 g

Volume made up to 100 ml, and pH adjusted to 8.8 with HCl.

Stacking gel buffer stock

Tris base.....5.7 g

Volume made up to 100 ml and pH adjusted to 6.9 with concentrated phosphoric acid (H_3PO_4)

Reservoir Buffer stock (x50)

Tris base.....30.2 g

glycine.....144.0 g

Volume made up to 1 l

The gel mixtures were prepared as follows from the above stocks:

15% (w/v) acrylamide solution

30% acrylamide stock.....10.0 ml

lower gel buffer.....2.5 ml

75% glycerol (w/v).....7.5 ml

Total final volume 20 ml. The mixture was degassed as usual, and polymerisation initiated with the addition of 4 µl TEMED and 40 µl 10% (w/v) ammonium persulphate.

4% (w/v) acrylamide solution

30% (w/v) acrylamide solution .....6.70 ml

lower gel buffer.....6.25 ml

distilled water.....37.00 ml

Total final volume 50 ml. The mixture was degassed as usual, and polymerization initiated with the addition of 10 µl TEMED and 100 µl 10% (w/v) ammonium persulphate.

The gel was poured as described in 2.13.1 and overlayed with butan-2-ol. The stacking gel mixture was prepared as follows:

4% (w/v) stacking gel acrylamide solution

stacking gel acrylamide stock.....2.50 ml

40% (w/v) sucrose.....5.00 ml

stacking gel buffer stock.....1.25 ml

distilled water.....1.25 ml

Total final volume 10 ml. The mixture was degassed as usual, and polymerization initiated by the addition of 5 µl TEMED and 100 µl 10% (w/v) ammonium persulphate.

The gel was run at 4°C overnight at 20 mA, the running buffer being the stock running buffer diluted 1 in 50. After removal from the tank the gel was taken out from the plates, dried and autoradiographed. Tracks were then sliced out in strips with a scalpel. A standard 10-30% gradient SDS polyacrylamide gel (2.13.1) was prepared with the exception that no comb was inserted into the stacking gel, the surface being overlayed with butan-2-ol instead to produce a flat surface. The gel strips were first re-hydrated in 40% (v/v) methanol, 7% (v/v) acetic acid and then soaked twice for 15 minutes each in equilibration buffer (62

mM Tris/HCl pH6.8, 2% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol). The gel strip was then layed on top of the 10-30% gradient denaturing gel stacker, and sealed in place with 1% (w/v) agarose in equilibration buffer. The gel was run as normal, then dried and autoradiographed.

2.14 Gel Staining

Two methods of gel staining were tried, silver staining and PAGE Blue 83 staining. PAGE Blue 83 staining was adopted as the method of choice as silver staining resulted in excessive quenching of the radioisotope emissions, and the sensitivity of the silver stain was not really required for locating non-radioactive standards (the main requirement for staining in these experiments). The staining procedure involved soaking the gel overnight in the staining solution:

Coomassie blue staining solution

PAGE Blue 83	2.5 g
methanol	500 ml
glacial acetic acid	70 ml
distilled water	430 ml

Total final volume 1 l. The PAGE Blue 83 was first dissolved in the methanol before the addition of the acetic acid and water. The solution was filtered before use.

After staining, the gel was initially destained in 45% methanol (v/v) 10% (v/v) glacial acetic acid for 4-6 hours with 2-3 changes. The gel was then transferred to the second destain (20% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid) and left overnight. Finally the gel was transferred to 10% (v/v)

propan-2-ol, 10% (v/v) glacial acetic acid until the background was satisfactory before photographing.

For gels of ^{32}P-labelled phosphopolypeptides it was usually just a case of visualizing the standards for Mr estimations on the autoradiographs. In this case a simplified procedure was adopted. The gel was soaked in the staining solution for two hours and then transferred to the TCA phosphoprotein gel treatment protocol (se below). The heat treatment in TCA was found to be an extremly effective destain.

2.15 Phosphoprotein Gel Treatment

The visualisation of phosphoproteins on polyacrylamide gels was complicated by several factors. Many of these could be overcome using an adaptation of the hot TCA treatment for polyacrylamide gels developed by Bhorjee & Pederson (1976). The gel was placed in a Pyrex dish with 800 ml 16% (w/v) TCA. This was then incubated for 40 minutes in a water bath that had been pre-heated to 95°C. After incubation the dish was taken out and immersed in a sink of cold water for cooling. The gel could then be removed and placed in the washing buffer (5% (w/v) TCA, 50 mM Na_2HPO_4, 35% (v/v) methanol). The gel was washed for 24 hours with three changes of buffer before being dried down and autoradiographed. While it was theoretically possible to stain gels after the TCA treatment (Manai & Cozzone, 1979a) it was found that the staining was poorer, and the background unacceptably high. Thus as a matter of routine gels were stained prior to the TCA treatment, which was found to be an extremely efective destain.

2.16 Gel Drying and Autoradiography

Gels were mounted on a double layer of Whatman 3MM chromatography paper and dried under vacuum at 80°C on a BioRad dual temperature slab gel dryer (1125B). For the autoradiography of ^{32}P-labelled gels the dried gel was placed in an X-ray cassette (X-Ray accessories Ltd, 16 Rudolph Rd., Bushey, Herts.) with Fuji RX X-Ray film sandwiched between two Dupont lightning-plus intensifier screens. The film was exposed at -70°C initially for 24 hours, and then a second exposure was taken for 3-14 days, the time depending on the intensity of bands on the 24 hour exposure. ^{35}S-labelled gels were autoradiographed by placing the dried gel in an X-Ray cassette with Amersham Hyperfilm-ßmax, a film specially sensitized to ß particle emissions. Autoradiography of ^{32}P-, ^{35}S- double labelled gels was carried out by the method described by Cooper & Burgess (1982). The cassette was loaded in the following order.

1) One sheet Fuji RX X-Ray film for detection of low energy ß-emissions from ^{35}S-label.

2) Dried down gel (face down on film).

3) One sheet of aluminium foil for screening out the low energy ^{35}S-emissions.

4) One sheet of Fuji RX X-Ray film sandwiched between two Dupont Cronex intensifier screens for the detection of high energy ß particle emissions.

The presence of the aluminium foil results in no detection of the ^{35}S-emissions on the ^{32}P film, and it was reported (Dasmarquets et al., 1984) that only a few percent of the high energy ^{32}P-emissions are detectable on the ^{35}S film. Thus discrimination of the two isotopes is virtually complete. Autoradiographs were developed in Kodak LX-24 X-Ray developer and fixed in Kodak FX-40 X-Ray fixer.

2.17 Alkaline Hydrolysis of SDS-Polyacrylamide Gels

As phosphotyrosine is known to be resistant to alkaline hydrolysis while phosphoserine and phosphothreonine are susceptible, this technique was used to attempt to distinguish between these residues on polyacrylamide gels. A similar method was used by Vallejos et al. (1985). A ^{32}P-labelled late exponential phase culture was harvested and subjected to SDS polyacrylamide gel electrophoresis as described in section 2.13. The gel was stained, the tracks marked by puncturing the gel with a hypodermic needle and then treated in hot TCA (as above). The acidity was then neutralized by soaking the gel in 1M NaOH for 15 minutes at 7°C. The first track was then sliced from the gel and the gel incubated at 40°C in 1M NaOH and gel slices removed at intervals from 15 minutes to 260 minutes. On removal slices were transferred to 35% (v/v) methanol, 10% (v/v) glacial acetic acid and soaked overnight. The slices were then dried and autoradiographed.

2.18 Preparation of Protein for Phosphoamino acid analysis

^{32}P-orthophosphate (5 mCi) was added in early exponential phase to a 4 ml culture grown in the absence of added phosphate with buffering capacity supplied by 50 mM HEPES. Cells were harvested in late exponential phase by centrifugation for one minute in an Eppendorf microcentrifuge. The cells were resuspended in 750 µl DNase I buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM 2-mercaptoethanol) and disrupted by sonication. The sonicate was incubated with 30 µg DNase I, 3.6 µg RNase T_1 and 60 µg RNase A for 1 hour at 30°C and then extracted once with 750 µl chloroform/methanol (1:1 v/v). Sufficient 50% (w/v) TCA was then added to the aqueous phase to obtain a final concentration of 10%

(w/v) TCA. The sample was vortexed, kept on ice for 10 minutes, and TCA-precipitable material collected by centrifugation in an Eppendorf microcentrifuge. The proteins in the sample were solublized by heating at 100°C for 5 minutes in 400 µl sample buffer, and insoluble material removed by centrifugation. Ice cold 1 M KCl (100 µl) was then added to the supernatant to precipitate protein-SDS complexes and after 10 minutes on ice the precipitated proteins were collected by centrifugation. The protein pellet was washed once with 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM KCl, once with 95% (v/v) ethanol and finally once with ice-cold acetone before drying. The proteins were then resuspended in 200 µl 6 M HCl and hydrolyzed at 121°C for 3 hours.

2.19 Phosphoamino Acid Analysis

Phosphoamino acids were analyzed by high voltage two-dimensional thin layer electrophoresis by the method of Manai & Cozzone (1982b). HPTLC cellulose plates (BDH, 10x10 cm, 0.1 mm thickness) were used. A cooling plate was required to prevent evaporation of the running buffers at the high voltages used. Separation in the first dimension was caried out at pH 1.9 using 7.8% (v/v) acetic acid, 2.5% (v/v) formic acid. This was carried out at 1600 volts for 15-20 minutes. Second dimension separation was carried out in 5% (v/v) acetic acid, 0.5% (v/v) pyridine (pH 3.5) at 1100 volts for 12-15 minutes. 40 nmoles each of authentic O-phospho-L-serine, O-phospho-L-tyrosine and O-phospho-DL-threonine were mixed with the samples and run simultaneously. These were then revealed by spraying with 0.5% (w/v) ninhydrin in acetone. The plate was then clamped against a similar plate with a sheet of X-Ray film trapped between them.

After autoradiography the film was developed and the positions of the spots on the X-Ray film compared with the ninhydrin stained spots on the plate. The spots were then scraped from the plates with a scalpel into scintillation vials and overlayed with 5ml Beckman EP scintillation cocktail. ^{32}P emissions were counted in an LKB Minibeta scintillation counter.

2.20 Determination of Radiolabel Incorporated in to Whole Cells

A sufficient volume of 50% (w/v) TCA was added to the sample in siliconized Eppendorf tubes to produce a final concentration of 10% (w/v) TCA. The samples were filtered through GF/C glass filter papers, and the tubes rinsed out with 5% (w/v) TCA, the wash also bei[g passed through the glass filters. The filters were washed once with 5% (w/v) TCA, once with ethanol and once with ethanol/ether (1:1 v/v). The filters were dried in an oven at 60°C, put into scintillation vials and overlayed with Beckman EP scintillation cocktail. The samples were counted in an LKB minibeta scintillation counter.

2.21 Immunoprecipitation of Proteins

Immunoprecipitations were carried out by two methods. The main method used was carried out as described by Pringle (1985). A 10% (w/v) suspension of protein A was prepared by suspending 0.1 g of protein A (insoluble, prepared from lyophilized cell powder of Staphylococcus aureus, Cowan strain) in 1 ml 50 mM potassium phosphate buffer (pH 7.5), and vortexed well. The protein was then pelleted in a microfuge and resuspended in the same buffer. This was repeated twice more. Cell extract (450 µl) was taken and 150 µl quadruple strength immunobuffer (150 µl 10% (w/v) protein A in 50 mM potassium phosphate buffer supplemented with 4

mM phenylmethylsulfonyl fluoride (PMSF)) added along with 30 µl pre-immune serum. The sample was incubated on ice for 30 minutes, pelleted in an Eppendorf microfuge for 1 minute and the supernatant collected. The antiserum was diluted fourfold (25 µl antiserum, 50 µl water, 25 µl 4x immunobuffer), and then serially diluted in 1x immunobuffer in 1/2 steps (thus 1/4, 1/8, 1/16, 1/32 e.t.c.). Diluted antiserum (50 µl) was added to 50 µl of pre-immune precipitated extract and the sample incubated on ice for 3 hours. Protein A (50 µl) was added to each sample and the sample incubated on ice for 30 minutes. The sample was then pelleted (1 minute) in a microfuge and the pellet resuspended in 500 mM LiCl, 100 mM Tris-HCl pH 8.5 at 0°C. The sample was then pelleted and washed a minimum of three times. The final time the pellet was resuspended in 100µl Laemmli sample buffer and subjected to SDS polyacrylamide gel electrophoresis.

An alternative, rapid method of immune precipitation was used for some preliminary experiments. This was described by Goswami & Russell (1983). Sample (200 µl) was incubated with 200 µl of antiserum for 1 hour at 4°C. Protein A (100 µl, 10%) prepared as above was added and the sample incubated at 4°C for 30 minutes. After pelleting in an Eppendorf microfuge for 10 minutes the sample was washed three times in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P40 (NP40), 10% (w/v) sucrose before finally resuspending in Laemmli sample buffer as above for polyacrylamide gel electrophoresis.

2.22 Partial Purification of RuBisCo

The partial purification of RuBisCo (Ribulose-1,5-bisphosphate carboxylase-oxygenase) was carried out by centrifugation on a step sucrose gradient as reported by Sani (1985). A step sucrose gradient consisting of equal volumes of 0.2, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8M sucrose in TEMMB buffer (20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2.6H_2O$, 50 mM $NaHCO_3$, 1 mM EDTA, 5 mM 2-mercaptoethanol) was prepared in a 25 ml polycarbonate tube, with the total volume of the gradient being less than 15 ml. In some cases a shelf of 1.75 M sucrose was included at the bottom of the gradient. A 2 ml volume of soluble cell extract (prepared preferably by French pressing) was loaded on top of the gradient and the tube topped with liquid paraffin. The gradients were centrifuged at 240,000 g in a 8x25 titanium angle rotor at 4°C for 2.5 hours. After centrifugation the gradient was maintained in an upright position for 10 minutes before piercing and collecting 1ml fractions from the bottom up. Fractions were assayed for protein using the BioRad protein assay, assayed for RuBisCo activity and run on polyacrylamide gels as described in section 2.13.1.

2.23 RuBisCo Assay

RuBP carboxylase assays were performed by determination of the incorporation of $^{14}CO_2$ from $NaH^{14}CO_3$ (Amersham) into acid stable products (Taylor & Dow, 1980). Assay buffer (150 µl, 100 mM Tris-HCl pH 8.2, 16 mM $MgCl_2.6H_2O$) was added to 5 µl $NaH^{14}CO_3$ (5 µmol at 0.8 $\mu Ci\mu mol^{-1}$), and this mixture then added to 50 µl of extract under investigation. The reaction mixture was incubated for 5 minutes at 30°C before addition of 50 µl of 5 mM ribulose bisphosphate to start the reaction. After a 5 minute

incubation at 30°C the reaction was stopped by the addition of 100 µl 12 M formic acid. A 200 µl sample of the assay mixture was removed and evaporated to dryness at 100°C in a fume hood. The sample was then resuspended in 200 µl of water, overlayed with 3 ml of Beckman EP scintillation fluid and ^{14}C emissions from stable product counted in an LKB minibeta scintillation counter.

CHAPTER 3.

PROTEIN PHOSPHORYLATION IN VIVO

This chapter will be concerned with several aspects of protein phosphorylation in vivo in Rm. vannielii. Initially the development of the protocol for examining protein phosphorylation in this organism shall be described. Later, the results obtained from experiments designed to investigate protein phosphorylation in two major aspects of the physiology of Rm. vannielii, the growth cycle and the differentiation of the swarmer cell, will be presented. It will become apparent that there are numerous difficulties associated with the investigation of protein phosphorylation in Rm. vannielii. Some of these are common to all workers in the field of protein phosphorylation and concern such factors as the characterization of ^{32}P-labelled bands on autoradiographs as being ester-linked phosphopolypeptides, and not DNA, RNA, acyl- or amido-phosphates, polyphosphates, phospholipids or ADP-ribosylated polypeptides. Techniques will be described that achieve this distinction. Other problems are typical of Rm. vannielii, and concern the evidence for a physiological role for protein phosphorylation and the multiplicity of physiological changes undergone by Rm. vannielii through the growth curve and differentiation. In particular, the difficulty in attributing changes in phosphopolypeptide patterns to the stage of growth when substantial changes in the light intensity incident on the individual cells, and on the types of cell present in the culture are also changing. These problems are addressed in more detail later in this chapter.

3.1 ^{32}P Labelling Regime For Rhodomicrobium vannielii

Rm. vannielii was grown in the pyruvate/malate medium of Whittenbury & Dow (1977), but with altered phosphate concentration. The visualization of phosphoproteins required the highest possible specific activity of ^{32}P-orthophosphate, and the establishment of an optimum labelling method. In addition the involvement of protein phosphorylation in metabolic regulation made it desirable to use one system for as much of the work as possible. Thus the system used had to be able to cope with all routine situations. The specific activity of the radiolabel could be increased by three methods:

1) An increase in the amount of radiolabel utilized.
2) A decrease in the culture volume utilized.
3) A decrease in the amount of unlabelled phosphate added to the culture.

The upper constraint on the amount of radiolabel able to be utilized was supplied by safety considerations. In effect 5 mCi or in exceptional circumstances 10 mCi ^{32}P-orthophosphate per experiment was chosen as the upper limit for routine experimental use. The minimum culture volume that could be utilized was limited by several factors, most of which were a consequence of the preparation of homogeneous swarmer cell populations. The minimum culture volume required to obtain a reliable homogeneous swarmer cell preparation was found to be 100 ml. In addition, for the analysis of phosphoproteins in the swarmer cell, 100 ml of culture was required to ensure sufficient cell material with which to work. As a result 100 ml was the minimum culture volume found to be suitable for all experiments. These two factors being determined, the only other method for increasing the specific

activity of the radioactive label was reducing the amount of added cold (unlabelled) phosphate. Whittenbury & Dow (1977) found that cells grown under phosphate limitation spent a long period of time in the filament formation stage of the cell cycle, lengthening the generation time to 18-20 hours. Russell (1984) found that cells grown without phosphate (in addition to that supplied by the 1% inoculum), but with buffering capacity supplied by 5 mM Tris-HCl pH 6.8 showed unchanged morphology and generation time, but only grew to half the cell density attained by cells grown in normal PM medium. Neither of these options were considered satisfactory for the experiments to be performed. What was required was the lowest possible concentration of phosphate capable of supporting growth with unchanged morphology and generation time, and of permitting at least one round of synchronous differentiation in homogeneous preparations of swarmer cells. To determine this phosphate concentration the following experiment was carried out.

3.1.1 Minimum phosphate requirement

Cells were grown in PM medium (Whittenbury & Dow, 1977) with phosphate added to a final concentration of 2.5 mM (the normal concentration), 1.25 mM, 0.625 mM, 0.313 mM, 0.25 mM, 0.125 mM, 0.1 mM and 0.05 mM. Growth was monitored by microscopic observation and optical density measurements at 540 nm. On attaining an OD_{540} of 0.8-1.0 swarmer cells were prepared by filtration through a glass wool column. Homogeneous swarmer cell populations were exposed to the light and allowed to differentiate. The synchrony of the differentiaton was monitored by microscopic observation and cell volume distribution analysis.

Results indicated that at phosphate concentrations of 0.05 mM

the growth rate was considerably reduced, the generation time lengthening to 12 hours and the cells developed extremely long, bipolar prosthecae. In addition, attempted synchronous differentiation of isolated swarmer cells resulted in loss of motility and, in many cases, the formation of short prosthecae, but no apparent entry into the reproductive cycle (Fig 3.1). A phosphate concentration of 0.1 mM resulted in almost normal morphology and generation time (prosthecae are still slightly elongated), and swarmer cell preparations will undergo partial, asynchronous, differentiation (Fig 3.2). A portion of the swarmer cell population still appears not to enter the reproductive cycle. At higher phosphate concentrations culture morphology and ability to undergo synchronous differentiation appears normal. Repetition confirmed these observations, and as a result the standard phosphate concentration for most experiments was fixed at 0.313 mM. This ensured reliable performance (Fig 3.3) and allowed a small safety margin over the reliable 0.25 mM phosphate concentration.

3.1.2 Growth in the absence of phosphate

For phosphoamino acid analysis it was found that considerably lower phosphate concentrations than those determined in section 3.1.1 were required. The prime requisite for this experiment was the highest possible specific activity of ^{32}P-orthophosphate. No ability to undergo synchronous differentiation was required, and as the whole culture was to be used a much smaller volume than was usual could be utilized. It was still necessary, however, that the culture should grow as normal (as determined by growth rate and morphological observations) as possible. Russell (1984) reported that cultures could be grown in the absence of added

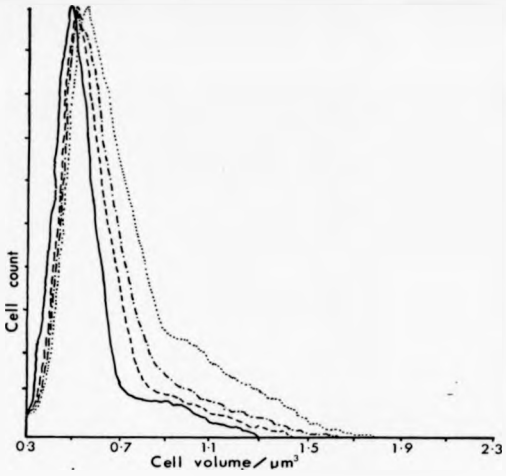


Fig 3.1 Coulter counter profile of the differentiation of a homogeneous population of swarmer cells prepared from a late exponential phase culture grown in the presence of a reduced phosphate concentration of 0.05 mM. The lines indicate profiles taken at different times after the initiation of differentiation, the times being given by / = 0 hours, / = 2 hours, / = 4 hours and / = 6 hours.

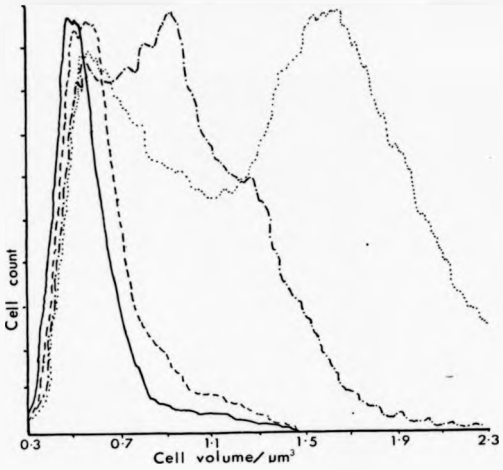


Fig 3.2 Coulter counter profile of the differentiation of a homogeneous population of swarmer cells prepared from a late exponential phase culture grown in the presence of a reduced phosphate concentration of 0.1 mM. The lines indicate profiles taken at different times after the initiation of differentiation, the times being given by / = 0 hours, / = 2 hours, / = 4 hours and / = 6 hours.

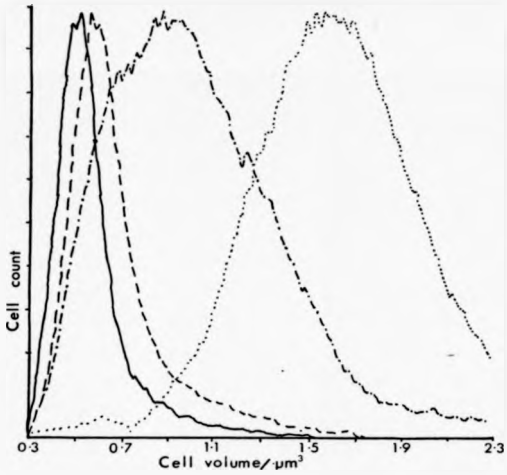


Fig 3.3 Coulter counter profile of the differentiation of a homogeneous population of swarmer cells prepared from a late exponential phase culture grown in the presence of a reduced phosphate concentration of 0.313 mM. The lines indicate profiles taken at different times after the initiation of differentiation, the times being given by / = 0 hours, / = 2 hours, / = 4 hours and / = 6 hours.

phosphate (apart from that contained in the inoculum) and still exhibit normal growth morphology, though such cultures could only attain half the final optical density of cultures grown in the presence of exogenous phosphate. The following experiment was conducted to determine the optimum buffer conditions and inoculum size.

Cultures (4 ml) were grown in the absence of added phosphate and in the absence of replacement buffering capacity. The only exogenous phosphate was supplied by the inoculum which was variable over the range 0.2-10% (Table 3.1a). A control culture was given the standard (Whittenbury & Dow, 1977) concentration of 5 mM phosphate. Growth was monitored after 3 days by microscopic observation, measurement of the culture density (OD_{540}) and measurement of the final pH. The results indicate that even with a 10% inoculum growth was inadequate in the absence of phosphate. In addition the final pH was higher than would be expected for the cell density or the time after inoculation (compared to the graph of pH and absorbance against time of Whittenbury & Dow, 1977). Microscopically the cultures grown in the absence of added phosphate had bipolar, elongated, prosthecae. The experiment was repeated with an inoculum range of 1-2%, with the addition of 5 mM Tris-HCl buffering (Table 3.1b). Though the optical density of the cultures after 3 days was higher and the pH changes not so great with the addition of Tris, the cell density was still far too low for our purposes. In addition, microscopically the culture looked very poor, containing cells with short prosthecae, dark inclusions, a large proportion with distorted morphology, and a considerable amount of cell debris present in the medium. The experiment was again repeated with an inoculum range of 1-2%, with the addition of an alternative buffer, 50 mM

A)

INOCULUM	OD_{540} after 3 days	pH after 3 days
10%	0.192	7.50
5%	0.112	7.40
2%	0.166	7.15
1%	0.110	7.30
0.2%	0.006	6.70

B)

INOCULUM	OD_{540} after 3 days	pH after 3 days
2%	0.26	7.50
1.33%	0.17	7.40
1%	0.26	7.40

C)

INOCULUM	OD_{540} after 3 days	pH after 3 days
2%	1.260	7.10
1.33%	0.325	6.90
1%	0.235	6.90

Table 3.1 Table of the inoculum, final OD_{540} after 3 days and final pH after 3 days in cultures grown in the absence of phosphate. A) No replacement buffering capacity. B) Buffering capacity replaced with 5 mM Tris pH 6.8. C) Buffering capacity replaced with 50 mM HEPES pH 6.8. See section 3.1.2.

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The results indicated (Table 3.1c) that growth rate in the presence of 50 mM HEPES using a 2% (v/v) inoculum was normal and the pH remained extremely stable. Microscopic observation revealed cells of basically normal morphology, the only variation being in the length of the prosthecae which were up to twice the normal length. These results appear to indicate that the transfer of a minimum amount of phosphate in the inoculum, together with the provision of some replacement buffering capacity (though not Tris) are required to permit normal growth. The conditions thus selected for growth in the absence of phosphate were 50 mM HEPES buffering and a 2% (v/v) inoculum.

3.2 Demonstration of Protein Phosphorylation in Rm. vannielii

Cells were harvested and washed in 10 mM Tris pH 8.0, 5 mM $MgCl_2$, 1 mM $CaCl_2$ before breaking by sonication. PMSF and 2-mercaptoethanol were then added to 1 mM and the extract was treated for 1 hour at 37°C with 30 $\mu g ml^{-1}$ DNase I, 3.6 $\mu g ml^{-1}$ RNase T_1 and 60 $\mu g ml^{-1}$ RNase A. Samples were then taken and treated with 16% (final w/v) TCA at 90°C for 5 minutes, 0.1 M NaOH at 37°C for 2 hours, 200 units alkaline phosphaase at 37°C for 2 hours, 100 $\mu g ml^{-1}$ snake venom phosphodiesterase at 37°C for 2 hours and alkaline phosphatase and snake venom phosphodiesterase as above. One sample remained untreated and another was treated with 100 $\mu g ml^{-1}$ protease K at 37°C for 2 hours without prior nuclease treatment. Samples were then precipitated by the addition of 5 volumes of ice cold acetone to remove phospholipids and the samples dried and solubilized in SDS loading buffer for 10-30% gradient SDS polyacrylamide gel electrophoresis (PAGE). After electrophoresis the gel was dried

and autoradiographed before rehydrating and treating in hot TCA (see 2.15). The gel was then dried and autoradiographed a second time. Fig 3.4 shows the autoradiograph of the gel before TCA treatment. The background was clearly too high to reveal detail, but the following general points can be made. RNase and DNase treatments alone were clearly insufficient to remove all the background. This could have been due to incomplete degradation of the nucleic acids, protection of some oligonucleotide sequences by bound proteins, the presence of residual phospholipid in the samples, the presence of loosely bound unincorporated orthophosphate in the gel and/or the presence of polyphosphates in the sample (as Wang & Koshland (1978) hypothesized). The actual reason is almost certainly a combination of these factors, as hot TCA treatment of the sample while greatly reducing the background did not remove it altogether. TCA treatment of samples (as used in some preliminary experiments) was clearly unsatisfactory for sample preparation as it appeared to remove all bands. This may have been due to acid hydrolysis of the proteins in the sample, as it is known that phosphate-ester bonds are stable under these conditions (Cozzone, 1984). Alkaline hydrolysis with 0.1 M NaOH, to which phosphotyrosine should be resistant and other phosphate esters and most ADP-ribosyl linkages susceptible appeared to result in the loss of most radiolabelled bands with the exception of a Mr 20,000 band which possibly indicates that it is phosphorylated on a tyrosine residue (though acyl- and amidophosphate linkages can not be excluded at this stage). Alkaline phosphatase treatment eliminated the majority of the bands visible in the control track, strong evidence for the presence *in vivo* of ester-linked phosphopolypeptides in this organism. In contrast, the addition

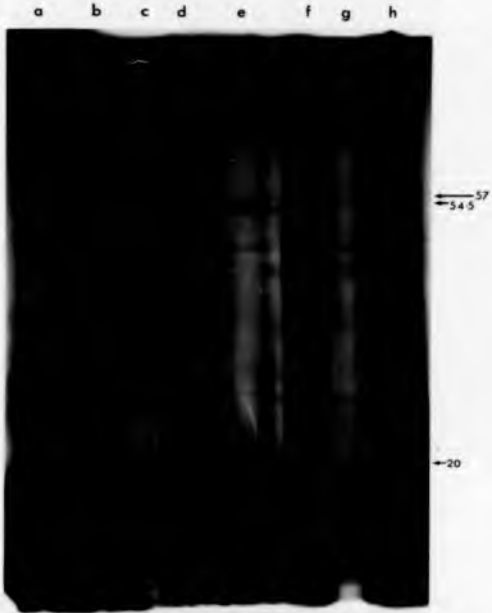


Fig 3.4 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-labelled samples prepared from mid-exponential phase cultures. Treatments are as indicated. a) untreated; b) 30 μgml^{-1} DNase I, 3.6 μgml^{-1} RNase T$_1$ and 60 μgml^{-1} RNase A for 1 hour at 30°C; c) as (b) followed by 16% (w/v) TCA at 90°C for 5 minutes; d) as (b) followed by 0.1 M NaOH at 37°C for 2 hours; e) as (b) followed by 200 units of alkaline phosphatase at 37°C for 2 hours; f) as (b) followed by 100 μgml^{-1} snake venom phosphodiesterase; g) as (b) followed by alkaline phosphatase as (e) and snake venom phosphodiesterase as (f); h) 100 μgml^{-1} protease K at 37°C for 2 hours. Figures on the right indicate Mr values x 10^{-3}.

of snake venom phosphodiesterase resulted in the loss of only a few bands, but the corresponding presence of these bands in the alkaline phosphatase treated track is evidence for the presence of ADP-ribosylated polypeptides. Indeed, it is known that the activity of the nitrogenase iron protein of Rs. rubrum is regulated by ADP-ribosylation (Pope et al., 1985), so it is possible that the iron protein of the Rm. vannielii nitrogenase could be similarly regulated. Treatment of gels with hot TCA is a standard technique used in protein phosphorylation studies (Bhorjee & Pederson, 1976). It has the advantage of eliminating nucleic acids and polyphosphates, two of the major phosphorylated products of ^{32}P-labelling in vivo. In addition it eliminates acylphosphates and amidophosphates, so anything still visible after such treatment must be an ester linked phosphopolypeptide or possibly an ADP-ribosylated polypeptide (though Fig 3.4 suggests that these are uncommon in the cell). Fig 3.5 shows the gel from Fig 3.4 after rehydration, hot TCA treatment, drying and autoradiography. The removal of nucleic acids and polyphosphates by this technique greatly reduced the background and clearly demonstrated the effectiveness of the technique. Despite the capacity of this method to eliminate nucleic acids, the amount of ^{32}P label incorporated into nucleic acids is so large relative to the incorporation into phosphoproteins that DNase and RNase treatment prior to autoradiography resulted in a much reduced background. Treatment of the sample directly with hot TCA clearly resulted in a loss of polypeptides while it can be seen that the Mr 20,000 phosphopolypeptide visible in the alkali hydrolyzed sample was in fact absent in the untreated control. In addition the alkali hydrolyzed sample had lost the Mr 54,500 polypeptide, so it is conceivable that the Mr 20,000 phosphopolypeptide is the

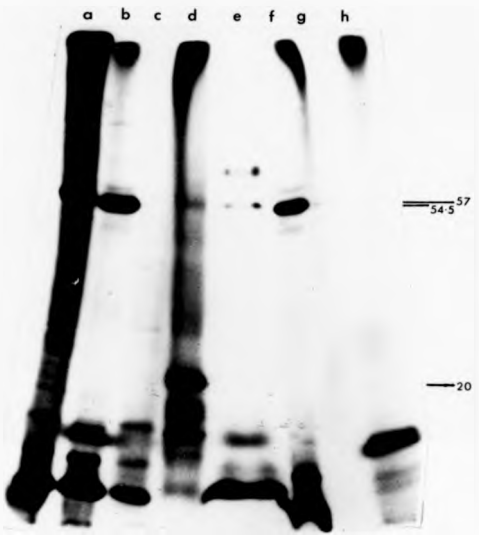


Fig 3.5 Autoradiograph of the 10-30% SDS polyacrylamide gel from Fig 3.4 after treatment with 16% (w/v) TCA at 90°C for 40 minutes. Treatments are as indicated. a) untreated; b) 30 μgml^{-1} DNase I, 3.6 μgml^{-1} RNase T_1 and 60 μgml^{-1} RNase A for 1 hour at 30°C; c) as (b) followed by 16% (w/v) TCA at 90°C for 5 minutes; d) as (b) followed by 0.1 M NaOH at 37°C for 2 hours; e) as (b) followed by 200 units of alkaline phosphatase at 37°C for 2 hours; f) as (b) followed by 100 μgml^{-1} snake venom phosphodiesterase; g) as (b) followed by alkaline phosphatase as (e) and snake venom phosphodiesterase as (f); h) 100 μgml^{-1} protease K at 37°C for 2 hours. Figures on the right indicate Mr values x 10^{-3}.

product of partial hydrolysis of the Mr 54,500 polypeptide. The resistance of this band to alkaline hydrolysis would indicate that the 54,500 polypeptide was phosphorylated on a tyrosine residue. It is also noticable that the loss of the Mr 54,500 phosphopolypeptide revealed a ^{32}P-labelled polypeptide of Mr 57,000. The label on this polypeptide was resistant to alkaline phosphatase, and so is possibly in an ADP ribosyl-protein linkage. This illustrates that under normal circumstances the prominent band visible as the Mr 54,500 phosphopolypeptide may conceal other labelled bands. A second alkaline phosphatase resistant phosphate-labelled polypeptide of Mr 65,000 did not appear in any other track, and is thus possibly associated with the alkaline phosphatase treatment. Phosphodiesterase treatment had little effect on the labelled polypeptide pattern and protease K treatment resulted in the loss of all polypeptides of Mr greater than 14,000. Overall, this experiment demonstrated two things. Firstly that the hot TCA treatment of polyacrylamide gels is a highly effective way of eliminating unwanted phosphate-labelled components and secondly that by far the greatest proportion of bands remaining after such a treatment are ester-linked protein phosphates.

3.3 Alkaline Treatment of Polyacrylamide Gels

In view of the fact that the phospho-amino acid bond of phosphotyrosine residues are resistant to alkaline hydrolysis while the equivalent bonds of phosphoserine and phosphothreonine are susceptible, it was decided to attempt to distinguish between the two susceptibility classes of phosphate-amino acid bond by treating polyacrylamide gels with hot NaOH based on the method of Vallejos et al. (1985). Samples were prepared from a late

exponential phase culture grown in the presence of ^{32}P-orthophosphate. Samples were run on 10-30% SDS-PAGE, and the gels treated with hot TCA as described in 2.15. The gel was then treated with hot NaOH as described in 2.17, with gel strips being removed at 0, 15, 30, 45, 80, 200 and 260 minutes after transfer to hot NaOH. The gel strips were soaked in the same vessel containing 35% (v/v) methanol, 10% (v/v) glacial acetic acid overnight to try and hydrate all the strips to the same size before laying the strips onto filter paper (leveling them at the 10% end of the gel) and drying them under a vacuum. Autoradiography was carried out as described in 2.16.

The autoradiograph (Fig 3.6) revealed firstly that the attempt to hydrate all gel strips to the same size was only partially successful. However there were sufficient bands visible in all gel strips for bands to be correctly correlated. It was possible to divide the detectable phosphopolypeptides into three classes based on susceptibility to the NaOH treatment, namely susceptible, partially susceptible and insusceptible.

The insusceptible class comprised polypeptides of Mr 21,500, 25,500, 29,000 and 33,000. These are almost certainly polypeptides phosphorylated on tyrosine residues. It is interesting to compare these results with those of Vallejos et al. (1985). They identified three polypeptides that appeared to be phosphorylated on tyrosine residues in Rs. rubrum of Mr 27,000, 30,000 and 33,000. The similarity in Mr of the phosphotyrosine containing polypeptides from these two organisms is even more interesting when it is considered that there is little resemblance in the overall pattern of protein phosphorylation between them (Vallejos et al. (1985); Holuigue et al. (1985); Holmes & Allen (1986) and section 3.5 of this

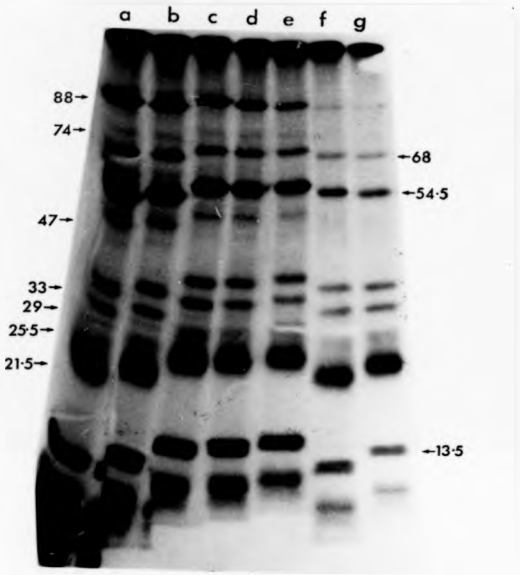


Fig 3.6 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-labelled samples prepared from late exponential phase cultures. The gel was treated with 1 M NaOH at 40°C for the following times. a) 0 minutes; b) 15 minutes; c) 30 minutes; d) 45 minutes; e) 80 minutes; f) 200 minutes; g) 260 minutes. Figures on the left of the picture indicate the Mr values x 10^{-3} of the NaOH susceptible and insusceptible classes of phosphopolypeptide, figures on the right indicate the Mr values x 10^{-3} of the NaOH partially susceptible class of phosphopolypeptides.

thesis). It is possible that the reason for this resemblance is that the three phosphopolypeptides are subunits of a protein that is conserved in both organisms. A possible candidate for such a protein could be the reaction center (RC), which in Rm. vannielii has subunits of Mr 26,000, 28,000 and 31,000, well within the margin for error in Mr determinations on polyacrylamide gels. The published Mr's of the RC of Rs. rubrum, however, are 22,000, 26,000 and 29,000 (see Drews, 1985). The difference between the Mr of these proteins and the observed triplet of phosphopolypeptides is probably too great to be attributed to the differences in the electrophoresis systems, and so the phosphotyrosine containing polypeptides of Rs. rubrum are probably not RC components.

The susceptible class of phosphopolypeptides contained a considerable number of labelled polypeptides including polypeptides of Mr values 88,000, 74,000 and 47,000. Most of them appeared to be present only in small amounts. These polypeptides are probably phosphorylated on serine or threonine, but some, for example the Mr 57,000 polypeptide from evidence described in 3.2, may be labelled with an ADP-ribosyl group.

The partially susceptible class (eg. the Mr 68,000, 54,500 and 13,500 polypeptides) all appeared to loose some label, but after 200 minutes no further loss was observed. There are two possible explanations for this result. Firstly the polypeptides could be phosphorylated on more than one residue, some of which were stable tyrosine residues and some of which were labile serine or threonines. Secondly there could be more than one phosphopolypeptide comigrating to the same position on the gel. If these polypeptides were phosphorylated on different residues the same effect would be seen.

3.4 Phosphoamino Acid Analysis

This experiment was carried out to confirm the presence of ester-linked phosphoamino acids and to determine the relative proportions of the phosphoamino acids present in the cell under the growth conditions used. The experiment was carried out as described in 2.18 and the phosphoamino acid analysis (based on the optimum method determined by Manai & Cozzone (1982)) as described in 2.19. Fig 3.7 shows the ninhydrin stained HPTLC (high performance thin layer chromatography) plate alongside its autoradiograph. ^{32}P-labelled spots could be seen comigrating with the ninhydrin stained authentic phosphoserine, phosphothreonine and phosphotyrosine. This confirms the presence of ester-linked phosphoproteins in Rm. vannielii. The removal and counting of ^{32}P emissions from the spots gave relative proportions of 73% phosphoserine, 13.5% phosphothreonine and 13.5% phosphotyrosine. On comparison with the autoradiograph this result is perhaps somewhat surprising as visually it appears that there is more phosphotyrosine than phosphothreonine. Whatever the exact proportions, it is clear that under these growth conditions phosphoserine is the major phosphoamino acid in the cell. It is also interesting to note that, like Manai & Cozzone (1982), we observed an unidentified spot (labelled X on Fig 3.7).

3.5 Protein Phosphorylation Through The Growth Curve

Enami & Ishihara (1984) reported variation in the pattern of protein phosphorylation through the growth cycle of Escherichia coli. This experiment was designed to look for evidence of growth cycle-dependent changes in phosphopolypeptide patterns in Rm. vannielii. The situation in Rm. vannielii is, however, more

Fig 3.7 a) Photograph of the ninhydrin stained HPTLC plate. The stained spots correspond to the authentic phosphoserine, phosphothreonine and phosphotyrosine standards as indicated. b) Autoradiograph of the plate shown in Fig 3.7a showing the ^{32}P-labelled phosphoamino acids prepared from a protein hydrolysate.

complicated than in Escherichia coli, for example. Under normal photoheterotrophic growth conditions the light intensity within a culture decreases markedly as cell density increases. Thus, care must be taken to distinguish between the effects of progression through the growth cycle and the effects of light limitation. Care must also be taken to ensure that the removal of samples through the growth cycle does not result in a change in light intensity within the culture. Other factors that must be considered are the need to utilize a constant specific activity of radiolabel when pulse labelling, the need to radiolabel an identical number of cells in each sample and the requirement for minimal perturbation of the culture during sampling. In many cases fulfilment of these conditions are mutually exclusive, so three experiments had to be performed.

3.5.1 Pulse labelling through the growth curve

Cultures (500 ml) were grown and samples taken in mid log, late log and stationary phase (Fig 3.8). All samples were diluted back to an OD_{540} of 0.285 in filter-sterilized medium removed from the culture at the same time as the sample. Duplicate 10 ml volumes of the diluted samples were pulse labelled with ^{32}P-orthophosphate for 2 hours to a specific activity of 10 $\mu Ci ml^{-1}$, one for 2 hours in the light, the other spending the second hour in the dark. The cells were harvested by centrifugation and prepared for 10-30% SDS-PAGE as described in 2.9. Fig 3.9 shows the autoradiograph of this gel. During the growth curve there was an apparent increase in the Mr 54,500 phosphopolypeptide, mainly between mid and late log phase. A Mr 14,000 phosphopolypeptide was most prevalent in late log phase and a Mr 12,700 phosphopolypeptide was lost through the growth

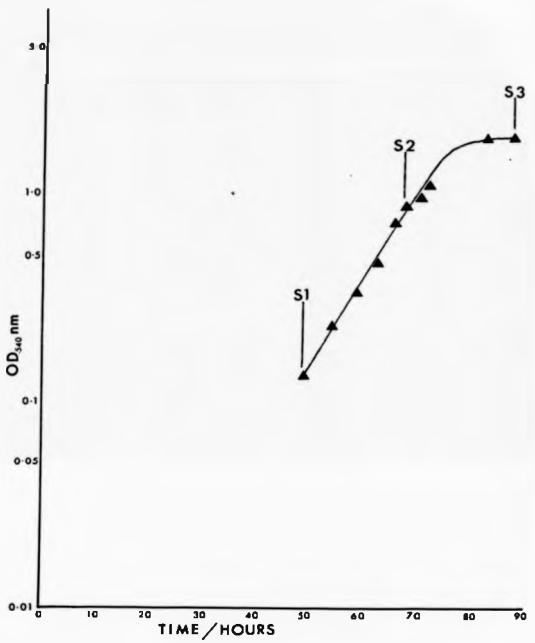


Fig 3.8 Plot of a growth curve with sampling points as indicated. The autoradiograph of the gel on which these samples were run is shown in Fig 3.9.

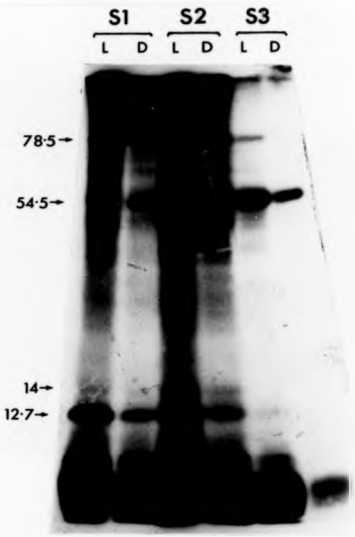


Fig 3.9 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-labelled samples taken at the points on the growth curve indicated in Fig 3.8. L indicates that the sample was pulse-labelled for 2 hours in the light, D indicates that the sample was pulse-labelled for 1 hour in the light, and then left in the dark for 1 hour. Figures on the left indicate Mr values x 10^{-3}.

curve, mainly between late log and stationary phase. The overall picture showed that the level of phosphorylation within the cell is at a maximum during late log phase. As regards the dark shift, it appeared that during exponential growth the only phosphopolypeptide effected was the Mr 12,700 phosphopolypeptide, the amount of which decreased considerably when cells were shifted into the dark. In stationary phase a shift in to dark conditions resulted in a substantial decrease in the levels of all phosphopolypeptides. Changes in the amounts of phosphopolypeptides cannot be fully interpreted as a result of this experiment. As the cultures under investigation were a heterogeneous mix of different cell types, the relative proportions of which vary through the cell cycle, we cannot exclude the possibility of changes in the phosphopolypeptide pattern being due to variations in the phosphopolypeptide composition of the different cell types. This problem is, however, addressed in section 3.7. In addition, a change in the intensity of a band between two pulse-labelled samples could be indicative of a change in the amount of protein available to be phosphorylated, a change in the activity or amounts of a kinase or phosphatase or even a change in the rate of turnover of the polypeptide. The apparent decrease in phosphorylation of stationary phase polypeptides may well have been partially attributable to the latter factor, though the continued high rate of labelling of the 54,500 polypeptide suggests that there is no reduction in the rate of non-specific turnover. Another factor that must be considered is that by labelling a constant number of cells at the same radioactive isotope concentration (by diluting all samples to the same optical density) an element of variable relief from light limitation had been introduced which could have

had a major effect on the rate of phosphorylation, especially if the light harvesting complexes should be phosphorylated and involved in the regulation of photosynthesis (Holmes et al., 1986). This is a possibility, as it is known that the four light-harvesting proteins from Rm vannielii have apparent Mr's of 11,000-14,000 (Kelly & Dow, 1985). Holmes et al. (1986) demonstrated that the phosphorylation state of the equivalent proteins in Rb sphaeroides were under redox control, a factor that may well be influenced by the light regime the cells are exposed to. Alone, then, this experiment is very difficult to interpret so further experiments were performed to verify the results.

Firstly the experiment was repeated, sampling more frequently over a greater area of the growth curve (Fig 3.10), and excluding the dark shift. Fig 3.11 shows the autoradiograph of these samples. There was a clear transition between mid/late log phase (S3) and stationary phase, involving a substantial decrease in the rate of phosphorylation of all polypeptides except the Mr 54,500 polypeptide. This confirmed the results obtained in the previous experiment, and all the comments made for that experiment apply here.

The second pulse-labelling experiment was intended to eliminate the effects of relief from light limitation. Four separate cultures were grown under identical conditions and each harvested at a different point in the cell cycle. Cultures were labelled with identical amounts of ^{32}P-orthophosphate 1 hour prior to harvesting. Fig 3.12 illustrates the superimposed growth curves and indicates the sampling points. Sample volumes were all of equal OD x volume, thus cells were labelled at the same radioactive concentration, and an identical number of cells

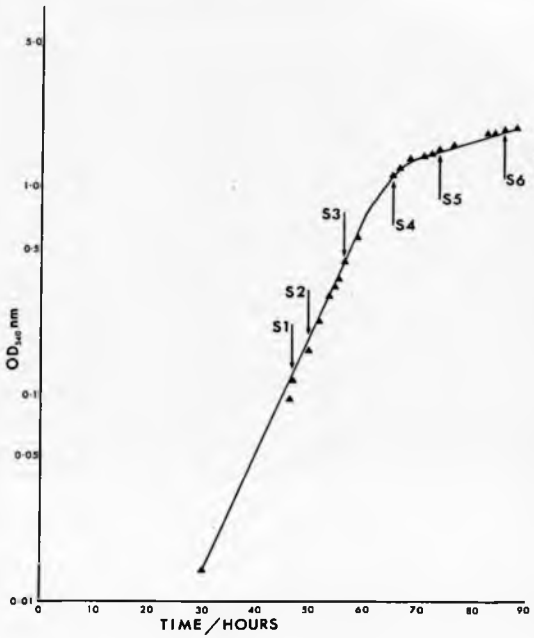


Fig 3.10 Plot of a growth curve with sampling points as indicated. The autoradiograph of the gel on which these samples were run is shown in Fig 3.11.

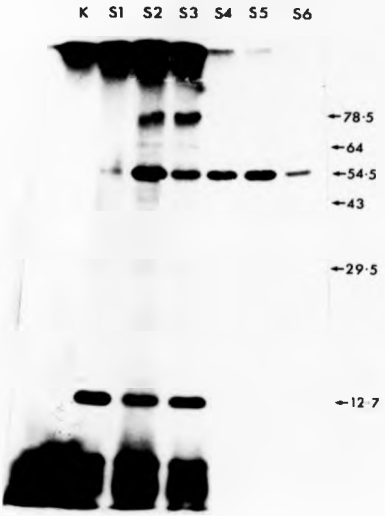


Fig 3.11 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples taken at the points on the growth curve indicated in Fig 3.10. Samples were pulse labelled with ^{32}P-orthophosphate for 1 hour. Figures on the right indicate Mr values x 10^{-3}.

harvested. Samples were prepared and run on SDS-PAGE as described in chapter 2. The autoradiograph of this gel is shown in Fig 3.13. The same trend as in Fig 3.11 was apparent, with the Mr 54,500 polypeptide beginning to be phosphorylated in mid-log phase and subsequently maintaining a high rate of incorporation of ^{32}P. The Mr 12,700 polypeptide was phosphorylated from early log phase, but this ceased between mid and late log phase. This indicates that the results observed in the first experiment were not a result of relief from light limitation as this experiment maintained the cultures in an undisturbed condition while labelling. Protease K treatment was used to confirm that the bands seen were, in fact, proteins. So, these experiments demonstrate that Rm. vannielii phosphorylates proteins at different rates through the growth cycle. While this gives us information regarding kinase activities through the growth curve, it would also be useful to know the phosphorylation state of proteins through the growth curve. To determine this the following experiment was performed.

3.5.2 Continuous labelling through the growth curve

This experiment required that a culture should be grown with ^{32}P-orthophosphate present from early log phase and samples removed at intervals. As a result, the way in which the culture was grown had to be carefully controlled. A culture volume of at least double the sum total of sample volumes to be removed was used, to minimize any perturbations of the culture by the introduction of a large gas space and to help prevent an abnormally high apparent light intensity per cell during stationary phase due to an excesive decrease in the length of the light path through the culture. This last factor was aided by the

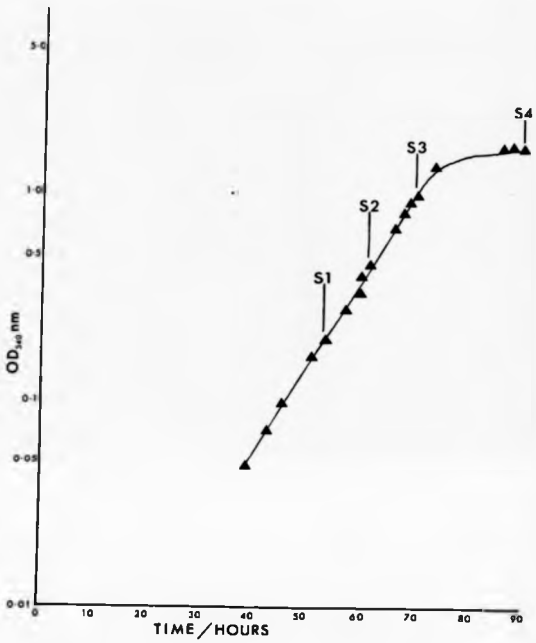


Fig 3.12 Plot of the superimposed growth curves of 4 cultures. The points indicated are the points at which individual cultures were harvested. The autoradiograph of the gel on which these samples were run is shown in Fig 3.13.

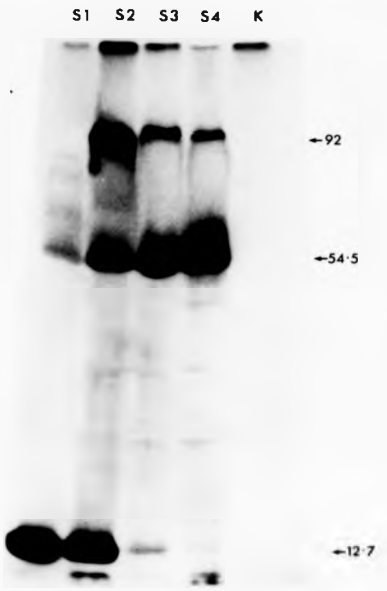


Fig 3.13 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from cultures taken at the points on the growth curve indicated in Fig 3.12. Cultures were pulse-labelled with ^{32}P-orthophosphate for 1 hour prior to harvesting. Figures on the right indicate Mr values x 10^{-3}.

use of the apparatus illustrated in Fig 3.14. The Churchill pump provided accurate temperature control, and the choice of a parallel-sided culture vessel with a perpendicular light source maintained a constant light path length throughout sampling. The culture was labelled in early log phase, sampled as indicated (Fig 3.15) and samples run on 10-30% SDS-PAGE. Fig 3.16 is a long exposure autoradiograph of the resulting gel. This showed a phosphopolypeptide pattern showing the same trends as that obtained by pulse labelling (Fig 3.11). There was a fairly constant amount of phosphopolypeptides during the growth curve, the exceptions being the Mr 12,700 phosphopolypeptide, the amounts of which appeared to decline in late log phase and the Mr 54,500 phosphopolypeptide the amounts of which increased in mid log phase. The longer exposure obscured these points somewhat, but showed that there are over 30 polypeptides labelled by ^{32}P-orthophosphate through the growth curve. Many of these phosphopolypeptides were only visible at particular times in the growth curve (eg. the Mr 19,000 and 84,000 phosphopolypeptides). This experiment revealed nothing about the causes of the changes seen, but if it is considered together with the results of 3.3.1 a clearer picture can be obtained.

So, considering the results of 3.5.1 and 3.5.2 together, the following points can be made:

1) Phoshorylation of a Mr 54,500 polypeptide commenced around mid log phase. Active phosphorylation was occuring throughout the growth curve without any detectable increase in the steady state amount of the polypeptide (after mid-log phase), so the phosphopolypeptide must be subject to a relatively high rate of turnover or to a bidirectional

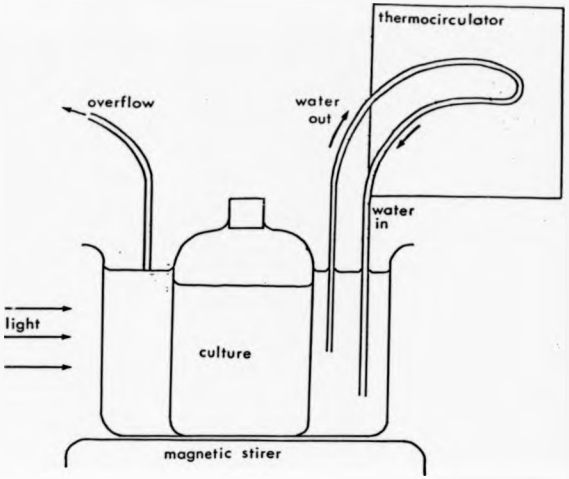


Fig 3.14 Apparatus used for the growth of cultures for ^{32}P-orthophosphate continuous labelling through the growth curve.

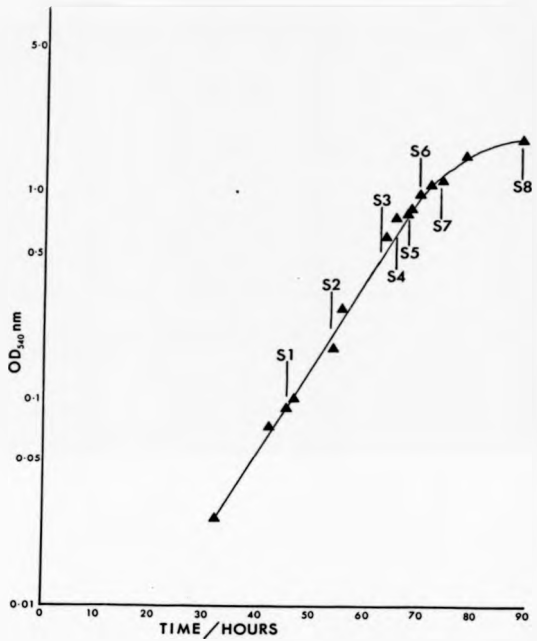


Fig 3.15 Plot of a growth curve with sampling points as indicated. The autoradiograph of the gel on which these samples were run is shown in Fig 3.16.

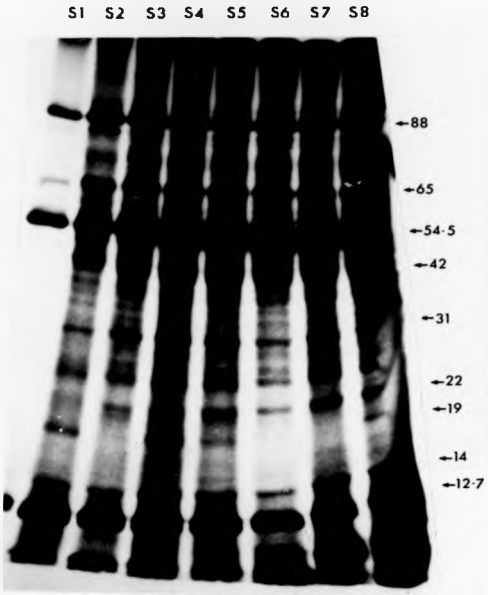


Fig 3.16 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from cultures taken at the points on the growth curve indicated in Fig 3.15. Cultures were continuous-labelled with ^{32}P-orthophosphate from early log phase. Figures on the right indicate Mr values x 10^{-3}.

phosphorylation/dephosphorylation reaction.

2) The Mr 12,700 polypeptide ceased to be actively phosphorylated in late log phase. At this point steady state labelling shows a steady decline in the amount of this phosphopolypeptide, indicating a phosphatase activity or turnover in the absence of kinase activity.

3) Rm vannielii was shown to contain a large number of phosphopolypeptides all through the growth curve. Many of these were only present in small amounts. Some polypeptides labelled prominently under pulse labelling conditions were not detectable as major phosphopolypeptides under continuous labelling conditions (eg. the Mr 78,500 polypeptide in Fig 3.11). This is possibly due to high kinase activity and a high turnover rate resulting in rapid incorporation under pulse labelling conditions, but only a low level of accumulation under continuous labelling conditions. Conversely some major phosphopolypeptides visible after continuous labelling were undetectable under pulse-labelling conditions (eg. the Mr 88,000 phosphopolypeptide in Fig 3.16). This may be due to a low kinase activity phosphorylating a major polypeptide in the cell. Eventually there will be large amounts of the phosphopolypeptide, but it may not show up over the pulse-labelling time scale.

This illustrates how complex the interpretation of these experiments can be. There are always two possible sources of variation, one involving the degree of phosphorylation of a polypeptide and a second involving the actual amount of a polypeptide present. An additional complication is that the rate of protein turnover can affect the phosphorylation pattern in

pulse-labelling experiments. As a result, it is convenient to consider results in terms of changes in the levels of phosphopolypeptides, and changes in the rate of production of phosphopolypeptides.

Regarding the results of phosphorylation through the growth curve the changes in amount and rate of synthesis of the Mr 12,700 polypeptide are of particular interest. If this is a component of light-harvesting complex II, it is useful to compare the phosphorylation of this polypeptide with the phosphorylation of the equivalent polypeptide in Rs rubrum (Holmes and Allen, 1986). They reported the phosphorylation of a Mr 13,000 polypeptide (B880-α) under cooperative conditions and phosphorylation of a Mr 10,500 polypeptide (B880-β) under non-cooperative conditions. The light-harvesting complex II of Rm. vannielii consists of two poypeptides of Mr 11,000 and 13,000 while the reaction center itself is associated with a B885 complex of two polypeptides of Mr 12,000 and 14,000. There is no direct parallel between Rm. vannielii light harvesting subunits and Rs. rubrum light harvesting subunits, as the latter organism only possesses one type of light harvesting complex (see Drews, 1985), however we can see that the observed Mr 12,700 phosphopolypeptide could be a candidate for at least two of the light harvesting complex subunits of Rm. vannielii. In this context the changes in amounts of this phosphopolypeptide through the growth curve and on a shift to dark conditions could be viewed in terms of possible changes in cooperativity (see 1.2.2.4). Loach et al. (1984) suggested that a decrease in cooperativity in the dark was necessary to prevent energy leakage to energy requiring systems associated with the photosynthetic units (eg. alanine transport) in the absence of light. The Mr

12,700 phosphopolypeptide is present in decreasing amounts as stationary phase is entered, and the rate of production of the phosphopolypeptide is virtually zero after late log phase. Thus dephosphorylation of the Mr 12,700 polypeptide would appear to be associated with a decrease in cooperativity analagous to the effect of dephosphoryation of the B880-α polypeptide of Rs. rubrum. A possible alternative to the suggestion of Holmes & Allen (1986) as to how phosphorylation can give rise to an increase in cooperativity can be postulated. If photosynthetic units were arranged so that there was no direct pathway from one reaction center to another via light harvesting complexes cooperativity would be low. Under these circumstances an increase in the lateral mobility of light harvesting complexes by mutual electrostatic repulsion could result in an increase in cooperativity by the interconnection of photosynthetic units. There is evidence that pigment-protein complexes in Rp. palustris are laterally mobile in the membrane and the mobility is responsive to the ionic environment (Holmes & Allen, 1986).

3.6 The Effect of a Protease Inhibitor on the Phosphopolypeptide Pattern

This experiment was intended to investigate the following:

1) It is apparent from the experiments described so far that consideration of the effects of protein turnover can make the interpretation of results difficult. M-aminophenylboronic acid (APBA, a serine protease inhibitor) was used in this experiment to look for phosphoproteins that were being rapidly turned over.
2) The use of two-dimensional non-denaturing/denaturing PAGE was intended to determine the Mr of any proteins of which the

phosphopolypeptides visible on 10-30% SDS-PAGE are subunits.

3) As discussed in 3.2, it is suspected that some bands on one-dimensional gels may be composed of more than one phosphopolypeptide. The use of a two-dimensional gel was intended to help resolve some of these.

Cells were grown in the presence of ^{32}P-orthophosphate. Samples were taken anaerobically at late log phase and incubated for a further hour, one culture having APBA added to 1 mM beforehand. Samples were harvested, resuspended and extracts prepared as described in 2.11c. Two-dimensional non-denaturing/denaturing PAGE was carried out as described in 2.13.4, and the gels treated before autoradiography as described in 2.15.

Fig 3.17 shows the autoradiograph of the non denaturing gel. At this stage there had been no hot TCA treatment, so it was not possible to distinguish between acyl- and amido-phosphate and ester-phosphate linkages. It could be seen that there were two phosphoproteins visible only in the APBA-treated sample, of approximate Mr 130,000 and 99,000. The addition of APBA also appeared to have increased the intensity of some of the bands, in particular the protein of approximate Mr 320,000. Fig 3.18 shows the autoradiograph of the two dimensional gels, Fig 3.18a and b being the autoradiographs of samples prepared in the absence and presence of APBA respectively. The most immediate observation was that in both cases the majority of phosphopolypeptides appeared to be subunits of complexes of approximate Mr 320,000. This was very surprising, but the distribution of minor spots in Fig 3.18a argued against an artifact of gel running. It was also noticable that the amount of label incorporated into phosphopolypeptides was higher in the APBA treated sample. In addition there were some phosphopolypeptides (eg. Mr 80,000, 45,000) for which the

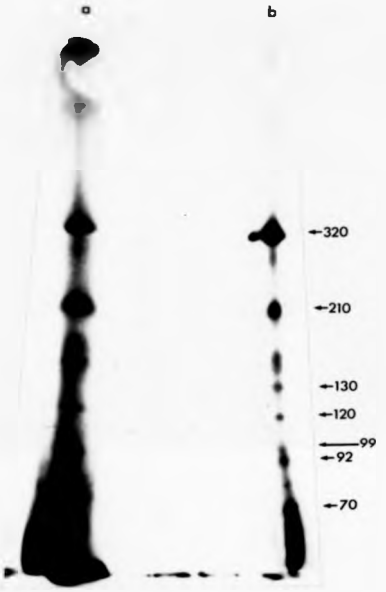


Fig 3.17 4-30% non-denaturing polyacrylamide gel of ^{32}P-labelled extracts prepared from cells incubated for one hour prior to harvesting in the absence (a) and presence (b) of m-aminophenylboronicacid. Figures on the right indicate Mr values x 10-3.

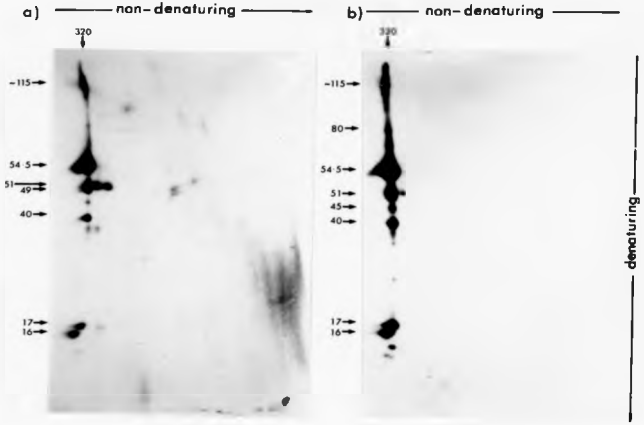


Fig 3.18 Autoradiographs of two dimensional 4-30% non-denaturing, 10-30% denaturing polyacrylamide gels of ^{32}P-labelled extracts prepared from cells incubated for one hour prior to harvesting in the absence (a) and presence (b) of m-aminophenylboronicacid. Figures on the left indicate Mr values x 10-3.

presence of a serine protease inhibitor resulted in a very marked increase in the amount present in the cell. This indicated that the normal level of kinase activity within the cell was considerably higher than that indicated in 3.5, the observation normally being obscured by the effects of protein turnover. It was noticable that, contrary to expectation, there were more visible spots on the autoradiograph of the non-APBA treated sample (Fig 3.18a) than there were on the autoradiograph of the treated sample. This was surprising as we might have expected accumulation of normally turned over phosphopolypeptides in the APBA-treated sample, but why should there have been so many more phosphopolypeptides visible in the untreated sample? There is a possible explanation for this. If the autoradiograph of the non-APBA treated sample is examined (Fig 3.18a), it can be observed that the phosphopolypeptides obtained from the two proteins running immediately below the Mr 320,000 protein(s) on the non-denaturing gel were all subsets of the the phosphopolypeptides from the band of immediately higher Mr. We can interpret this by suggesting that the tracks immediately to the right of the Mr 320,000 protein track represent a Mr 320,000 protein undergoing proteolysis. Initially one specific target for a protease, or the most exposed/susceptible polypeptide, is degraded. This may cause disassociation of further polypeptides or be followed by further proteolysis. The effect is a series of bands on a non denaturing gel, each containing a subset of the polypeptides of the corresponding higher Mr band. The presence of APBA inhibits serine proteases, maintaining the protein intact and resulting in fewer phospholypeptides being visible on the autoradiograph of the APBA-treated sample gel. Evidence for multiple phosphopolypeptides comigrating at Mr 54,500 on SDS-PAGE

could be seen in the presence of the Mr 54,500 phosphopolypeptide in the track derived from the Mr 320,000 protein and in a track of a slightly higher Mr. However, this was inconclusive evidence as it could be argued that this was another case of partial proteolysis of a protein, though this time by a protease other than a serine protease as the double spot is seen in both Fig 3.18a and b.

In conclusion, this experiment demonstrated that the phosphopolypeptides of Rm. vannielii exist mostly as subunits of proteins of Mr 320,000. This figure, due to the nature of non-denaturing PAGE is only an approximation. It also indicates that there is a considerable amount of turnover of phosphoproteins in this organism, and thus the statements made in 3.5 regarding the consideration of results in terms of the rate of synthesis and amounts of phosphopolypeptides were justified. Some phosphopolypeptides seem to be subject to a very high rate of turnover, including phosphopolypeptides of Mr 80,000, 70,000, 45,000, 17,000 and 16,000.

3.7 Cell-Type Specificity of Protein Phosphorylation

It is known that there are a small number of polypeptides unique to either swarmer cells or reproductive cells in Rm. vannielii (Dow et al., 1983). It was observed (section 3.5) that there were changes in the pattern of protein phosphorylation through the growth curve. As the relative proportions of the different cell types being produced varies through the growth curve, it is possible that the changes in phosphopolypeptides seen are a reflection of a different phosphopolypeptide composition in the different cell types. This experiment was intended to investigate the phosphorylation state of polypeptides

in the swarmer and reproductive cells.

Cells were grown in a shaken flask in the presence of ^{32}P orthophosphate to late log phase. Three samples were taken, one of heterogeneous culture, one for preparation of vegetative multicellular arrays (as described in section 2.6) and one for the preparation of swarmer cells (as described in section 2.5). Equal OD_{540} x volume samples were processed for 10-30% SDS-PAGE, and the gel was treated as described in 2.15.

Fig 3.19 shows clearly that despite similar protein loadings, there was apparently no detectable phosphorylation of proteins in swarmer cells prepared from cultures grown under standard photoheterotrophic conditions. Prolonged exposure of the film still revealed no phosphopolypeptides in the swarmer cell extract. This experiment was repeated seven times, with the growth regime being varied between a shaken flask and a stirred bottle. Some cultures showed traces of phosphopolypeptides in the swarmer cell track, but it was found that the amount of phosphopolypeptides seen was inversely proportional to the quality of the swarmer cell preparation. That is, the greater the homogeneity of the swarmer cell preparation (as determined by Coulter Counter analysis and microscopic observation) the fewer and less intense were the visible phosphopolypeptides. Thus it seems that any phosphopolypeptides seen in extracts prepared from swarmer cells were the result of contaminating vegetative cells. It was found that the shaken flask growth regime gave superior swarmer cell preparations, possibly because there was less disturbance of the multicellular arrays by this method resulting in larger arrays that were more readily filtered out. The extracts of the heterogeneous culture and of the multicellular arrays (Fig 3.19) were very similar in their phosphopolypeptide

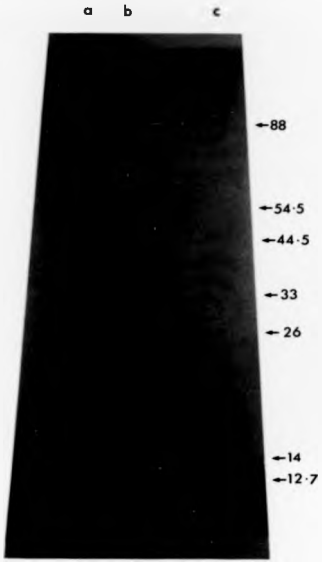


Fig 3.19 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from a ^{32}P continuous-labelled culture harvested in late exponential phase (a) and separated into homogeneous populations of chain cells (b) and swarmer cells (c). Figures on the right indicate Mr values x 10^{-3}.

content, the only visible difference being the presence of slightly greater amounts of a Mr 14,000 phosphopolypeptide in the extract prepared from multicellular arrays. This may be due to the method of isolation of the arrays. The repeated dilutions and concomitant increase in light intensity may have resulted in the phosphorylation of this polypeptide. This experiment also demonstrates that it is safe to ignore the variation in cell type expression through the growth curve in section 3.5, as the swarmer cells that are produced in increasing numbers later in the cell cycle have no influence on the phosphopolypepide pattern. What is the significance of the lack of protein phosphorylation in the swarmer cell? This may prove very difficult to determine. It is possible that the phosphorylation state of polypeptides is important in the onset and control of differentiation. Without the aid of specific inhibitors of protein phosphorylation it may prove impossible to separate cause and effect and thus confirm or deny this suggestion. It is interesting to speculate that if Rm. vannielii possesses any opposingly driven phosphorylation/dephosphorylation control systems of the type used to control isocitrate dehydrogenase activity in E. coli, then they must be abandoned in the swarmer cell in favour of either an alternative, less energy intensive, control mechanism or in favour of no control at all. If the latter alternative is the case, then the dephosphorylated form of the polypeptides must represent the state in which the polypeptide is found in the swarmer cell. Thus proteins with an activity regulated by protein phosphorylation must be active in the dephosphorylated form if they are required to be active in the swarmer cell.

3.8 Protein Phosphorylation During the Differentiation of the Swarmer Cell

The observation (section 3.7) that there are no phosphopolypeptides in the swarmer cell but a substantial number in the vegetative cell implies that at some stage during differentiation protein kinases must be activated or induced. The following experiments were carried out to investigate protein phosphorylation through swarmer cell differentiation.

3.8.1 Pulse labelling through differentiation

Cells were grown and harvested as described in section 2.9. A homogeneous population of swarmer cells was prepared by filtration into a covered, pre-gassed bottle (as described in section 2.5). Synchronous differentiation was initiated by exposing the swarmer cell population to light, and samples were removed at 1.5, 3.4 and 5.5 hours after the initiation of differentiation. Samples were incubated for a further hour under the same conditions as the culture from which they were derived in the presence of 10 $\mu Ci.ml^{-1}$ ^{32}P-orthophosphate. A sample taken before the initiation of differentiation was incubated for 1 hour in the dark under the same labelling conditions to provide a zero time sample. Identical volumes were taken for all samples. Samples were harvested (2.9) and subjected to 10-30% SDS-PAGE. The gel was then treated with hot TCA (2.15) before drying and autoradiography (2.16). The autoradiograph (Fig 3.20) shows how there was a substantial increase in the rate of phospholypeptide production during differentiation, with the rate of production of the Mr 55,000 phosphopolypeptide showing the greatest increase. As with the studies of protein phosphorylation through the growth curve, there are numerous complications with the interpretation

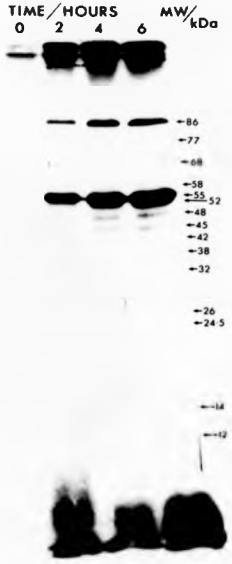


Fig 3.20 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from differentiating swarmer cells pulse-labelled with ^{32}P-orthophosphate one hour before harvesting. Time indicates the number of hours into differentiation half way through the labelling period, figures on the right indicate Mr values x 10-3.

of these results. The first factor that must be accounted for is that as the sample treatments and gel loadings were based on using an equal number of cells, the protein loading in the track of the 6 hour sample would be approximately double that of the 0 hour sample. Sample times were designated as being the mid-point of the labelling duration, thus the sample labelled from 5.5-6.5 hours post-initiation of differentiation is designated the 6 hour sample. However, this can only account for a two-fold difference in the rate of ^{32}P incorporation, a more serious problem is that the rate of ^{32}P uptake through the cell cycle is unknown. Experiments carried out (data not shown) indicate that ^{32}P uptake does occur in the swarmer cell, but bearing in mind the effect of even slight heterogeneity of swarmer cell preparations on the demonstration of the absence of phosphopolypeptides in the swarmer cell (3.7) it must be possible that contaminating vegetative cells are responsible for the uptake. Indeed, it is possible that slight heterogeneity of the swarmer cell preparation is the reason for the trace levels of polypeptide phosphorylation visible in the dark-incubated swarmer cell (0 hour) track of Fig 3.20. It can be suggested that, being grown under conditions of low phosphate, the differentiating swarmer cell is taking up phosphate at the maximum possible rate from the start. However, the rate of ^{32}P incorporation into phosphopolypeptides is dependent on the internal specific activity of ^{32}P. This is not only dependent on the rate of uptake of ^{32}P, but also on the presence or absence of stored cold phosphate, and the rate of release of the cold phosphate from these stores. These factors are normally not so important due to the presence of internal controls, phosphopolypeptides that are phosphorylated to a constant degree against which comparisons can

be made. On the basis of this principle, using the Mr 86,000, 77,000, 68,000, 42,000, 38,000, 32,000 and 24,500 phosphopolypeptides as internal controls, on the basis that they all show a steady increase in kinase activity through differentiation, we can draw the following, tentative, conclusions:

a) There was little or no protein kinase activity in the inhibited (dark-incubated) swarmer cell in *vivo*.

b) Within two hours of the induction of differentiation by light, the activity of a majority of the kinases was at a maximum.

c) The rate of phosphorylation of the Mr 55,000 polypeptide continued to increase throughout differentiation. This may be a reflection of a high rate of synthesis of the substrate polypeptide.

d) One phosphopolypeptide of Mr 26,000 appeared to be exclusively produced towards the end of the reproduction phase, and may thus be concerned with specific events at this time.

e) As in d), one polypeptide of Mr 24,500 was being predominantly phosphorylated at four hours after the initiation of differentiation.

This experiment was repeated using an alternative method of sample preparation. In this case, the experiment was performed as described above, but samples were prepared for gel electrophoresis by the method used for the preparation of proteins for phosphoamino acid analysis (2.18), with the exception that rather than being resuspended in 6 M HCl, samples were finally resuspended in Laemmli (1970) sample buffer. Fig 3.21 shows the autoradiograph of this gel. Some of the 6 hour

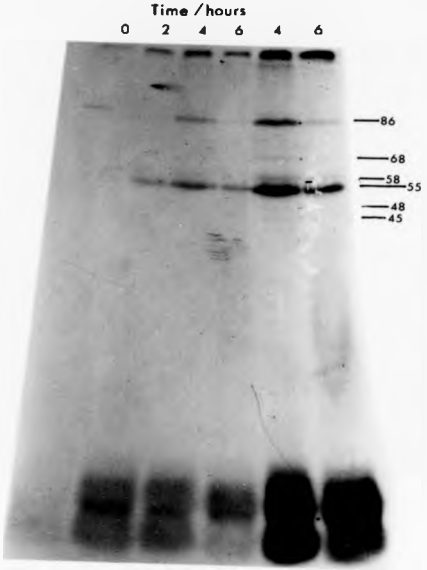


Fig 3.21 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from differentiating swarmer cells pulse-labelled with ^{32}P-orthophosphate one hour before harvesting. Time indicates the number of hours into differentiation half way through the labelling period. The second set of 4 and 6 hour samples have 4 times the loading of the other samples. Figures on the right indicate Mr values x 10-3.

sample was lost during the processing, which explains the reduced band intensity of the 6 hour track in comparison to the 4 hour track. The extensive processing of these samples, and the inevitable loss of protein at each subsequent stage mean that it would probably be extremely difficult to get a stronger signal using this method than was obtained here. The picture is, however, clear enough to observe that (except for the 6 hour sample) the autoradiograph corresponded very well with Fig 3.20, thus confirming the observations made there. Secondly, this illustrates that the method used for the preparation of proteins for phosphoamino acid analysis (section 3.4) does not appear to give rise to the purification of a subset of the phosphopolypeptides in the cell, all the major phosphopolypeptides are represented in (as far as can be determined from the autoradiographs) the correct proportions.

In order to eliminate problems due to the variation in the internal specific activity of ^{32}P label, and to obtain a picture of the phosphopolypeptide composition of cells at various stages through the cell cycle, experiments were carried out utilizing a continuous labeling regime.

3.8.2 Continuous labelling through the cell cycle

These experiments were carried out to examine the phosphopolypeptide composition of swarmer cells through differentiation. Cells were grown in the presence of ^{32}P-orthophosphate, and swarmer cells prepared when the culture reached late log phase (see 2.5). The covered collection vessel was exposed to the light, and samples taken at intervals for preparation for SDS-PAGE (see 2.9). Fig 3.22 shows the autoradiograph of an experiment in which samples were removed

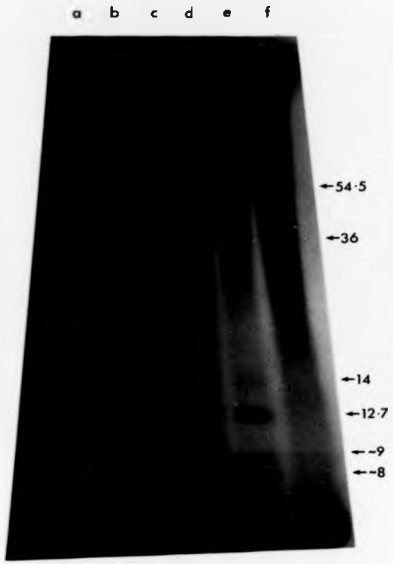


Fig 3.22 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from differentiating swarmer cells continuous-labelled from early exponential phase with ^{32}P-orthophosphate. Time into differentiation on sampling is given as a) 0 hours, b) 2 hours, c) 4 hours, d) 6 hours, e) 6 hours differentiation in the presence of 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole, f) a mixture of all samples treated with 100 $\mu g ml^{-1}$ protease K. Figures on the right indicate Mr values x 10-3.

immediately after synchronization and at 2, 4 and 6 hours after the initiation of differentiation. In addition, one sample was removed after synchronization and allowed to differentiate for 6 hours before preparation for SDS-PAGE in the presence of 1 mM 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole, a suspected inhibitor of protein kinases in eukaryotes (Egyhazi et al., 1984). The autoradiograph shows that, as was observed in section 3.7, there was little or no protein phosphorylation in the undifferentiating swarmer cell. After the initiation of differentiation protein phosphorylation commenced, and the phosphopolypeptide content of the cell increased, attaining a maximum steady level by 4 hours into differentiation. The eukaryotic protein kinase inhibitor, 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole, had no apparent effect on protein phosphorylation in Rm. vannielii. Whether this was due to a lack of uptake by the organism or the lack of susceptibility of the kinases to this compound is not known. The Mr 26,300 phosphopolypeptide reported to be produced only at the end of the reproduction phase (3.8.1) could be seen as a very faint band in the 6 hour track. The Mr 24,500 phosphopolypeptide being produced only around 4 hours into differentiation could not be seen. It is possible that the four low Mr bands at the bottom of the gel are the four polypeptides of light-harvesting complex (LHC) I and II. The Mr determinations noted for the lower three of these phosphopolypeptides in Fig 3.22 are estimations based on an extrapolation of the standard curve, and thus are only very approximate. If these are the LHC polypeptides then it can be seen that the polypeptides corresponding to the LHC II polypeptides undergo a change in phosphorylation state through differentiation, the lower Mr polypeptide showing the greatest

degree of phosphorylation early in differentiation and the higher Mr polypeptide showing the greatest degree of phosphorylation late in differentiation. We can compare this to the changes in the phosphorylation state of the light harvesting polypeptides of Rs. rubrum observed by Holmes & Allen (1986). They observed that the lower Mr polypeptide was phosphorylated under non-cooperative (dark-incubated) conditions and the higher Mr polypeptide was phosphorylated under cooperative conditions. As the Rm. vannielii swarmer cells are produced under conditions of light limitation it is possible that the LHC II protein in Rm. vannielii is behaving in an analagous fashion to the LHC protein of Rs. rubrum, and is similarly involved in the regulation of excitation energy distribution.

This experiment was repeated several times with the result that:

1) The above results were confirmed.
2) Once again it became apparent how vitally important it was to obtain high quality (i.e. with very low heterogeneity) swarmer cell preparations.

The highly phosphorylated state of vegetative cells in comparison with the swarmer cells meant that even small amounts of contamination of the swarmer cell preparation with vegetative cells obscured the details described above.

The above experiment demonstrated that phosphorylation in the swarmer cell began sometime before 2 hours after the initiation of differentiation. The following experiment was intended to examine more closely the early stages of differentiation by sampling before initiation, and at 15, 30, 60, 180 and 360 minutes after the initiation of differentiation. A further modification to this experiment was that in an attempt to improve

the homogeneity of the swarmer cell preparations, cultures were covered and kept in the dark for two hours prior to selective filtration. The principle behind this was that this may allow those cells already committed to differentiation to do so, increasing their size and altering their morphology (by the development of prosthecae and buds) with the result that they would be more easily trapped during the filtration. Fig 3.23 shows the autoradiograph obtained from this experiment. From this it appeared that the principle of covering the culture for a period of time prior to swarmer cell preparation is sound. Negligible phosphopolypeptide content (except for a Mr 36,000 phosphopolypeptide) was observed in the undifferentiating swarmer cell. This can be attributed to an effect of the synchrony quality rather than the effects of a dark incubation period on all cell types as it was observed that swarmer cells contain no phosphopolypeptides (section 3.7). Fig 3.23 demonstrated that phosphopolypeptides only started to accumulate in the cell around 1 hour after the initiation of differentiation, and reached maximum amounts by three hours. The Mr 36,000 phosphopolypeptide, whilst visible in Fig 3.22, did not show the prominence and specificity for the early half of differentiation seen here. Because of this unusual prominence, it may be that this phosphopolypeptide can be attributed to the extreme light limitation imposed on the culture pior to swarmer cell preparation. As before, there was a large increase in the Mr 12,700 phosphopolypeptide through differentiation. This experiment, then, confirms and expands on the result of the previous experiment.

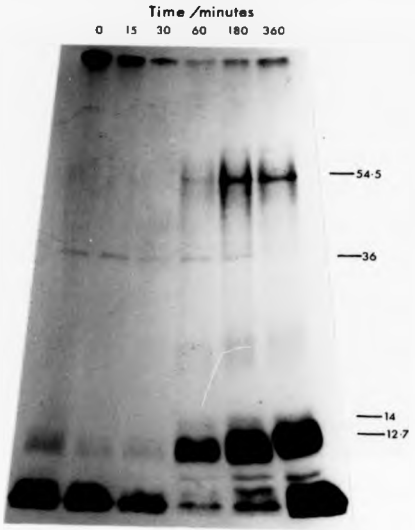


Fig 3.23 Autoradiograph of a 10-30% SDS polyacrylamide gel of samples prepared from differentiating swarmer cells continuous-labelled from early exponential phase with ^{32}P-orthophosphate. Time into differentiation on sampling is given in minutes, figures on the right indicate Mr values x 10-3.

Considering the results of the pulse labelling and continuous labelling experiments together, we can draw the following conclusions:

1) There were no detectable phosphoproteins in the non-differentiating swarmer cell.
2) Phosphopolypeptides did not begin to accumulate in the swarmer cell until 1 hour into differentiation.
3) The lack of accumulation of phosphopolypeptides earlier in differentiation, the lack of phosphopolypeptides in the inhibited swarmer cell and the lack of observable kinase activity in the inhibited swarmer cell suggested that there was no kinase activity in dark-incubated swarmer cells in vivo.
4) As differentiation proceeded, and cells moved from being under conditions of light limitation to a high light intensity, there was a gradual change in the distribution of the low Mr phosphopolypeptides. The low Mr phosphopolypeptides are approximately the right Mr to correspond to the light-harvesting complex polypeptides. This could correlate with the hypothesis of Allen & Holmes (1986), involving phosphorylation of the light harvesting complexes with cooperativity changes in response to changing light conditions.
5) The rate of phosphorylation of the Mr 54,500 polypeptide continued to increase through differentiation. However, no increase in the accumulated amount of the phosphopolypeptide was seen after 3 hours into differentiation. This implies that from 3 hours onward during differentiation there must be an increasing amount of turnover of the Mr 54,500 phosphopolypeptide and/or an increasing amount of Mr 54,500

phosphopolypeptide phosphatase activity.

This chapter, then, demonstrates clearly that protein phosphorylation occurs in Rm. vannielii. It is also clear that there is an association between the phosphopolypeptide pattern and the physiological state of the cell. As is often the case, whether these changes are required for adaptation to the required physiological state or occur as a result of the change in physiological state can not be ascertained from these experiments. This chapter also illustrates the care needed in performing and interpreting the results of experiments that by necessity vary in more than one factor. Having generally examined protein phosphorylation in Rm. vannielii in vivo it was decided to complement these studies with an investigation into kinase activities in vitro, with the aim of being able to examine factors with the potential of controling kinase activities. These results are considered in chapter 4.

CHAPTER 4.
PROTEIN PHOSPHORYLATION IN VITRO

In this chapter work carried out on the activity of protein kinases in cell free extracts of Rm. vannielii will be described. Much of this work was concerned with the effect of cations on kinase activities, and the development of a system for studying this.

The advantages associated with in vitro studies are numerous. Firstly, it eliminates the problems associated with the incorporation of radiolabel into nucleic acids and phospholipids, resulting in cleaner gels and the opportunity to work with much smaller amounts of radioisotope with all the corresponding safety advantages. Secondly, the reaction conditions can be modified, allowing studies on factors modulating kinase and phosphatase activities to take place. Thirdly, the experiments themselves are usually much easier and faster to perform than in vivo studies and finally the ability to carry out a whole series of experiments on one previously prepared extract has the advantage of a level of consistency probably unobtainable in vivo.

The singular major disadvantage with in vitro studies is that the phosphopolypeptide patterns visualized rarely show much resemblance to the patterns observed from experiments performed in vivo (see section 1.4). As discussed in section 1.4, there are several possible explanations for this. Firstly, all the results obtained in vitro could be artifacts, produced by non-specific phosphorylation of proteins normally compartmentalized away from the kinases. This is unlikely as prokaryotic kinases appear to be

highly specific, with no generalized phosphorylation as is observed for protein kinase C in eukaryotes. A second possibility is that they are polypeptides not normally phosphorylated (or phosphorylated to a very low level) in vivo due to the growth conditions used. The conditions used for in vitro kinase assays are non-physiological, and this could lead to the activation and inactivation of a different set of kinases from those seen in vivo. A third possibility is that the proteins seen to be phosphorylated in vitro are proteins that are phosphorylated in vivo, the differences in apparent Mr arising from the further processing (e.g. proteolytic cleavage) of the protein in vivo. This would only apply in cases where phosphorylation takes place early post-translationally (before further modification). Other possibilities include, during the extract fractionation procedure, the loss of regulatory polypeptides, the separation of the kinase or phosphatase and their substrate (if the substrate is both phosphorylated and dephosphorylated simultaneously in vivo the removal of one enzyme will have a marked effect on the phosphopolypeptide pattern) and the degradation of particularly unstable kinases or phosphatases. In conclusion, I believe that the phosphorylation observed in vitro is significant, though not representative of the condition in which the cells are grown.

4.1 The Effects of cAMP and cGMP on Protein Phosphorylation In Vitro

The aim of this experiment was to determine whether any cAMP- or cGMP-dependent protein kinase activities could be observed in cell-free extracts of *Sm. vannielii*. Cyclic-AMP and cGMP were used in conjunction with added Ca^{2+} or Mg^{2+} in case these cations were required as cofactors. Crude cell-free extracts were

prepared by the method described in section 2.11a, with the exception that the Mg^{2+} was absent from the kinase buffer and the 38,000 g spin after cell breakage to remove particulate material was substituted by a 5 minute 12,000 g spin in an Eppendorf 5412 microcentrifuge to remove whole cells, cell debris and large particulate material. Kinase assays were carried out in the presence of the following supplements to the standard (minus Mg^{2+}) kinase buffer:

a) 20 μM cAMP

b) 20 cAMP, 1 mM $MgCl_2$

c) 20 cAMP, 1 mM $CaCl_2$

d) No additions

e) 20 cGMP

f) 20 cGMP, 1 mM $MgCl_2$

g) 20 cGMP, 1 mM $CaCl_2$

The kinase assay was stopped by the addition of double strength Laemmli sample buffer and 10-30% gradient SDS-PAGE was carried out on the samples. The gel was treated with hot TCA as described in section 2.15. The autoradiograph obtained is shown in Fig 4.1. The first observation was that, as in so many other cases (see section 1.4) the pattern of phosphopolypeptides appeared to bear little, if any, resemblance to the phosphopolypeptide pattern observed from *in vivo* experiments. This, as was discussed in section 1.4, is perhaps not surprising. It does, however, set the pattern for much of this chapter as many of the subsequent experiments were designed to examine the effect of various additions to the kinase assay buffer, especially with regards to the activation of kinase activities producing a similar phosphopolypeptide pattern *in vitro* to that seen *in vivo*. On further examination of Fig 4.1 it could be seen that there appear

Fig 4.1 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled cell free extracts of Rm. vannielii suplemented with: a) 20 µM cAMP; b) as (a) plus 1 mM $MgCl_2$; c) as (a) plus 1 mM $CaCl_2$; d) no supplement; e) 20 µM cGMP; f) as (e) plus 1 mM $MgCl_2$; g) as (e) plus 1 mM $CaCl_2$. Figures on the right indicate Mr values x 10^{-3}.

to be few if any differences between the tracks, except for those of the assays carried out in the presence of Mg^{2+}. In these cases the labelling and background appears to be heavier but still with no specific changes in the phosphopolypeptide pattern. We were, then, unable to detect any cAMP or cGMP-dependent protein kinase activity in Rm. vannielii cell-free extracts. It is perhaps not surprising that little effect was observed with the addition of Ca^{2+} and Mg^{2+}. In the crude extracts used the amount of endogenous Mg^{2+} released on cell breakage may have been considerable. Bacteria are known to contain high levels of Mg^{2+} (see Walker, 1986). Much greater control over the constituents of the kinase assay mix is required if the effects of the addition of cations are to be meaningfully investigated.

4.2 The Effect of GTP on Protein Phosphorylation In Vitro

GTP-binding proteins are well documented in eukaryotes, possibly the best characterized example being eukaryotic initiation factor-2 (eIF-2) (Proud, 1986). This protein is involved in the control of translation (see section 1.2.2.2), the transfer of guanine nucleotides, and the prevention of this transfer by the phosphorylation of eIF-2, being an essential part of the control mechanism. Prokaryotic initiation factor-2 is also known to bind GTP, along with other proteins involved in protein synthesis (eg EF-Tu, EF-G) (see Hughes, 1983), and GTP is thought to be involved in controlling Bacillus subtilis sporulation (Ochi et al., 1982). GTP-binding proteins (G-proteins) have been suggested as a distinct class of regulatory proteins (Hughes, 1983), and on this basis this experiment was performed to test the effect of GTP on the in vitro kinase activity of Rm. vannielii.

Crude cell-free extracts were prepared by the method described in section 2.11a. Kinase assays were carried out in the presence of GTP suplements to the standard (2.11a) kinase buffer to a concentration of 0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μM. Kinase assays were stopped as usual by boiling the sample in double strength Laemmli sample buffer. The samples were then subjected to electrophoresis on 10-30% gradient SDS-polyacrylamide gels. The gel was subsequently treated with hot TCA as described in section 2.15. Fig 4.2 shows the autoradiograph of the gel obtained. Under the conditions used there was no demonstrable effect of concentrations of GTP up to 10 μM. As a negative result this does not tell us very much. It may be that the concentration of GTP required to elicit a detectable response in kinase/phosphatase activities is higher, or that some other factor in addition to GTP is required. What is noticable is that the phosphopolypeptide pattern is very similar to that observed in Fig 4.1, the difference between the two extracts used being in the degree of "cleaning up" of the extract that took place after breakage. This seems to indicate that the amount of particulate material in the extract has no effect on the phosphopolypeptide pattern under the conditions used, and thus that there are no active membrane bound kinases or phosphatases in the extracts, and no membrane bound substrate polypeptides.

4.3 The Effect of Different Extraction and Assay Conditions on Protein Phosphorylation In Vitro

The results presented in the previous two experiments have been discussed in terms of the observed effects being specific to the conditions used to prepare and assay the kinase activies of the extracts used. Enami & Ishihama (1984) found that the preparation

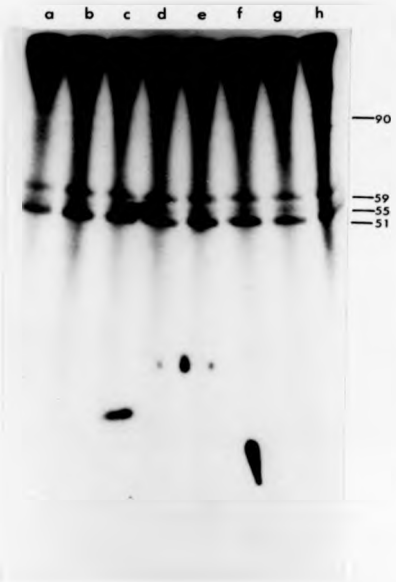


Fig 4.2 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled cell free extracts of Rm. vannielii suplemented with GTP to the following concentrations: a) no supplement; b) 0.1 μM; c) 0.2 μM; d) 0.5 μM; e) 1 μM; f) 2 μM; g) 5μM; h) 10 μM. Figures on the right indicate Mr values x 10^{-3}.

of a cell lysate after prior treatment with Brij-58 and lysozyme resulted in much greater kinase activity, and the phosphorylation of a different set of polypeptides when compared to extracts prepared in the absence of lysozyme and Brij-58. Moreover, the pattern of phosphopolypeptides observed on SDS-polyacrylamide gels compared well with those observed from in vivo labelling experiments. This experiment was intended to examine the effect of cell lysate preparation after prior treatment with lysozyme and Brij-58 on the kinase activity of the extracts. In addition, assay conditions were varied to include a system similar to that used by Enami & Ishihama (1984).

Cell free extracts were prepared by the method described in section 2.11b. Kinase assays were subsequently carried out under various conditions, as follows:

All samples contained the following components, 20 mM Tris-HCl pH 8.1, 12.5% (w/v) sucrose, 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM EDTA. In addition, individual samples were further treated as follows:

a) No additions.

b) Centrifugation in an Eppendorf microfuge at 12,000 g for 10 minutes to remove large particulate material.

c) 1 mM magnesium acetate, 0.1 mM APBA, 0.4 mM $MnSO_4$.

e) 55mM EDTA (net concentration after chelation of known metal ion content 6 mM)

f) 1 mM magnesium acetate, 0.1 mM APBA, 0.4 mM $MnSO_4$, 5 mM $CaCl_2$.

g) 1 mM magnesium acetate, 0.1 mM APBA, 0.4 mM $MnSO_4$ for kinase assay, 8 µg protease K added after boiling of sample.

The kinase assays were stopped as usual by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot

TCA as described in section 2.15. The autoradiograph of this gel is shown in Fig 4.3. The most striking change in the kinase activities of extracts could be seen in track 4, the EDTA treated track. Most bands were lost altogether and others (eg. the Mr 55,000 phosphopolypeptide) were greatly reduced in their labelling intensity. This might be expected, as ATP is used as a Mg^{2+}-ATP complex (see Knowles, 1980), and EDTA results in the chelation of the metal ions in the extract. However, in addition to the bands lost on EDTA treatment two new bands became apparent, one of Mr 35,500 and a second, very intense band, of Mr 16,000. The residual phosphorylation of some proteins on removal of Mg^{2+} could be explained by the presence of some kinases with a high affinity for their substrate and ATP being able to phosphorylate proteins to a small extent in the absence of Mg^{2+}, or alternatively, the mechanism of phosphorylation might involve some form of high affinity Mg^{2+} binding protein that still retained bound Mg^{2+} after its removal with EDTA. This does not, however, explain the phosphorylation of two new polypeptides. One possible explanation is that there are kinases capable of phosphorylating proteins at the expense of ATP, in a manner idependent of Mg^{2+}. The reason for the absence of any visible band in the other tracks could be attributed to the presence of a ATP.Mg^{2+}-dependent phosphoprotein phosphatase activity such as is observed in rabbit skeletal muscle tissue (Vandenheede, 1980). Another alternative is that we are looking at a protein with a very high affinity for ATP, and the band on the gel is a ^{32}P-γ-ATP-protein complex. This seems unlikely, as the sample was boiled in SDS loading buffer to denature the proteins prior to loading on the gel, and the gel was subsequently subjected to treatment in hot TCA. It is unlikely that any non-covalent

Fig 4.3 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled cell free extracts of Rm. vannielii suplemented with, or subjected to, the following prior to labelling: a) No additions; b) Centrifugation at 12,000 g for 10 minutes; c) 1 mM magnesium acetate, 0.1 mM APBA, 0.4 mM $MnSO_4$; d) 55 mM EDTA; e) as (c) plus 5 mM $CaCl_2$; f) as (c) followed by treatment with 8 μg protease K after labelling. Figures on the right indicate Mr values x 10^{-3}.

linkages could survive such treatments. The nature and source of the new bands remains unknown, save for the fact that they are not nucleic acids, polyphosphates, acylphosphates or amidophosphates. As regards the other tracks on the autoradiograph, protease K treatment resulted in the loss of all bands, indicating that the bands observed were proteins. The addition of magnesium acetate, manganese sulphate and a serine protease inhibitor (AFBA) to resemble the conditions used by Enami & Ishihama (1984) had no effect on the phosphopolypeptide pattern, and the clearing of the extract only gave a marginal improvement in the heavy background observed. The addition of Ca^{2+} also resulted in no change in the phosphopolypeptide pattern.

Comparing generally the extraction procedure and the kinase assay mixtures used here to those used in the previous two experiments, it can be seen that while the labelling intensity and number of visible phosphopolypeptides are slightly greater when using the lysozyme-Brij-58 extraction (probably due to more complete extraction, though the addition of K^+ or some other component to the kinase assay buffer can not be excluded), the background on the autoradiograph is also much increased. It was concluded that the small improvement in the labelling intensity of the phosphopolypeptides was not worth the deterioration in the clarity of the autoradiographs, and henceforth cell extracts were prepared as described in section 2.11a.

4.4 The Effect of Ca^{2+} and Mg^{2+} on In Vitro Kinase Activities

This section is concerned with the effects of divalent cations, in particular Ca^{2+} and Mg^{2+}, on the kinase activity of cell-free extracts. In view of the related nature of the experiments in

this section, a brief general introduction is provided here to facilitate the discussion of the results in the context of the current knowedge of the role of these cations in prokaryotic cells.

4.4.1 Introduction

Metal ions play a central role in biology, as Williams (1985) asserted, "...biology without metal ions does not exist any more than biology exists without DNA or proteins." Calcium has long been recognized as a modulator of many fundamental processes in eukaryotic cells, working in many cases through the ubiquitous calcium-binding protein, calmodulin (see Pallen et al., 1986, section 1.2.2 and section 1.2.3.3). In recent years it has become apparent that calcium also has an important role to play in prokaryotes. Ordal (1977) demonstrated that the internal concentration of calcium ions regulated chemotactic behaviour in Bacillus subtilis by reversing the direction of flagellar rotation. Ca^{2+} was presumed to bind to a switch controlling the direction of flagellar rotation. When bound, clockwise rotation of the flagella is favoured inducing tumbling. When unbound, anticlockwise rotation and thus swimming is favoured. Evidence was also presented for a role for Mg^{2+} in the control of flagellar rotation. The suggestion was that Mg^{2+} and Ca^{2+} were antagonists at the switch, Mg^{2+} promoting swimming and Ca^{2+} tumbling. Ca^{2+} was considered to be the regulating cation, however, as the high internal Mg^{2+} concentration in the cell (20-40 mM) can only undergo fractional changes in response to chemotactic stimuli. Ca^{2+}, on the other hand, is normally exported from the cell resulting in a very low internal concentration (10-100 nM) which can be potentially dramatically

influenced by chemotactic stimuli. Ca^{2+} has also been shown to stimulate stalk development and promote phosphate acquisition in Caulobacter crescentus (Poindexter, 1984), to be required for sporulation in Bacillus subtilis and to be involved in the synthesis of cell wall structures (see De Vrij et al., 1985). Interest in the role of Ca^{2+} in prokaryotes has been further stimulated by the discovery of high-affinity calcium binding proteins and calmodulin-like activities in various prokaryotes. Iwasa et al. (1981) first presented evidence for a calmodulin-like protein in Escherichia coli. This protein had similar properties to calmodulin, and was capable of activating various eukaryotic calmodulin-dependent enzymes in a calcium-dependent fashion. Subsequently other organisms have been shown to possess calmodulin-like proteins. The development-specific protein S from Myxococcus xanthus was shown to have a high level of homology with calmodulin at the structural level, in its physical properties and at the level of the DNA sequence (Inouye et al., 1983). A calmodulin-like protein has been identified by radioimmunoasay in the cyanobacteria Oscillatoria limnetica and phosphate uptake has been shown to be stimulated by Ca^{2+} and inhibited by calmodulin antagonists (Kerson et al., 1984). A calmodulin-like protein has also been found in Bacillus subtilis (Fry et al., 1986) and Streptomyces erythreus (Leadlay et al., 1984). Harmon et al.(1985) reported other high-affinity calcium binding proteins, unrelated to calmodulin, in E. coli. These above facts, and the recognition that bacteria are probably able to regulate their internal calcium ion concentrations through the operation of two, opposing Ca^{2+} transport systems (Rasch et al., 1986) provide evidence for a potentially important role for Ca^{2+} in the regulation of

cellular processes. It is interesting to note that the Ca^{2+} export system in the purple sulfur bacterium Chromatium vinosum is inhibited by phenothiazine drugs, notable calmodulin antagonists (Peach et al., 1986). One cannot overlook, however, the possible influence of other known effects of phenothiazine drugs on this process and on the inhibition of phosphate uptake in Oscillatoria limnetica (Kerson et al., 1984), notably the demonstration that potassium ion leakage from Staphylococcus aureus cells is induced in the presence of phenothiazines (Shibl et al., 1984).

The establishment of a role for Mg^{2+} in bacteria is much further advanced. There is accumulating evidence for a universal role for Mg^{2+} in cell cycle control (Walker, 1986). Mg^{2+} appears to be an absolute requirement for cell proliferation in both heterotrophic and autotrophic bacteria. Evidence from cyanobacteria has been used to suggest that Mg^{2+} controls the cell cycle by controlling the rate of synthesis of a cell division initiator protein, "protein X". In addition, Mg^{2+} is required for protein kinase activity (Knowles, 1980), is involved in the molecular mechanism of ribosome assembly in E. coli (Allen & Wong, 1986) and has a role in the control of bacterial chemotaxis (Ordal, 1977).

4.4.2 Kinase activities in cell extracts prepared from early and late exponential phase cultures

The aim of this experiment was to examine the kinase activities of cell-free extracts prepared from early and late exponential phase cultures, looking in particular for qualitative differences in the patterns of polypeptides being phosphorylated. In addition, the effect of Ca^{2+} on the phosphopolypeptide pattern

was to be examined.

Samples were taken at two points in the growth curve, early and late exponential phase, and crude cell-free extracts were prepared by the method described in section 2.11a without the final clearing centrifugation. Simultaneously with this sample, samples were taken from a parallel culture labelled *in vivo* with ^{32}P-orthophosphate. The sample sizes taken were of identical OD_{540} x volume. *In vitro* kinase assays were carried out as described in section 2.12, samples of each extract being supplemented with 10 mM Ca^{2+} or 10 mM EGTA (a chelating agent with a high affinity for calcium ions). The samples can thus be considered as containing no Ca^{2+} (EGTA treated), trace ammounts of Ca^{2+} (from the cell free extracts) and high Ca^{2+} (10 mM addition). The kinase assays were stopped as usual by boiling the sample in double strength Laemmli sample buffer. The samples, along with the *in vivo* labelled samples, were subjected to electrophoresis on 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15. Fig 4.4 shows the autoradiograph of this gel. The first point to be made is that the extracts prepared in late exponential phase appear to have considerably greater kinase activity than extracts prepared from early exponential phase extracts. This corresponds well with observations made *in vivo* (chapter 3), where it was demonstrated that a similar phenomenon could be observed. The kinase activity observed was considerably greater than had previously been observed in a cell-free extract, with at least 12 polypeptides being labelled in the late exponential phase extracts. The reason for this could be the use of extremely crude extracts, with all paticulate matter retained and no loss of trace elements. Apart from the considerably

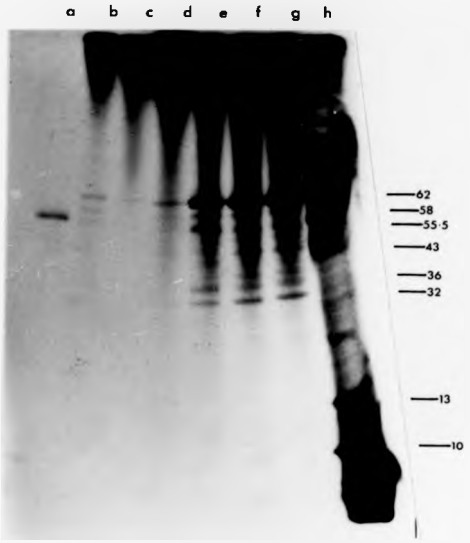


Fig 4.4 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled cell free extracts of Rm. yannielii harvested in early and late exponential phase, and of ^{32}P in vivo continuous-labelled samples from parallel cultures harvested simultaneously with the cells for the cell-free extracts. Tracks are as follows: a) Early log phase in vivo labelled sample; b) early log phase in vitro labelled sample supplemented with 10 mM EGTA; c) early log phase in vitro labelled sample; d) as (c) plus 10 mM Ca^{2+}; e) late log phase in vitro labelled sample supplemented with 10 mM EGTA; f) late log phase in vitro labelled sample; g) as (f) plus 10 mM Ca^{2+}; h) late log phase in vivo labelled sample. Figures on the right indicate Mr values x 10^{-3}.

greater kinase activity in the late exponential phase extract, there are no discernable qualitative differences in the patterns of polypeptides being phosphorylated. This seems to indicate that the same kinases may be present throughout the growth curve, and changes in phosphopolypeptide patterns in vivo through the growth curve may be due to changes in the available substrate polypeptides or to changes in the activation/ deactivation of protein kinases or phosphatases. This should not be overstressed, however, as it can be seen from Fig 4.4 that the patterns of phosphopolypeptides in vitro bear little resemblance to the phosphopolypeptide patterns seen in vivo. The only phosphopolypeptides with similar Mr values in vitro to phosphopolypeptides observed in vivo are those of Mr 55,500, 13,000 and 10,000. As a result, in vitro observation are difficult to interperet in terms of what is actually occurring in vivo. As regards the effects of Ca^{2+} a number of observations can be made. The sample treated with EGTA showed some differences from the tracks possessing trace Ca^{2+} and added Ca^{2+} (some of the early exponential phase, untreated, sample was lost during preparation, so this track should be ignored). The addition of Ca^{2+} to the late exponential phase extract resulted in no discernable change from the untreated extract. The track with Ca^{2+} removed by EGTA showed increased phosphorylation of the Mr 55,500 and 36,000 polypeptide and decreased phosphorylation of the Mr 43,000, 39,000 and 32,000 polypeptides. It appears, then, as though Ca^{2+} is capable of exerting an influence on the phosphopolypeptide patten. Whether this influence is mediated through protein kinases, phosphoprotein phosphatases or even through the activation of Ca^{2+} dependant proteases can not be determined in this experiment.

4.4.3 The effect of Ca^{2+}, Mg^{2+} and Zn^{2+} on kinase activities in cell free extracts

This experiment was intended to try and clarify the effect of the addition of various cations on the kinase activities observed in cell free extracts. The experiment described in section 4.4.2, whilst demonstrating a possible calcium effect in cell free extracts, was difficult to interperet due to the use of crude extracts. As a result, the extract would contain many elements in trace amounts, any of which could potentially influence the activities of kinases in vitro. This experiment was designed to more thoroughly test the effects of addition of Ca^{2+} and Mg^{2+} (both cations with an established role in bacteria, see section 4.4.1), and in addition to test zinc ions for an ability to moderate kinase activities. It has been shown in eukaryotes that zinc is capable of activating kinases in vitro (Sakakihara & Volpe, 1985).

Cell-free extracts were prepared from a late exponential phase culture by the method described in section 2.11a. Eight samples of extract were taken, one was untreated, one was treated with 75 μgml^{-1} chlorpromazine , a calmodulin antagonist, and the remaining six samples were treated with 10 mM EDTA, the aim being to remove all cations from solution. The EDTA treated samples were then supplemented with Mg^{2+}, Ca^{2+} and Zn^{2+}, sufficient cation being added to saturate the residual EDTA and leave a final concentration of approximately 5 mM cation. The samples, additions and estimated final compositions are outlined below:

a) Untreated kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol).

b) Kinase buffer + 10 mM EDTA (net ~5 mM EDTA)

c) Kinase buffer + 10 mM EDTA + 15 mM $CaCl_2$ (net ~5 mM $CaCl_2$).

d) Kinase buffer + 10 mM EDTA + 15 mM $MgCl_2$ (net ~5 mM $MgCl_2$).

e) Kinase buffer + 10 mM EDTA + 15 mM $CaCl_2$ + 15 mM $MgCl_2$ (net ~10 mM $CaCl_2$, 10 mM $MgCl_2$).

f) Kinase buffer + 10 mM EDTA + 15 mM $ZnCl_2$ (net ~5 mM $ZnCl_2$).

g) Kinase buffer + 10 mM EDTA + 15 mM $ZnCl_2$ + 15 mM $MgCl_2$ (net ~10 mM $ZnCl_2$, 10 mM $MgCl_2$).

h) Kinase buffer + 75 $\mu g ml^{-1}$ chlorpromazine.

Where more than one addition was to be made to one sample (after the chelation of endogenous cations with EDTA), the additions were mixed and added to the sample in two parts. The first addition (to a final concentration of 5 mM added cations) was intended to be sufficient to saturate the EDTA present, the second addition consisted of the remainder. This precaution was taken to avoid imbalances arising in the concentrations of added cations due to differental affinities and stability constants of EDTA for different cations. All final concentrations given are approximate due to the unknown contribution of cations from the cell-free extract. The figures given should be taken as minimum concentrations except for sample 2, for which the EDTA concentration is a maximum. Kinase assays were carried out as described in section 2.12 and stopped as usual by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on a 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15. The autoradiograph of this gel

is shown in Fig 4.5. It is clear from this picture that the use of a chelating agent resulted in considerably less background on the autoradiograph. Chlorpromazine had no discernible effect on the kinase activity of an untreated cell extract. The effect of EDTA on the cell extracts was to prevent the phosphorylation of a Mr 53,000 polypeptide and to stimulate the phosphorylation of an Mr 15,500 polypeptide. This effect is less dramatic than the effect seen in Fig 4.3. This may be due, in some unknown fashion, to the use of cleared extracts in this experiment or to the use of the Brij-58/lysozyme extraction in experiment 4.3. The effect observed on the addition of Ca^{2+} to the extract was striking. The Mr 60,000 phosphopolypeptide band is lost, and the Mr 15,500 phosphopolypeptide band greatly reduced in intensity. This could be due to the deactivation of a kinase, or the activation of a phosphatase or calcium-dependent protease. The addition of Mg^{2+} to the extract stimulated the phosphorylation of the Mr 60,000 polypeptide, activated the phosphorylation of a Mr 60,500 polypeptide and completely abolished the phosphorylation of the Mr 15,500 polypeptide. When Mg^{2+} and Ca^{2+} were added together, the effect of Ca^{2+} appeared to dominate with the phosphopolypeptide pattern appearing very similar to the track of the Ca^{2+} treated extract. Zinc appeared to stimulate the phosphorylation of the Mr 56,500 polypeptide, though this effect was not quite so marked in the presence of Ca^{2+}. None of the cations added resulted in the phosphorylation of the Mr 53,000 polypeptide seen in the untreated extract. As the only difference between samples is in the cation content, this suggests that there is some unidentified component that is cation-dependent exerting an effect in the untreated extract. The abolition of the Mg^{2+} effect by Ca^{2+} could be due to several reasons. It could be

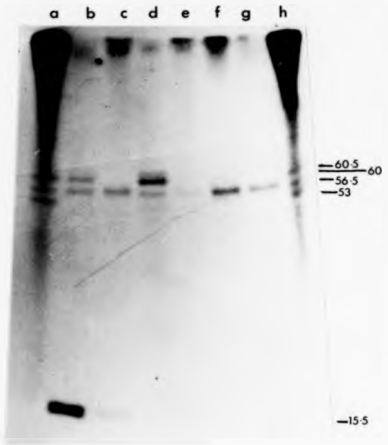


Fig 4.5 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-ß-ATP labelled cell free extracts of Rm. vannielii suplemented with: a) No additions; b) 10 mM EDTA; c) as (b) plus 15 mM $CaCl_2$; d) as (b) plus 15 mM $MgCl_2$; e) as (b) plus 15 mM $CaCl_2$ and 15 mM $MgCl_2$; f) as (b) plus 15 mM $ZnCl_2$; g) as (b) plus 15 mM $ZnCl_2$ and 15 mM $MgCl_2$; h) 75 $\mu g ml^{-1}$ chlorpromazine. Figures on the right indicate Mr values x 10^{-3}.

that the observed magnesium effect is a function of enhanced kinase activity through the formation of the Mg^{2+}-ATP complex, rather than through any direct modulation of kinase activities. If Ca^{2+} were the regulating factor, it would thus be able to overide the effects of Mg^{2+}. Alternatively, if the calcium effect is due to the activation of a calcium-dependent protease, again the activation of kinases by Mg^{2+} (by any means) will be overidden by the effects of Ca^{2+}. A third possibility is that there exists a system analagous to that operating in the control of flagellar rotation in Bacillus subtilis (Ordal, 1977). Here Mg^{2+} and Ca^{2+} are antagonists at the regulatory point and the Ca^{2+}/Mg^{2+} ratio is the controlling factor. In vivo, for the system to be regulated by the preferential binding of Ca^{2+} or Mg^{2+}, the binding affinity of the switch for Ca^{2+} would have to be very much higher than its affinity for Mg^{2+} due to the very much higher concentration of Mg^{2+} in the cell compared to the Ca^{2+} concentraton (see 4.4.1). As a result, any system regulated by the opposing actions of Ca^{2+} and Mg^{2+} will be totally dominated by the effect of Ca^{2+} in the assay conditions used (equal concentrations of Ca^{2+} and Mg^{2+}).

This experiment demonstrated that Ca^{2+}, Mg^{2+} and to some extent zinc were capable of altering the phosphorylation pattern produced in vitro by cell-free extracts, though the manner in which this modulation was effected is unknown.

4.4.4 The effect of Ca^{2+}, Mg^{2+} and chlorpromazine on kinase activities in cell free extracts

This experiment was carried out to confirm the calcium and magnesium effects observed in 4.4.3, and also to more thoroughly investigate the effect of chlorpromazine on phosphopolypeptide

patterns in in vitro kinase assays. Experiment 4.4.3 showed no demonstrable effect of chlorpromazine, but it is possible that prokaryotic calmodulin-like molecules respond differently to eukaryotic calmodulins on metal ion and antagonist binding. Eukaryotic calmodulin undergoes extensive structural changes on binding Ca^{2+} (see Mills & Johnson, 1985). These changes expose hydrophobic sites thought to be the sites of interaction of calmodulin with its target proteins and with inhibitory drugs (calmodulin antagonists). It may be that in prokaryotes, calmodulin antagonists block the site of metal ion binding. Thus it could be important, over the short period of an in vitro kinase assay, in which order the hypothetical calmodulin-like molecule encounters Ca^{2+} and the calmodulin antagonists. Furthermore, in addition to Ca^{2+}, calmodulin has been shown to interact with other cations, namely La^{3+}, Tb^{3+}, Pb^{2+}, Sm^{3+}, Sr^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+} (see Mills & Johnson, 1985). Some of these metals compete for the calcium-binding site, while others can effect the conformation of calmodulin beyond the effect produced by Ca^{2+} alone. As a result of this, it was considered necessary to examine the effects of calmodulin antagonists under more carefully controlled conditions than those described in section 4.4.3.

Cell-free extracts were prepared from a late exponential phase culture by the method described in section 2.11a. Eight samples of extract were taken and the samples, additions and estimated final compositions are outlined below:

a) Untreated kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol).

b) Kinase buffer + 10 mM EDTA (net ~5 mM EDTA)

c) Kinase buffer + 10 mM EDTA + 15 mM $CaCl_2$ (net ~5 mM $CaCl_2$).

d) Kinase buffer + 10 mM EDTA + 15 mM $CaCl_2$ + 75μgml^{-1} chlorpromazine (net ~5 mM $CaCl_2$, 75μgml^{-1} chlorpromazine).

e) Kinase buffer + 10 mM EDTA + 15 mM $MgCl_2$ (net ~5 mM $MgCl_2$).

f) Kinase buffer + 10 mM EDTA + 15 mM $MgCl_2$ + 75μgml^{-1} chlorpromazine (net ~5 mM $MgCl_2$, 75μgml^{-1} chlorpromazine).

g) Kinase buffer + 10 mM EDTA + 10 mM $CaCl_2$ + 10 mM $MgCl_2$ (net ~5 mM $CaCl_2$, 5 mM $MgCl_2$).

h) Kinase buffer + 10 mM EDTA + 10 mM $CaCl_2$ + 10 mM $MgCl_2$ + 75 μgml^{-1} chlorpromazine (net ~5 mM $CaCl_2$, 5 mM $MgCl_2$, 75 μgml^{-1} chlorpromazine).

Where more than one addition was to be made to one sample (after the chelation of endogenous cations with EDTA), the additions were mixed and added to the sample as described in 4.4.3. Chlorpromazine (when added) was always added after EDTA and before the addition of metal ions before begining the assay with the addition of the ATP. As described in 4.4.3, all final concentrations given are approximate due to the unknown contribution of cations from the cell-free extract. Kinase assays were carried out as described in section 2.12 and stopped by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on a 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15 before being subjected to autoradiography. Fig 4.6 shows the autoradiograph obtained. Again, the untreated track showed the typical pattern obtained

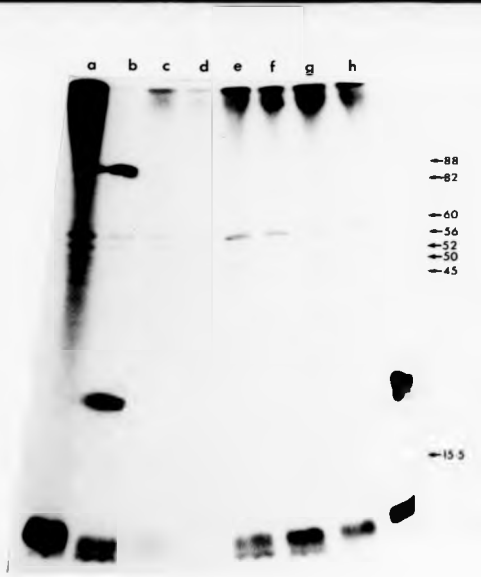


Fig 4.6 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled cell free extracts of Rm. vannielii suplemented with: a) No additions; b) 10 mM EDTA; c) as (b) plus 15 mM $CaCl_2$; d) as (c) plus 75 mM μgml^{-1} chlorpromazine; e) as (b) plus 15 mM $MgCl_2$; f) as (e) plus 75 μgml^{-1} chlorpromazine; g) as (b) plus 15 mM $CaCl_2$ and 15 mM $MgCl_2$; h) as (g) plus 75 μgml-1 chlorpromazine. Figures on the right indicate Mr values x 10^{-3}.

from kinase assays of cell-free extracts of three bands in the Mr range 50,000-60,000. However, this extract appeared to be able to phosphorylate a number of minor bands of Mr 88,000, 82,000 and 45,000. The EDTA treated sample again lost the Mr 52,000 phosphopolypetide, but the stimulation of the Mr 15,500 band was less pronounced than was seen in section 4.4.3. The possibility cannot be excluded that the added EDTA was insufficient to completely chelate the metal ions from the cell extract. The addition of Ca^{2+} did not appear to have the same effect of eliminating the Mr 60,000 phosphopolypeptide as was seen in section 4.4.3, though when preceded by the addition of chlorpromazine the pattern was slightly altered. The addition of Mg^{2+} again resulted in the enhancement of the Mr 60,000 band, but rather than also inducing the phosphorylation of a Mr 60,500 polypeptide, a Mr 50,000 phosphopolypeptide was seen instead. With the prior addition of chlorpromazine, however, this band was not seen. Adding Mg^{2+} and Ca^{2+} to the sample, in direct contrast to the experiment described in 4.4.3, resulted in the effect of Mg^{2+} being dominant though again the prior addition of chlorpromazine prevented the appearance of the Mr 50,000 phosphopolypeptide.

There are a number of conclusions that can be drawn from this experiment. Firstly, the fact that there are considerable discrepancies between this experiment and the experiment described in section 4.4.3 illustrates that the degree of control over the assay conditions offered by the addition of EDTA followed by the addition of saturating concentrations of appropriate cations is still insufficient. A possible reason for this is that whilst removal of cations is being effected, other low molecular weight compounds with the potential to influence

the phosphopolypeptide pattern (e.g. cAMP) are not, and thus variation in the levels of these compounds between extracts could effect kinase activities. Another possibility is that all the cations may not have been removed from the sample. The amount of EDTA added may have been insufficient. Some cations are capable of exerting an effect at very low concentrations (e.g. Ca^{2+}, Ordal, 1977), and it is possible that some elements present in only trace amounts may still be able to moderate kinase activities. Thus it is clear that further experiments, under more precisely defined conditions, are necessary. Despite these doubts, it is again clear that the addition of metal ions to cell-free extracts can effect the pattern of phosphopolypeptides produced. The inhibition of the Mr 50,000 phosphopolypeptide production by chlorpromazine is of especial interest. The mechanism of this inhibition is unknown. On speculation, however, various suggestions can be made. It could be that the hypothetical calmodulin-like molecule, stimulates the production of the Mr 50,000 phosphopolypeptide by interacting with a kinase or phosphatase. This calmodulin-like molecule may have an affinity for Mg^{2+} rather than Ca^{2+} and if, as detailed above, the interaction with chlorpromazine results in blockage of the Mg^{2+} binding site, the calmodulin-like molecule will be unable to bind the subsequently added Mg^{2+} and will thus remain unable to stimulate the production of the Mr 50,000 phosphopolypeptide. This is not, of course, the only possible explanation, the system could operate as described above with the chlorpromazine blocking the site of protein binding that was exposed on the binding of Mg^{2+}. This may be more likely as this is how calmodulin antagonists bind to calmodulin (Mills & Johnson, 1985). The evidence does appear to suggest a role for Mg^{2+} rather than

Ca^{2+}, but the possibility of trace amounts of metal ions remaining after the EDTA treatment cannot be ruled out, and these residual amounts may be sufficient for modulating proteins with a high affinity for their cation being able to sequester enough to exert an effect on the phosphopolypeptide pattern. Thus the conclusion is that, while this experiment was inconclusive, there are apparent effects worthy of further investigation under more carefully controlled conditions.

4.4.5 The effect of Mg^{2+} and chlorpromazine on kinase activities in purified cell free extracts

Previous experiments in this section (4.4) have demonstrated that the divalent cations Mg^{2+} and Ca^{2+} are capable of altering the pattern of phosphopolypeptides produced in kinase assays of cell-free extracts. However, as yet no clear definition of what this effect is has emerged. The reasons for this are thought to be due to the presence of residual amounts of metal ions after EDTA treatment, the presence of other effector molecules which varied in concentration between extracts, and the inability to define the exact conditions in the assay due to the unknown residual concentration of free EDTA after removal of the metal ions from the cell free extract. In addition, there is evidence that a calmodulin antagonist (chlorpromazine) can also effect the phosphopolypeptide pattern in cell-free extracts. This experiment was designed to allow the investigation of the effects of metal ions and calmodulin antagonists on the phosphopolypeptide pattern produced by cell-free extracts under rigidly defined conditions.

Cell extracts were harvested in late log phase, washed and resuspended in 10 mM Tris pH 7.5. The cells were broken by sonication and immediately afterwards m-aminophenylboronic acid

(APBA, a serine protease inhibitor) was added to 1 mM, and DNase I and RNase A were added each to 20 $\mu g ml^{-1}$. The extract was cleared by centrifugation in an Eppendorf microfuge at 12,000 g and 4°C for 10 minutes. Metal ions were removed from the extract by dialyzing a 2 ml volume against 4 l 20 mM Tris pH 7.5, 10 mM EDTA, 1 mM APBA, 1 mM 2-mercaptoethanol. Dialysis was carried out for 16 hours at 4°C, with 2 changes of the dialysis buffer. All buffers and solutions used in this experiment were prepared using the highest quality purified water available, prepared from a Millipore Super Q filtration system. After the removal of low molecular weight components, cations e.t.c. the extract was dialyzed as above against 20 mM Tris pH 7.5, 1 mM APBA, 1 mM 2-mercaptoethanol to remove all EDTA from the extract. Thus the calculated final concentration of EDTA in the cell-free extract was 1.25 pM. Extract not being immediately used was drop-frozen in liquid nitrogen and stored at -80°C for later experiments.

The experiment was carried out by supplementing the extract with various concentrations of Mg^{2+}, and with chlorpromazine as follows:

a) Extract, no additions.
b) Extract + 1 mM $MgCl_2$.
c) Extract + 5 mM $MgCl_2$.
d) Extract + 10 mM $MgCl_2$.
e) Extract + 20 mM $MgCl_2$.
f) Extract + 50 mM $MgCl_2$.
g) Extract + 10 mM $MgCl_2$ + 75 $\mu g ml^{-1}$ chlorpromazine.
h) Extract + 75 $\mu g ml^{-1}$ chlorpromazine + 10 mM $MgCl_2$.

The order in which components are listed is the order in which they were added to the extract. As described above, all solutions were prepared using only water from a Millipore Super Q filtration system to prevent the reintroduction of unknown quantities of metal ions. Kinase assays were carried out as described in section 2.12 and stopped by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on a 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15 before being subjected to autoradiography. Fig 4.7 shows the autoradiograph obtained. 13 phosphopolypeptides could be seen on the autoradiograph, though most were very faint. This was a considerable improvement on previous experiments, and was possibly a result of maintaining the extract in the presence of a serine protease inhibitor. The triplet usually estimated as being in the Mr range 50,000-60,000 appeared to have slightly lower Mr values of Mr 55,000, 53,000 and 47,000. These Mr estimations match well with a triplet seen at the same position from phosphorylation experiments in vivo (see Fig 3.16). There was a clear Mg^{2+}-dependent effect on the phosphopolypeptide pattern observed. In the absence of Mg^{2+} a prominent doublet of Mr 15,500 and 15,000 could be seen, as well as other phosphopolypeptides of Mr 55,000, 40,000 and 10,000. 1 mM Mg^{2+} was sufficient to drastically reduce the amounts of the Mr 15,500, 15,000 doublet, dramatically increase the amount of the Mr 55,000 phosphopolypeptide and to stimulate the phosphorylation of a Mr 53,000 polypeptide and a lesser degree of phosphorylation of a Mr 47,000 polypeptide. 5 mM Mg^{2+} resulted in the reduction of the phosphorylation of the Mr 15,500, 15,000 phosphopolypeptide doublet to a level beyond which no further

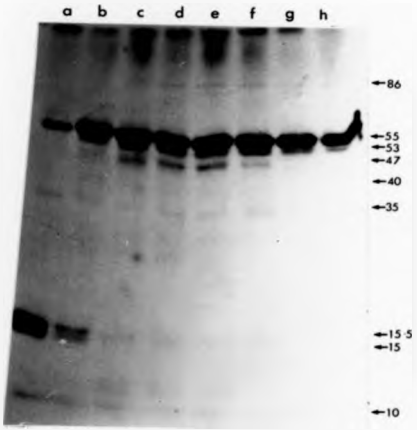


Fig 4.7 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled dialysed cell free extracts of Rm. vannielii suplemented with $MgCl_2$ to the following concentrations: a) 0; b) 1 mM; c) 5mM; d) 10 mM; e) 20 mM; f) 50 mM; g) 10 mM followed by 75 μgml^{-1} chlorpromazine; h) 75 μgml^{-1} chlorpromazine followed by 10 mM $MgCl_2$. Figures on the right indicate Mr values x 10^{-3}.

increase in the Mg^{2+} concentration altered. In addition it induced a large increase in the degree of phosphorylation of the Mr 47,000 polypeptide and induced the phosphorylation of two other polypeptides of Mr 88,000 and 40,000. Higher concentrations of Mg^{2+} resulted in slightly greater phosphorylation of the Mr 47,000 polypeptide up to 20 mM Mg^{2+}, the estimated physiological concentration. At 50 mM Mg^{2+} phosphorylation of this, and other polypeptides was reduced. The effect of the addition of chlorpromazine was clear in this experiment, and confirmed the result observed in section 4.4.4. Chlorpromazine prevented the magnesium-dependent phosphorylation of the Mr 47,000 polypeptide. No other phosphopolypeptides were effected and the order of addition of the chlorpromazine and Mg^{2+} was irrelevant. One further point of interest was the high degree of labelling of the Mr 55,000 phosphopolypeptide. This level of phosphorylation had not been seen in previous, cruder, extracts.

This experiment clearly demonstrated that the pattern of phosphopolypeptides produced by cell-free extracts *in vitro* is dependent on the Mg^{2+} concentration. The observation of effects dependent on different Mg^{2+} concentrations is evidence that more than one protein kinase is active in cell-free extracts of *Rm. vannielii*. having different affinities for Mg^{2+}. Chlorpromazine clearly effects the phosphorylation of the Mr 47,000 polypeptide, though whether this is through some direct interaction with a kinase or its substrate protein, or through an intermediary calmodulin-like molecule is unknown. The latter is possibly more likely, as the phenothiazine drugs are known to be highly specific in their binding to calmodulin. This would, however suggest that the calmodulin-like molecule could interact with Mg^{2+} as well as/instead of Ca^{2+}.

4.4.6 The effect of Mg^{2+}, Ca^{2+} and chlorpromazine on kinase activities in purified cell free extracts

This experiment was designed to investigate the effects of Mg^{2+}, Ca^{2+} and chlorpromazine on the highly purified cell-free extract prepared as described in section 4.4.5. Thawed aliquots of the same extract used in the experiment described in section 4.4.5 were used for this experiment, the frozen samples having been stored at -80°C for 7 days since preparation. The experiment was carried out exactly as described in section 4.4.5, with only the additions to the extract being different. The experiment was carried out by supplementing the extract with various concentrations of Mg^{2+}, Ca^{2+} and chlorpromazine as follows:

a) Extract, no additions.
b) Extract + 75 $\mu g ml^{-1}$ chlorpromazine.
c) Extract + 5 mM $CaCl_2$.
d) Extract + 5 mM $CaCl_2$ + 75 $\mu g ml^{-1}$ chlorpromazine.
e) Extract + 75 $\mu g ml^{-1}$ chlorpromazine + 5 mM $CaCl_2$.
f) Extract + 5 mM $MgCl_2$.
g) Extract + 5 mM $MgCl_2$ + 75 $\mu g ml^{-1}$ chlorpromazine.
h) Extract + 75 $\mu g ml^{-1}$ chlorpromazine + 5 mM $MgCl_2$.
i) Extract + (5 mM $MgCl_2$, 5 mM $CaCl_2$).
j) Extract + (5 mM $MgCl_2$, 5 mM $CaCl_2$) + 75 $\mu g ml^{-1}$ chlorpromazine.
k) Extract + 75 $\mu g ml^{-1}$ chlorpromazine + (5 mM $MgCl_2$, 5 mM $CaCl_2$).

The order in which components are listed is the order in which they were added to the extract. Components listed within brackets were mixed together first and then added to the sample. Again, all solutions were prepared using only water from a Millipore

Super Q filtration system to prevent the reintroduction of unknown quantities of metal ions. The reason for the use of non-optimum Mg^{2+} concentrations was that the results for the previous experiment investigating the optimum Mg^{2+} concentration (4.4.5) were not available at this time. Kinase assays were carried out as described in section 2.12 and stopped by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on a 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15 before being subjected to autoradiography. Fig 4.8 shows the autoradiograph obtained. A similar range of phosphopolypeptides as was seen in Fig 4.7 could be seen, with one exception. Where, in Fig 4.7, there was a Mr 15,500, 15,000 phosphopolypeptide doublet, there was now only a single phosphopolypeptide of Mr 15,000. This suggests that the Mr 15,500 phosphopolypeptide was extremely unstable, and was lost over the intervening period of time between experiments despite being stored at -80°C. Whether this phosphopolypeptide was lost altogether, or whether a small fragment only was lost to produce the Mr 15,000 phosphopolypeptide is unknown. The latter is possibly more likely as the intensity of the Mr 15,000 phosphopolypeptide band in this experiment is somewhat higher, relative to other bands, than was observed in section 4.4.5. In the absence of added metal ions, the pattern of phosphopolypeptides produced corresponded with that observed in section 4.4.5, barring the difference between the Mr 15,000 phosphopolypeptide and the Mr 15,500, 15,000 phosphopolypeptide doublet discussed above. The addition of chlorpromazine in the absence of added metal ions had no effect on the phosphopolypeptide pattern. Ca^{2+} stimulated the phosphorylation

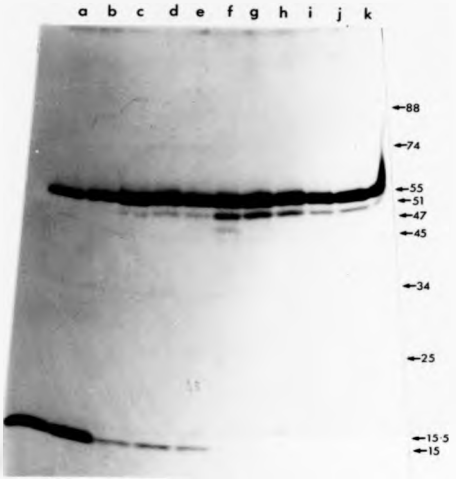


Fig 4.8 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled dialysed cell free extracts of Rm. vannielii suplemented with: a) No additions; b) 75 μgml^{-1} chlorpromazine; c) 5 mM $CaCl_2$; d) 5 mM $CaCl_2$ followed by 75 μgml^{-1} chlorpromazine; e) 75 μgml^{-1} chlorpromazine followed by 5 mM $CaCl_2$; f) 5 mM $MgCl_2$; g) 5 mM $MgCl_2$ followed by 75 μgml^{-1} chlorpromazine; h) 75 μgml^{-1} chlorpromazine followed by 5 mM $MgCl_2$; i) 5 mM $MgCl_2$, 5 mM $CaCl_2$; j) 5 mM $MgCl_2$, 5 mM $CaCl_2$ followed by 75 μgml^{-1} chlorpromazine; k) 75 μgml^{-1} chlorpromazine followed by 5 mM $MgCl_2$, 5 mM $CaCl_2$. Figures on the right indicate Mr values x 10^{-3}.

of the Mr 55,000 and 53,000 polypeptides, and inhibited the phosphorylation of the Mr 15,000 polypeptide. In addition, it induced the phosphorylation of a Mr 74,000 polypeptide that was not phosphorylated in the presence of Mg^{2+}, or Mg^{2+} and Ca^{2+}. Mg^{2+}, as observed in section 4.4.5, also stimulated the phosphoryation of the Mr 55,000 polypeptide to the same extent as Ca^{2+}, stimulated the phosphorylation of the Mr 53,000 phosphopolypeptide and inhibited the phosphorylation of the Mr 15,000 phosphopolyeptide to a greater extent than Ca^{2+}, and induced the phosphorylation of two polypeptides that were not phosphorylated in the presence of Ca^{2+} of Mr 88,000 and 47,000. When Ca^{2+} and Mg^{2+} were added together, the phosphopolypeptide pattern was identical to that seen in the presence of Mg^{2+} alone, except for the Mr 47,000 phosphopolypeptide which was absent. The addition of chlorpromazine had no effect on Ca^{2+} treated extracts, but prevented the phosphorylation of the Mr 47,000 polypeptide when added to a Mg^{2+}-treated extract. The order of addition of Mg^{2+} and chlorpromazine did not effect the phosphopolypeptide pattern.

It can be seen from these experiments that metal ions can influence the phosphorylation of polypeptides in cell-free extracts. The following summary of the results obtained will be drawn mainly from sections 4.4.5 and 4.4.6, as these results are considered to be the most significant and reliable due to the tight control exercised over the assay conditions in these experiments.

The major phosphopolypeptide produced in kinase assays carried out in cell-free extracts of Em. vannielii was a Mr 55,000 phosphopolypeptide. This polypeptide was phosphorylated in the

absence of all metal ions, but required Mg^{2+} or Ca^{2+} for maximum phosphorylation. It is possible that this phosphopolypeptide could correspond with the Mr 54,500 phosphopolypeptide seen to be phosphorylated in vivo. It is also possible that this phosphopolypeptide may correspond with the Mr 56,500 phosphopolypeptide observed in Fig 4.5, in which case phosphorylation of this polypeptide may be stimulated by Zn^{2+}. The second most prominant phosphopolypeptide observed was a Mr 15,000 phosphopolypeptide. In fresh extracts a doublet of Mr 15,500 and 15,000 could be seen in this region of the gel. The identical response of both the Mr 15,500 and the Mr 15,000 phosphopolypeptides to Mg^{2+} and the increase in intensity of the Mr 15,000 phosphopolypeptide in 1 week old extracts suggested that the Mr 15,000 phosphopolypeptide may be derived from the Mr 15,500 phosphopolypeptide by some proteolytic mechanism or by the loss of some modifying group. The phosphorylation of this polypeptide was greatly inhibited in the presence of 1 mM Mg^{2+} or 5 mM Ca^{2+}, and almost totally inhibited by =>5 mM Mg^{2+}. As the concentration of Mg^{2+} in vivo is 20-40 mM, this phosphopolypeptide will not occur in appreciable amounts in vivo unless there is some other controlling factor. What this controlling factor is, if in fact it does exist, is unknown. The Mr 15,000 phosphopolypeptide is the only polypeptide the phosphorylation of which is inhibited by Mg^{2+}. All others showing a response to Mg^{2+} demonstrated increased phosphorylation in the presence of Mg^{2+} concentrations of up to 20 mM. One such is the Mr 53,000 phosphopolypeptide which showed maximum phosphorylation in the presence of greater than 10 mM Mg^{2+}, but considerable phosphorylation even at 1 mM Mg^{2+}. Ca^{2+} also induced slight phosphorylation at 5 mM. There were a number of very minor

phosphopolypeptides the phosphorylation of which showed little or no regulation by Ca^{2+} or Mg^{2+} (e.g. the Mr 35,000 and 10,000 phosphopolypeptides), and a number that showed maximum phosphorylation at around 20 mM Mg^{2+} (e.g. the Mr 88,000 and 40,000 phosphopolypeptides). The most interesting phosphopolypeptide from a regulatory aspect, however, is the Mr 47,000 phosphopolypeptide. This polypeptide was only slightly phosphorylated in the presence of 1 mM Mg^{2+}, and was phosphorylated to the greatest extent in the presence of 20 mM Mg^{2+} (higher concentrations resulted in reduced phosphorylation). It was not phosphorylated at all in the presence of Ca^{2+}. In the presence of Ca^{2+} and Mg^{2+}, the effect of Ca^{2+} was dominant and no phosphorylation of the Mr 47,000 polypeptide was seen. This may be due to the presence of a Mg^{2+} activated kinase and a Ca^{2+} activated phosphatase or protease, with the Ca^{2+} activated enzyme having the highest activity. In addition if chlorpromazine (a calmodulin antagonist) was added to the assay in the presence of Mg^{2+}, again, the production of the Mr 47,000 phosphopolypeptide was inhibited. This could indicate the presence of a prokaryotic camodulin-like molecule with an affinity for Mg^{2+} rather than Ca^{2+} as described in section 4.4.5. There is, however, an altenative explanation. This explanation is based on the operation of the eukaryotic signal transduction system, which is explained here so that it can be shown how aspects of this system may apply to the cell-free kinase assay system of Rm. yannielii.

4.4.6.1 Protein kinases and signal transduction

The mechanisms involved in signal transduction are not totally understood, however the pivotal regulatory element is protein kinase C, a phospholipid- and calcium-dependent protein kinase

discovered by Nishizuka and coworkers (see Nishizuka, 1984a). Extracellular signals induce the cleavage of phosphoinositides releasing 1,2-diacylglycerol and inositol monophosphate and polyphosphates as part of inositol phospholipid turnover (Nishizuka, 1984a; Fig. 4.9). Inositol 1,4,5-trisphosphate, derived as above from the signal-dependent cleavage of phosphatidylinositol 4,5-bisphosphate, has been shown to mobilise intracellular Ca^{2+} (Berridge, 1984). Chauhan & Brockerhoff (1986) have proposed a mechanism for the maintainance of intracellular Ca^{2+} levels by means of the inositide shuttle.

$$PI \rightleftharpoons PIP \rightleftharpoons PIP_2$$

They suggest that Ca^{2+} is sequestered in a cage formed by the head groups of two acidic phospholipid molecules, e.g. phosphatidylserine and phosphatidylinositol. The cage is stabilised by hydrogen bonds which are disrupted, releasing the Ca^{2+}, on the phosphorylation of the inositol group. This could be important, as the diacylglycerol released by the phosphoinositide cleavage modulates protein kinase C by decreasing the amount of Ca^{2+} required to activate it (Kishimoto et al., 1980). Ganong et al. (1986) have proposed a mechanism for this activation. They derived a model based on the deduced minimal stoichiometry of monomeric protein kinase C, four molecules of phosphatidylserine, one molecule of diacylglycerol, and one calcium ion. The model proposes that four molecules of phosphatidylserine could form a structure with the four carboxyl groups in close proximity, chelating a calcium ion. The binding of protein kinase C to the membrane is Ca^{2+} dependent but diacylglycerol independent (Hannun et al., 1985), so at this point the protein kinase C binds to the surface complex in an inactive form. Activation of the molecule then occurs when a diacylglycerol molecule associates

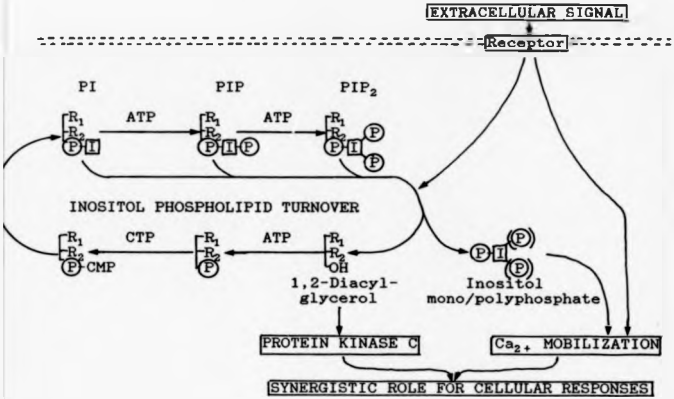


Fig 4.9 The role of Ca^{2+}, protein kinase C and inositol phospholipid tunover in the activation of cellular functions. After Nishizuka (1984).

with this surface bound complex. Ganong et al. (1986) suggest that the diacylglycerol molecule inserts within the phosphatidylserine molecules (Fig. 4.10) and forms a direct bond with the chelated calcium ion. This bond is proposed to be the key step in the activation of protein kinase C. It is thought possible that the ester carbonyl of the diacylglycerol may be the group that forms this bond (though the 3 hydroxyl group is also a candidate), in a manner analagous to the binding of Ca^{2+} to calmodulin (via four carboxyl groups and one carbonyl group). This possibility is interesting as it is known that calmodulin antagonists inhibit protein kinase C (Nishizuka, 1984b) and this model may suggest an explanation.

In addition to this positive control mechanism, a 'counteractive' or negative control mechanism is also in operation mediated by the second messenger cAMP. In most tissues, the receptor-linked events outlined above (inositol phospholipid breakdown, protein kinase C activation and Ca^{2+} mobilisation) can be blocked by extracellular signals that result in an increase in the concentration of cAMP. The molecular events in this 'counteraction' are unknown, but cAMP is thought to exert its effect through protein kinase A (Nishizuka, 1984a), a kinase with firmly established pleiotropic actions (Rosen & Krebs, 1981, as cited by Nishizuka, 1984a).

This system contains a number of aspects that ought to be considered when analysing the effect of Ca^{2+} and Mg^{2+} on the phosphorylation of the Mr 47,000 polypeptide in cell-free extracts of Rm. vannielii. Possibly the most significant is the principle that Ca^{2+} can be sequestered in a cage formed by the

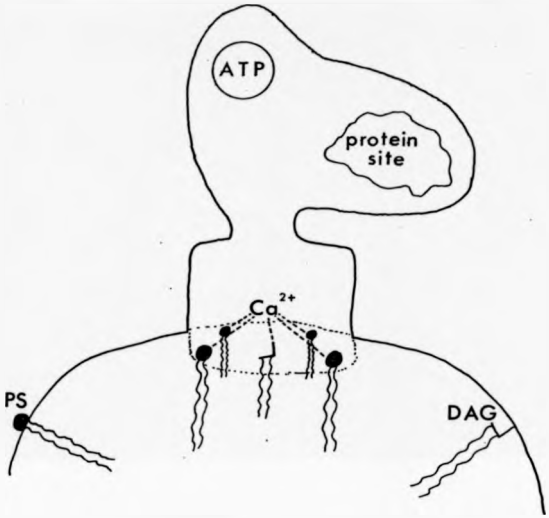


Fig 4.10 Model of protein kinase C activation by phospholipid, diacylglycerol and Ca^{2+}. PS represents phosphatidylserine, DAG represents diacylglycerol.

heads of acidic phospholipids, including phosphatidylinositol (Chauhan & Brockerhoff, 1986). The cell-free extracts used in these experiments, having only had the large cell debris and membrane fragments removed by a 10 minute 12,000 g centrifugation in a microfuge, would contain a great many small membrane fragments. If Rm. vannielii possesses such a system of Ca^{2+} sequestration, the Ca^{2+} bound within these cages may have been protected from chelation by EDTA. If this were to be the case, then the effects of chlorpromazine would have to be interpreted with some care, as it has been shown (Leli & Hauser, 1986) that chlorpromazine is capable of dramatically increasing the rate of phosphoinositide breakdown in rat C6 glioma cells. The mechanism by which this operates is currently unknown. This would result in the breakdown of the phospholipid cages, and the release of Ca^{2+} in to the media. This would explain why the effects of Ca^{2+} and chlorpromazine addition to a Mg^{2+}-containing extract were the same. In fact the same effect, the effect of Ca^{2+} addition, would be being observed in both cases. In one case the source of Ca^{2+} would be external, in the second it would be released from internal stores by phospholipid breakdown. Another alternative for calmodulin-independent inhibition of phosphorylation by chlorpromazine would depend on the Mr 47,000 polypeptide kinase operating in a manner analagous to protein kinase C (i.e. Ca^{2+} and phospholipid-dependent), though perhaps in a Mg^{2+}-dependent rather than a Ca^{2+}-dependent fashion. Chlorpromazine is known to inhibit protein kinase C, a calmodulin-independent enzyme (Nishizuka, 1984b). As described above, chlorpromazine is capable of causing the phosphorylation of phosphoinositides. While the phospholipid cage involved in the activation of protein kinase C suggested by Ganong et al. (1986) was shown to be composed

entirely of phosphatidylserine, other phospholipids are known to show positive or negative cooperativity with phosphatidylserine. If phosphatidylinositol was incorporated into this cage as it is in the model for the Ca^{2+} sequestering cages described by Chauhan & Brockerhoff (1986), then the breakdown of phosphatidylinositol as a result of the presence of chlorpromazine may be the cause of the observed inhibition of protein kinase C. This type of system could thus explain the observed inhibition of the Mr 47,000 polypeptide kinase by chlorpromazine. There is another implication of the breakdown of phosphoinositides as a result of the presence of chlorpromazine. The breakdown products of phosphoinositides are diacylglycerol and inositol phosphates. Inositol 1,4,5-trisphosphate (IP_3) is one of the products of breakdown of phosphatidylinositol 4,5-bisphosphate. This breakdown is greatly increased by chlorpromazine (Leli & Hauser, 1986), and it is known that IP_3 can mobilize Ca^{2+} from intracellular stores in pancreatic acinar cells (Streb et al., 1983). This provides another possible alternative for the inhibition of the Mr 47,000 polypeptide kinase by chlorpromazine in the absence of a calmodulin-dependent kinase.

The conclusion for this section is, then, that the inhibition of the kinase activity of the Mr 47,000 polypeptide kinase does not neccessarily imply a kinase the activity of which is dependent on a calmodulin-like molecule. However, neither do the above observations argue against a calmodulin-like molecule in Rm. vannielii. Though the eukaryotic protein kinase C is calmodulin independent and inhibitable by chlorpromazine, this may be due to the effect of enhanced phosphoinositide breakdown by chlorpromazine. The mechanism of this enhancement is unknown, and thus may potentially involve calmodulin. Thus inhibition of a

camodulin-independent enzyme by chlorpromazine does not argue for a non-specific action of chlorpromazine while the mechanism of inhibition remains unknown.

4.5 In Vitro Kinase Activities Through Differentiation

The purpose of this experiment was to examine the in vitro kinase activities of cell-free extracts prepared from homogeneous swarmer cell populations at different stages of differentiation. There were two points of particular interest under investigation.

1) There is no detectable protein phosphorylation in dark-incubated swarmer cells in vivo, but are there any protein kinases?
2) Does the pattern of protein kinase activities in vitro remain constant throughout differentiation?

A homogeneous population of swarmer cells was prepared as described in section 2.5 using a 20 l culture. 2 l samples were removed for preparation of cell-free extracts before exposure to light, and at 2, 4 and 6 hours in to differentiation. Cells were harvested as described in section 2.9, and cell-free extracts prepared by the method described in section 2.11a. Kinase assays on the prepared extracts were carried out as described in section 2.12 and the reaction was stopped by boiling the sample in double strength Laemmli sample buffer. The samples were subjected to electrophoresis on a 10-30% gradient SDS-polyacrylamide gel and the gel subsequently treated with hot TCA as described in section 2.15 before being subjected to autoradiography. Fig 4.11 shows the autoradiograph obtained from this experiment. The first point to note is that the extract prepared from dark-incubated swarmer cells appeared to contain protein kinases despite the lack of protein phosphorylation in the dark-incubated swarmer cell. This

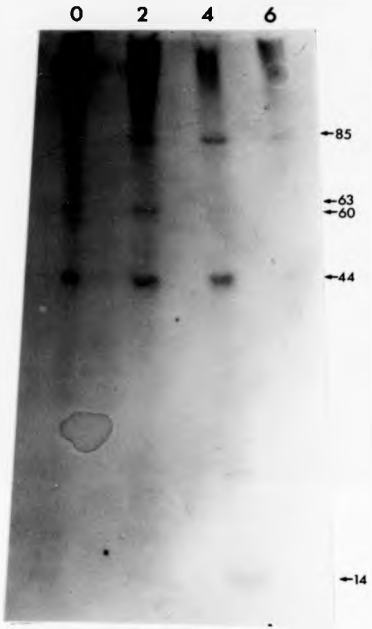


Fig 4.11 Autoradiograph of a 10-30% SDS polyacrylamide gel of ^{32}P-δ-ATP labelled dialysed cell free extracts of Rm. vannielii harvested at different stages during differentiation. Figures at the top of tracks indicate the number of hours of differentiation that had elapsed when that sample was harvested. Figures on the right indicate Mr values x 10^{-3}.

suggested that the protein kinases were present, but that they were not active. It is possible that they are maintained in an inactive state by some environmentally related factor, e.g. redox potential, and are activated only when differentiation commences. It is clear from the autoradiograph that there were differences in the polypeptides phosphorylated by cell-free extracts prepared from homogeneous populations of swarmer cells at different stages of differentiation. Specifically, the Mr 63,000 and 60,000 phsphopolypeptides were lost by four hours into differentiation, a Mr 85,000 phosphopolypeptide appeared at two hours and reached maximum intensity by four hours into differentiation, and a Mr 14,000 phosphopolypeptide appeared at four hours into differentiation. The track of the extract prepared after 6 hours of differentiation was distorted at the edge of the gel and is thus unclear. Whether the observed changes were due to a change in the species of protein kinases present, a change in the activities of the the same protein kinases, or a change in the substrate proteins available for phosphorylation is unknown. This experiment was carried out before the kinase assay method described in section 4.4.5 was developed, and thus all the problems of not having totally defined assay conditions apply. This poses particular problems with this experiment as the extracts were prepared from different points during differentiation. It is quite possible that changes in internal cation concentrations and other effector molecules could occur through differentiation, and so it is possible that each kinase assay could have been carried out under slightly different conditions. As has been noted above (section 4.4), small differences in cation concentrations are capable of having a dramatic effect on cellular processes. As a result, the changes

observed should be interpreted with some caution. What this experiment does show, however, is that there are potentially active protein kinases in the dark-incubated swarmer cell. This leads into many questions, such as how are these kinases inactivated?, at what point do they become active in vivo?, what is the activating factor? Further work is required to answer these questions.

CHAPTER 5.

THE IDENTIFICATION OF SPECIFIC PHOSPHOPROTEINS

This chapter describes the attempts that were made to identify specific phosphorylated proteins in Rm. vannielii. Few phosphoproteins have been identified as a result of phenomenological studies and in view of this, it was decided to specifically examine two proteins that were considered *a priori* to have good potential for regulation by protein phosphorylation. These are discussed separately in the following sections. Much of the work discussed in this chapter was carried out jointly with my supervisor, Dr N.H. Mann. Those parts of the experiments carried out by Dr Mann are duly designated as such in the experimental protocol.

5.1 The Phosphorylation of the DNA-Dependent RNA Polymerase of Rm. vannielii

One of the proteins chosen for further examination was the DNA-dependent RNA polymerase. The reasons for this were as follows. Zillig et al. (1975) first reported that phosphorylation was capable of altering the properties of a prokaryotic DNA-dependent RNA polymerase when they discovered that the phosphorylation of the β' subunit catalysed by a T7 phage coded protein kinase inhibited initiation (Ponta et al. 1974) and enforced termination of transcription (Hercules et al., 1976). It has also been observed (Enami & Ishihama, 1984) that the β, β' and σ subunits of the Escherichia coli RNA polymerase are phosphorylated *in vivo* and though this result was not

corroborated by the detailed analysis of the phosphoproteins of E. coli carried out by Cortay et al. (1986), the confirmation of the phosphorylation of the β and β' subunits by immunoprecipitation with anti-RNA polymerase antibody (Enami & Ishihama, 1984) does suggest that this is a genuine phenomenon. The role of RNA polymerase in gene expression means that it is an ideal control point for modulating gene expression. Changes in the RNA polymerase would be especially useful for the control of gross changes in the cell, where large numbers of genes need to be switched on or off. This could be brought about by changes in the template specificity of the RNA polymerase by modifying the subunit composition of the RNA polymerase (e.g., changing the σ subunit). A possible alternative, however, involves modulating the template specificity of the RNA polymerase by altering its phosphorylation state. It was with this possibility in mind with regard to the control of differentiation in Rm. vannielii that it was decided to investigate the phosphorylation of the RNA polymerase of this organism. The inhibited (dark maintained) swarmer cells of Rm. vannielii are known to synthesize mRNA and proteins, despite there being no increase in biomass (Scott & Dow, 1986a). In addition, it is known that the RNA polymerase undergoes changes in its susceptibility to rifampicin during differentiation, the maturation phase being sensitive to 10-20 μgml^{-1} whilst the reproductive phase is sensitive to only 1 μgml^{-1} (Whittenbury & Dow, 1977). As described by Dow et al. (1983), this poses the question as to whether alterations in the RNA polymerase could result in the recognition of different promoter sites, and hence the production of different cell types. The DNA-dependent RNA polymerase was partially characterized by Scott & Dow (1986b). The enzyme has a core of 3 subunits ($\alpha\beta\beta'$)

and a fourth subunit (σ) that may be required for promoter specificity as in other eubacteria. The β and β' subunit have very similar Mr values of between 150,000 and 160,000 making them hard to separate on SDS-PAGE. The α subunit has a Mr value of 38,000, and appears to have several forms of different isoelectric pH values. The σ factor, likewise, has a Mr value of 98,000 and again appeared to exist as more than one form with different isoelectric pH values. In addition to these major polypeptides, minor polypeptides of Mr 78,000 and 68,000 have been detected (Scott & Dow, 1986a). The Mr 78,000 polypeptide appears to be associated with the RNA polymerase extracted from chain cells, and the Mr 68,000 polypeptide with the RNA polymerase extracted from motile swarmer cells. The function of these polypeptides is unknown.

The analysis of the DNA-dependent RNA polymerase from Rm. vannielii was carried out by immunoprecipitation of the enzyme from radiolabelled cell-free extracts using anti-RNA polymerase antibody (kindly donated by Dr. N.W. Scott) prepared in rabbits injected with the purified RNA polymerase characterised by Scott & Dow (1986b).

5.1.1 Immunoprecipitation of the DNA-dependent RNA polymerase from radiolabelled cultures

An early log phase culture of Rm. vannielii was double labelled with ^{35}S-methionine at 1 μCiml^{-1} and ^{32}P-orthophosphate at 50 μCiml^{-1} and harvested in late log phase by centrifugation as described in section 2.9 (though without the addition of NaF to the culture). Cells were washed in 10 mM Tris-HCl pH 7.5, and broken by sonication, the sonication being carried out as described in section 2.11a. Extracts were cleared by

centrifugation in an Eppendorf microfuge. Immunoprecipitations were carried out by both of the methods described in section 2.21, the rapid method (Goswami & Russell, 1983) being used for the preliminary experiments and the longer, more precise, method (Pringle, 1985) being used for the main experiment. SDS-polyacrylamide gel electrophoresis was carried out on 20% non-gradient or 10-30% exponential gradient gels. Gels were treated as described in section 2.15 to eliminate acyl- and amido-phosphates before drying and autoradiography (as described in section 2.16).

Preliminary experiments carried out using only ^{32}P-orthophosphate label and the rapid immunoprecipitation technique described in section 2.21 demonstrated that phosphopolypeptides were being precipitated by the anti-RNA polymerase antiserum in an apparently antibody concentration-dependent fashion. The problem was that the phosphopolypeptides being precipitated did not correspond in their Mr values to the known Mr of any of the subunits of the RNA polymerase. In view of this, it was decided to carry out the full experiment as described in section 2.21, using double labelling to reveal the total pattern of precipitated polypeptides as well as phosphopolypeptides. In addition, an immunoprecipitation with pre-immune serum was carried out to remove those proteins binding non-specifically to the antibody before the immunoprecipitation with the anti-RNA polymerase antiserum was carried out. Fig 5.1 shows the autoradiograph exposed for ^{35}S emissions. It is clear from this autoradiograph that over 16 polypeptides were precipitated by the anti-RNA polymerase antibody. This was probably due to contaminants in the original RNA polymerase preparation with which the antiserum was prepared. Only very

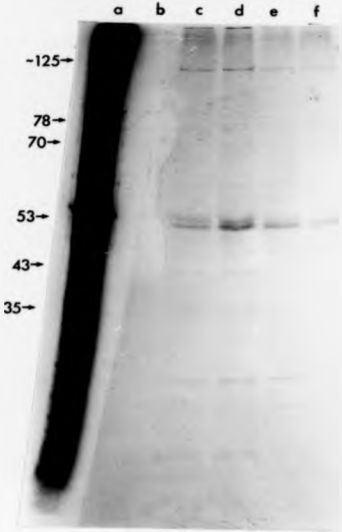


Fig 5.1 ^{35}S-exposure of an autoradiograph of a 10-30% polyacrylamide gel of ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts after the following treatments: a) Untreated; b) after precipitation with protein A; c) after immunoprecipitation with 1/4 dilution of anti-RNA polymerase antibody; d) after immunoprecipitation with 1/8 dilution of antibody; e) after immunoprecipitation with 1/16 dilution of antibody; f) after immunoprecipitation with 1/32 dilution of antibody. Figures on the left indicate Mr values x 10^{-3}.

small amounts of contaminating proteins would be required, providing they were highly antigenic, to produce a substantial immune response. It is also possible that there are a great many proteins associated with the RNA polymerase in the cell, though this seems unlikely as most of the polypeptides precipitated are more prominent than the RNA polymerase subunits. The track precipitated with protein A in the absence of antiserum indicates that there is no non-specific precipitation by the protein A. On examination of the autoradiograph for polypeptides with Mr values corresponding to the known Mr values of the RNA polymerase subunits, the following is observed. No polypeptides of Mr 150,000-160,000 could be seen. However, these Mr values are considerably higher than the Mr of the largest standard polypeptide used (phosphorylase b, with Mr 94,000). As a result any Mr values determined in Fig 5.1 that were higher than this are only approximate, as the linear relationship of $\log_{10}$ Mr against distance migrated is lost at the extremes of the gel. Bearing this in mind, the polypeptide of approximate Mr 125,000 could well represent the β and β' subunits of the RNA polymerase. Indeed, on closer inspection of this band (Fig 5.2) it could be seen that what at first appears to be a single polypeptide is in fact a doublet of very similar Mr values. This matches well with the description of the migratory properties of the β and β' subunits of the RNA polymerase given by Scott & Dow (1986b). This, along with the fact that these proteins were precipitated with antiserum prepared against the RNA polymerase and the Mr ~125,000 polypeptides are the only polypeptides visible on the gel with a Mr greater than 85,000, encourages an identification of the Mr ~125,000 polypeptides as the β and β' subunits of the RNA polymerase. The other core polypeptide, the α subunit, is

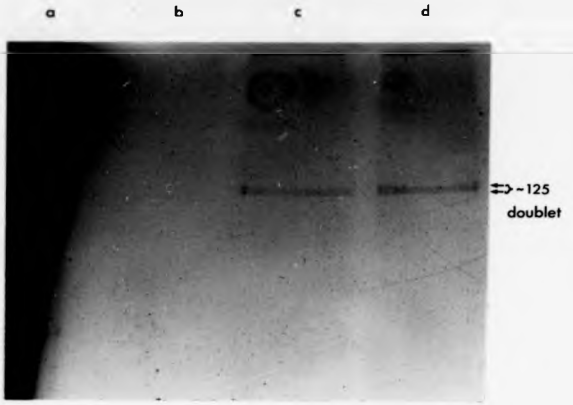


Fig 5.2 An enlargement of the upper region of figure 5.1, showing the suspected RNA polymerase ββ' subunits doublet. The Mr estimation on the right is determined through the extrapolation of a standard curve.

known to have a Mr value of 38,000. Examination of Fig 5.1 shows that the best candidate for this polypeptide was the Mr 35,000 polypeptide, though the presence of at least one other polypeptide in this region of the gel (Mr 43,000) and the lack of any distinguishing features as in the case of the ββ' doublet prevents any firm identification of this polypeptide. The σ factors of the Rm. vannielii RNA polymerase have been sized at Mr 98,000 (Scott & Dow, 1986b). Examining Fig 5.1 for polypeptides of this Mr range reveals no ^{35}S-labelled polypeptides of anything like this Mr value. There are three ways this may be explained. Firstly, it is possible that the σ factor is only weakly antigenic. Secondly, being readily dissociable, it may have been lost from the holoenzyme core during the immunoprecipitation. A third explanation is that the σ factor was precipitated, but contains no methionine residues in the amino acid sequence and thus was not labelled by the ^{35}S-methionine. Scott & Dow (1986b) also reported difficulties visualizing the σ factor by ^{35}S-labelling. As for the minor Mr 78,000 and 68,000 polypeptides reported to be associated with the RNA polymerase (Scott & Dow, 1986a), there are polypeptides precipitated by the anti-RNA polymerase antiserum of Mr 78,000 and 70,000 that may well correspond with these polypeptides. Fig 5.3 shows the ^{32} P exposure of the same gel shown in Fig 5.1. While it is apparent that a number of phosphopolypeptides not known to be associated with the RNA polymerase had been precipitated, it is immediately clear that the putative β and β' subunits of the RNA polymerase were phosphorylated. The resolution of the ^{32}P autoradiograph was not adequate to be able to attributed the phosphorylation to one (or both) subunit(s) from the photograph, though careful examination of the original autoradiograph suggests that both

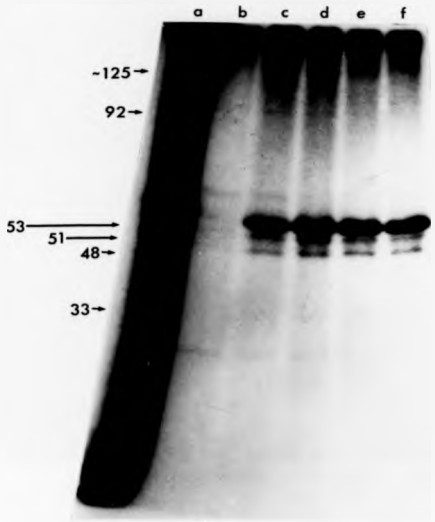


Fig 5.3 ^{32}P-exposure of an autoradiograph of a 10-30% polyacrylamide gel of ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts after the following treatments: a) Untreated; b) after precipitation with protein A; c) after immunoprecipitation with 1/4 dilution of anti-RNA polymerase antibody; d) after immunoprecipitation with 1/8 dilution of antibody; e) after immunoprecipitation with 1/16 dilution of antibody; f) after immunoprecipitation with 1/32 dilution of antibody. Figures on the left indicate Mr values x 10^{-3}.

subunits are phosphorylated. Several phosphopolypeptides were visible for which there are no corresponding ^{35}S-labelled bands in Fig 5.1, indicating that they were either present in very small amounts, or that the polypeptides contain no methionine. Some phosphopolypeptides appeared to be non-specifically precipitated by the protein A. The 92,000 phosphopolypeptide may be a σ factor, though this is unlikely as the Mr estimations at that point should still be fairly accurate. The Mr 33,000 phosphopolypeptide did not correspond with the Mr 35,000 polypeptide suspected of being an α subunit in Fig 5.1, and the discrepancy in Mr was again too high for this to represent an α subunit. The possible RNA polymerase associated polypeptides also appeared not to be phosphorylated.

The conclusions from this experiment can be stated as follows. The evidence for the phosphorylation of the β and β' subunits of the RNA polymerase is good. The lack of any immunoprecipitated phosphopolypeptides of the correct Mr for the other subunits suggests that these are probably not phosphorylated. The σ factor was not visible on either the ^{32}P or the ^{35}S autoradiograph exposures of the gel, thus suggesting that the subunit is poorly antigenic, or not phosphorylated and containing no methionine. The significance of the phosphorylation of the β and β' subunits of the RNA polymerase is not known, but possibilities include a role in defining the template specificity of the holoenzyme. There is clearly a need for further experimentation. Particular experiments that may be of interest include the immunoprecipitation of the RNA polymerase from cells at different stages through the differentiation, or under different growth conditions. A necessary requirement for such experiments would be the preparation of highly purified RNA polymerase with which

antisera with greater specificity could be obtained.

5.2 The Phosphorylation of the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase of Rm. vannielii

The second protein chosen for investigation into its potential for regulation by phosphorylation was the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The reasons for choosing RuBisCO for further examination are basically twofold. Firstly, there is considerable potential for a role for phosphorylation in the regulation of RuBisCO activity, assembly or distribution. Secondly, there exists in Rm. vannielii in vivo a very prominent phosphopolypeptide with a Mr value corresponding well with the known Mr of the large subunit of the RuBisCO. This phosphopolypeptide is, in fact, the most abundant phosphopolypeptide in Rm. vannielii.

RuBisCO is a key enzyme of the reductive pentose phosphate pathway (Calvin cycle, Fig 5.4) catalyzing the fixation of CO_2 on to the acceptor molecule ribulose-1,5-bisphosphate to produce an unstable 6-carbon intermediate which splits to produce two molecules of 3-phosphoglycerate. The enzyme can also catalyze an alternative reaction, the oxygenolysis of ribulose-1,5-bisphosphate resulting in the accumulation of glycolate which is subsequently either metabolized or excreted. The prokaryotic RuBisCO holoenzyme generally consists of large (L) and small (S) subunits arranged in the form L_8S_8, though there are exceptions (see Codd, 1984). The RuBisCO of Rs. rubrum lacks small subunits and exists as a simple dimer (L_2), Rp sphaeroides possesses two forms L_8 and L_8S_8 and Rm. vannielii has a L_8S_8 RuBisCO (Taylor & Dow, 1980). The carboxylase activity of RuBisCO requires magnesium ions and CO_2 for activation, the

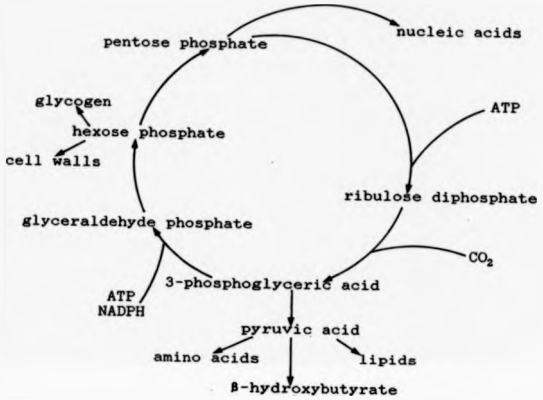


Fig 5.4 The reductive pentose phosphate cycle (Calvin cycle), illustrating the sources of major cellular constituents.

activation proceeding by a rate-limiting carbamate formation on a lysine residue within the large subunit followed by rapid association with Mg^{2+} (see Incharoensakdi et al., 1986). Tabita et al. (1984) found that the carboxylase activity of anaerobically grown Rs. rubrum was markedly and reversibly inhibited on bubbling cultures with air in the absence of any change in the amount of RuBisCO present. This evidence permits the idea of some form of reversible covalent modification to the RuBisCO. Foyer (1985) demonstrated that the large and small subunits of spinach RuBisCO were slightly phosphorylated in vivo and that the amount of phosphorylation was increased by CO_2 fixation. Taylor & Dow (1980) reported that two forms of large subunit of Rm. vannielii RuBisCO could be seen on denaturing polyacrylamide gels. It is also known that the phosphorylated and non-phosphorylated forms of isocitrate dehydrogenase can be separated by non-denaturing PAGE (Borthwick et al., 1984). In view of all these facts, it was considered possible that the RuBisCO of Rm. vannielii could be phosphorylated on its large subunit. The following experiments investigate this possibility.

The analysis of the RuBisCO from Rm. vannielii was carried out by immunoprecipitation of the enzyme from radiolabelled cell-free extracts using anti-RuBisCO antibody and by direct purification and examination of the enzyme. The antibodies used were kindly donated by Dr C.S. Dow.

5.2.1 The immunoprecipitation of RuBisCO from radiolabelled cultures

An early log phase culture of Rm. vannielii was double labelled with ^{35}S-methionine at 1 $\mu Ciml^{-1}$ and ^{32}P-orthophosphate at 50 $\mu Ciml^{-1}$ and harvested in late log phase by centrifugation as

described in section 2.9 (though without the addition of NaF to the culture). Cells were washed in 10 mM Tris-HCl pH 7.5, and broken by sonication, the sonication being carried out as described in section 2.11a. Extracts were cleared by centrifugation in an Eppendorf microfuge. Immunoprecipitations were carried out by the rapid method described by Goswami & Russell (1983) (see section 2.21), the antibody concentrations used being 1, 1/2, 1/4, 1/8, 1/16, 1/32 and 0. SDS-polyacrylamide gel electrophoresis was carried out on 10-30% exponential gradient gels. Gels were treated as described in section 2.15 to eliminate acyl- and amido-phosphates before drying and autoradiography (as described in section 2.16). Fig 5.5 shows the ^{35}S-exposure of the autoradiograph for this experiment. It is clear that only one polypeptide of Mr 53,000 was precipitated by the anti-RuBisCO antibody. There was no non-specific precipitation by protein A. This polypeptide corresponds well to the large subunit of RuBisCO sized at Mr 53,300 by Taylor & Dow (1980). The optimum concentration of antiserun for the precipitation appeared to be a 1/4 dilution of the serum. Fig 5.6 shows the ^{32}P exposure of the same gel shown in Fig 5.5. It was immediately apparent that the precipitated polypeptide seen in Fig 5.5, identified as a RuBisCO large subunit, was a phosphopolypeptide. This experiment clearly demonstrates that the major phosphopolypeptide seen in Rm. vannielii is the large subunit of RuBisCO. The reason for the observation of only one of the two RuBisCO large subunits in the immunoprecipitation (Fig 5.5) is unknown.

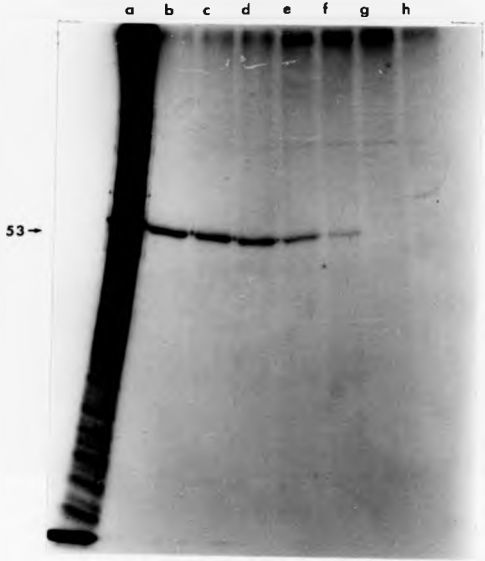


Fig 5.5 ^{35}S-exposure of an autoradiograph of a 10-30% polyacrylamide gel of ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts after the following treatments: a) Untreated; b) after immunoprecipitation with undiluted anti-RubisCO antibody; c) after immunoprecipitation with 1/2 dilution of antibody; d) after immunoprecipitation with 1/4 dilution of antibody; e) after immunoprecipitation with 1/8 dilution of antibody; f) after immunoprecipitation with 1/16 dilution of antibody; g) after immunoprecipitation with 1/32 dilution of antibody; h) after immunoprecipitation in the absence of antibody;. Figures on the left indicate Mr values x 10^{-3}.

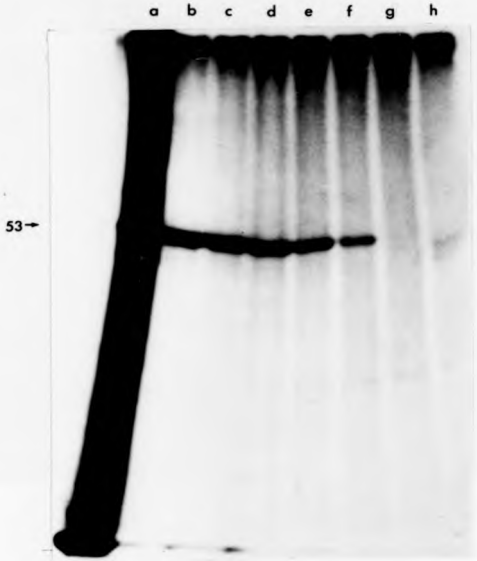


Fig 5.6 ^{32}P-exposure of an autoradiograph of a 10-30% polyacrylamide gel of ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts after the following treatments: a) Untreated; b) after immunoprecipitation with undiluted anti-RubisCO antibody; c) after immunoprecipitation with 1/2 dilution of antibody; d) after immunoprecipitation with 1/4 dilution of antibody; e) after immunoprecipitation with 1/8 dilution of antibody; f) after immunoprecipitation with 1/16 dilution of antibody; g) after immunoprecipitation with 1/32 dilution of antibody; h) after immunoprecipitation in the absence of antibody;. Figures on the left indicate Mr values x 10^{-3}.

5.2.2 The purification and examination of RuBisCO by gel electrophoresis

Having demonstrated that the large subunit of Rm. vannielii RuBisCO is phosphorylated, the question arises as to what function the phosphorylation performs? Is it involved in regulation?, or is it involved in assembly or in the distribution of RuBisCO between soluble and particulate forms? In order to begin to answer some of these questions more extensive characterization of the phosphorylation of the RuBisCO large subunit was required. This work was carried out in collaboration with Dr N.H. Mann. Dr Mann carried out the sucrose gradient centrifugation and fractionation, the immunoprecipitation and the RuBisCO assays, while I carried out the cell breakage for the RuBisCO assays, gel electrophoresis, protein assays, phosphoprotein gel treatments, autoradiography, scintillation counting and developing.

The experiment was carried out as follows. An early exponential phase culture of Rm. vannielii was double labelled with ^{35}S-methionine to 1 μCiml^{-1} and with ^{32}P-orthophosphate to 50 μCiml^{-1} and harvested in late log phase by centrifugation as described in section 2.9 (though without the addition of NaF to the culture). Cells were washed in 10 mM Tris-HCl pH 7.5, and broken by sonication, the sonication being carried out as described in section 2.11a. Extracts were cleared by centrifugation in an Eppendorf microfuge. The extract obtained was loaded on to a step sucrose gradient, centrifuged and fractionated as described in section 2.22. The fractions obtained were subjected to 4-30% non-denaturing PAGE (see section 2.13.3), 10-30% SDS-PAGE (see section 2.13.1) and immunoprecipitation (the

method described by Pringle (1985), see section 2.21) followed by 10-30% SDS-PAGE (2.13.1). An identical, unlabelled, culture was fractionated in the same manner except that French pressing was used in preference to sonication for cell breakage, and fractions were assayed for protein (2.10) and RuBisCO activity (2.23). The results obtained were as follows.

Fig 5.7 shows the autoradiograph of the 10-30% denaturing gel of the double-labelled samples from the bottom of the sucrose gradient. At the bottom of the gradient one protein predominated, of Mr 53,500. There were also traces of a protein of Mr 55,500 running just above this protein. As the RuBisCO large subunits are known to run at this position both on the sucrose gradient and on polyacrylamide gels, it is highly likely that these bands, in fact, represent the RuBisCO large subunits (LSU). If this is the case, then it can be seen that the two RuBisCO LSUs have a different distribution on the sucrose gradient, the Mr 55,500 LSU reached highest concentration in fraction 9 from the gradient, while the Mr 53,500 subunit attained highest concentration in fraction 7. In addition, the larger subunit appeared to be concentrated in a narrower band on the gradient than the smaller of the LSUs, and appeared to be present in much smaller amounts. Another band of interest was the Mr 15,500 polypeptide. The distribution of this subunit appeared to match that of the larger LSU, and it is thus possible that this may represent the small subunit of the RuBisCO. Fig 5.8 shows the ^{32}P exposure of the same gel autoradiographed for Fig 5.7. This clearly illustrated that, of the suspected RuBisCO subunits, only the smaller (Mr 53,500) was phosphorylated. The high intensity of the ^{32}P labelled LSU band must be taken into consideration when commenting on the relative amounts of the LSUs. The slight

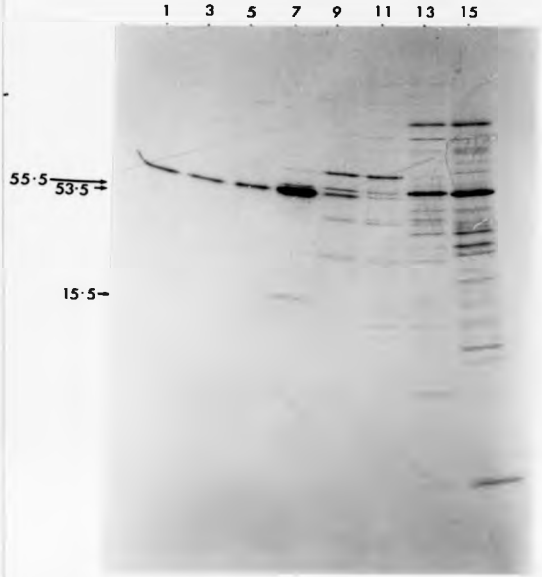


Fig 5.7 ^{35}S-exposure of an autoradiograph of a 10-30% polyacrylamide gel of alternate fractions from the bottom of a sucrose gradient on which had been run ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts. The numbers at the top of the tracks represent the number of the fraction (1=the bottom of the gradient). Figures on the left indicate Mr values x 10^{-3}.

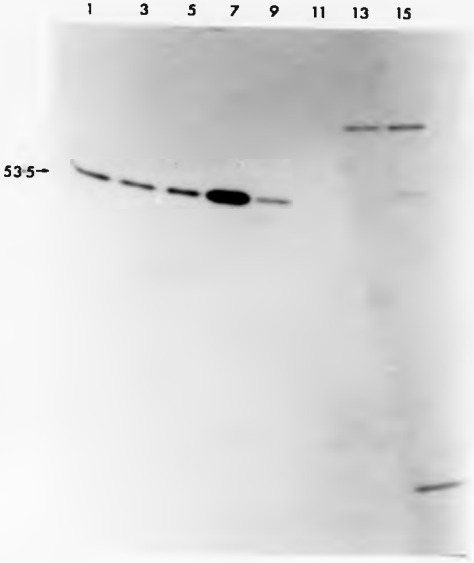


Fig 5.8 ^{32}P-exposure of an autoradiograph of a 10-30% polyacrylamide gel of alternate fractions from the bottom of a sucrose gradient on which had been run ^{32}P and ^{35}S double-labelled Rm. vannielii cell extracts. The numbers at the top of the tracks represent the number of the fraction (1=the bottom of the gradient). Figures on the left indicate Mr values x 10^{-3}.

'leakage' of ^{32}P emmissions blackening the ^{35}S exposed autoradiograph accounted in part for the high intensity of the Mr 53,500 polypeptide on the ^{35}S autoradiograph (Fig 5.7) and probably wholly for the diffuse appearance of the band on this picture. Fig 5.8 appears to confirm also the suspicion expressed in Chapter 3 that the Mr 54,500 phosphopolypeptide band comprised more than one phosphopolypeptide. These observations, however, can not be taken too far without some definite proof that the polypeptides being considered are, in fact, RuBisCO subunits. This evidence arises from two sources, assays of RuBisCO carboxylase activity from parallel sucrose gradients, and direct observation of the anti-RuBisCO antisera-precipitable polypeptides from the sucrose gradient fractions of the double-labelled samples. The immunoprecipitations gave the following results.

Fig 5.9 shows the ^{35}S exposed autoradiograph of a SDS-PAGE gel of proteins precipitated from the sucrose gradient fractions seen in Fig 5.7 with anti-RuBisCO antiserum provided by Dr C.S. Dow. Fig 5.10 shows the ^{32}P exposure of the same gel. Fig 5.9 clearly confirmed that the polypeptides designated as RuBisCO LSUs in Fig 5.7 were indeed genuine. In addition the Mr 15,500 polypeptide suspected of being the RuBisCO small subunit was also precipitated. This showed more clearly than Fig 5.7 how the distribution of the small subunit on the sucrose gradient appears to match the distribution of the Mr 55,500 LSU more closely than it does the Mr 53,500 LSU. Fig 5.10 showed that the Mr 53,500 LSU is a phosphopolypeptide. In both Fig 5.9 and 5.10 small amounts of a Mr 27,000 phosphopolypeptide could be seen. There are no known RuBisCO components of this Mr value, indicating that this may have been a contaminant. The possibility cannot be excluded,

Fig 5.9 ^{35}S-exposure of an autoradiograph of a 10-30% polyacrylamide gel of the alternate fractions from the bottom of a sucrose gradient seen in Fig 5.7 after immunoprecipitation with anti-RubisCO antibody. The numbers at the top of the tracks represent the number of the fraction (1=the bottom of the gradient). Figures on the left indicate Mr values x 10^{-3}.

Fig 5.10 ^{32}P-exposure of an autoradiograph of a 10-30% polyacrylamide gel of the alternate fractions from the bottom of a sucrose gradient seen in Fig 5.7 after immunoprecipitation with anti-RubisCO antibody. The numbers at the top of the tracks represent the number of the fraction (1=the bottom of the gradient). Figures on the left indicate Mr values x 10^{-3}.

however, that this polypeptide has some role to play in RuBisCO assembly, or perhaps in the formation of carboxysomes which may be present in Rm. vannielii (Whittenbury & Dow, 1977). The possibility of immunoprecipitation of non-RuBisCO carboxysome components necessitates a brief explanation as to why both the Mr 55,500 and the Mr 53,500 polypeptides are considered to be RuBisCO LSUs. The explanation is simply that the heterogeneity of the LSUs was observed in electrophoretically homogeneous soluble Mr 420,000 RuBisCO and is necessary because Holthuijzen et al. (1986) reported that the apparent heterogeneity of the LSUs of RuBisCO from carboxysome preparations from a variety of organisms was due to the presence of a glycoprotein in the outer layer of the carboxysomes having a similar Mr value to the RuBisCO LSU. The second line of evidence confirming the polypeptides observed in Fig 5.7 as RuBisCO subunits comes from assays of RuBisCO carboxylase activity from a parallel sucrose gradient. Fig 5.11 shows the results of a RuBisCO assay on the gradient fractions overlayed on the protein assay of the gradient fractions. This clearly showed that the peak of RuBisCO carboxylase activity occured in fraction 8. Considering only the odd numbered fractions corresponding to those run on the gels from the double labelling experiments (Figs 5.7-5.10), then the peak of RuBisCO carboxylase activity would be seen in fraction 7. This corresponds exactly with the fraction in which the maximum amounts of the suspected large subunits are observed. It is interesting to note that the odd numbered fractions containing the highest levels of RuBisCO carboxylase activity match well the levels of the LSU of the higher (55,500) Mr value, i.e. the non-phosphorylated form. A second point of interest is the small peak of both protein content and RuBisCO carboxylase activity in

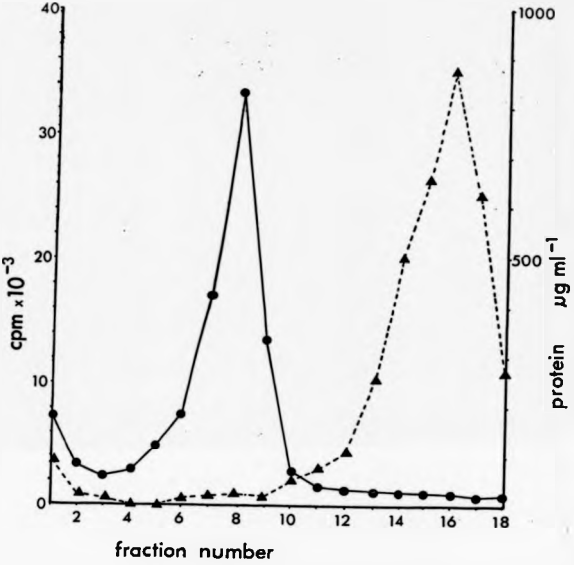


Fig 5.11 Plot of Ribulose bisphosphate carboxylase activity and protein content in sucrose gradient fractions of Rm. vannielii cell-free extracts. The solid line indicates RubisCO activity in cpm ^{14}C-bicarbonate incorporated into acid insoluble material, the dashed line indicates protein concentration.

fraction 1 from the bottom of the gradient. This suggests that there is a heavy component in the cell containing some RuBisCO activity. This could be interpreted in several ways. It may indicate the presence of carboxysomes in Rm. vannielii. or possibly be due to some form of aggregation between RuBisCO subunits. Another explanation would be that a portion of the RuBisCO is, in fact, particulate i.e. membrane associated.

The evidence is clear, then, that there are two forms of the large subunit of RuBisCO in Rm. vannielii. one of which is phosphorylated. Whether the two forms of the LSU are identical in all but their phosphorylation state or whether they are, in fact, two different polypeptides has yet to be established. It is also possible that one may be a precursor form of the other. The role of the phosphorylation of the RuBisCO LSU also remains to be established. Possibilities include a role in the assembly of the RuBisCO holoenzyme, a role in the formation or maintainance of carboxysomes or covalent regulation of the enzyme activity. The latter function introduces the possibility that phosphorylation may differentially effect the carboxylase and oxygenase activities of the RuBisCO. While these experiments show that there is phosphorylation of a RuBisCO LSU, it is very difficult to attribute the RuBisCO carboxylase activity to one LSU. As mentioned above, there are various circumstantial elements that suggest that the non-phosphorylated form may the active one, but the possibility can not be excluded that the phosphorylated form in conjunction with the small subunit (the distribution of which matches the non-phosphorylated LSU) may be the active form. Of course, both forms may be active and the phosphorylation may serve a different purpose altogether. Clearly, a great deal of work still remains to be done on the phenomenon of the phosphorylation of the RuBisCO LSU.

CONCLUSIONS

CHAPTER 6.

In this chapter I will first present an overview and summary of the results obtained in this project, and then go on to discuss the possibilities for future work.

6.1 An Overview

Rm. *vannielii* was shown to contain phosphopolypeptides, the synthesis of which varied through the growth curve. Care was taken to eliminate the possibility of changes in the phosphopolypeptide pattern due to the change in the incident light intensity as the culture density increased through the growth curve. Phosphoamino acid analysis demonstrated that the predominant phosphoamino acid was phosphoserine, though phosphotyrosine and phosphothreonine were also detected. The major phosphopolypeptide in the cell was shown to be the RuBisCO large subunit. Phosphorylation of this polypeptide was shown to be growth stage-dependent, commencing around mid log phase. The fact that this polypeptide is being actively phosphorylated through the growth curve, with no apparent change in the steady state-labelled amounts of this phosphopolypeptide suggests that the RuBisCO large subunit is subject to bidirectional phosphorylation-dephosphorylation. The role of RuBisCO large subunit phosphorylation is as yet undetermined, so the effect of changes in its phosphorylation state through the growth curve can not be ascertained. We can speculate, however, that if phosphorylation is associated with deactivation of the enzyme (as in isocitrate dehydrogenase) an active RuBisCO should not be

required in photoheterotrophically grown cells, and thus the RuBisCO is phosphorylated. However, after a long period of maintenance of the stock culture all organic carbon may have been utilized and the cells may be gaining carbon for their maintenance requirements by fixation of evolved CO_2. Thus in the inoculum, the RuBisCO may be dephosphorylated and active. This is probably not the case, as in the stock culture on the bench light limitation is extreme. Thus it is unlikely that a cell under these conditions would be able to obtain enough energy from light to maintain an active RuBisCO, and hence deactivation of the RuBisCO (by dephosphorylation) would be required to prevent the wasteful utilization of the limited available energy. A similar explanation can be applied to the swarmer cell. A swarmer cell is produced under conditions of low light and high CO_2 and is thus energy-limited. In addition, the supressed, non-differentiating swarmer cell does not increase its biomass, and thus has a limited requirement for the fixation of CO_2. So, again, the swarmer cell would appear to benifit from having an inactive RuBisCO. This tends to suggest that the RuBisCO is active in a phosphorylated form and inactive (or more correctly less active as it is probable that as with most allosteric enzymes the modification would not totally abolish activity) when dephosphorylated. Evidence from experiments since carried out by Dr N.H. Mann (personal communication) supports this hypothesis. A second phosphopolypeptide of Mr 12,700 was tentatively identified as a light harvesting chlorophyll binding protein. Analysis of phosphopolypeptides from swarmer and chain cells revealed that there were no detectable phosphopolypeptides in the non-differentiating swarmer cell. Analysis of phosphorylation during differentiation revealed that the accumulation of

phosphopolypeptides did not become detectable until 1 hour into differentiation and the phosphorylation of most polypeptides appeared to reach a maximum rate by 2 hours into differentiation. The rate of phosphorylation of the RuBisCO large subunit, however, continued to increase through differentiation despite there being no detectable increase in the observed amounts of the phosphorylated form of the RuBisCO large subunit beyond three hours into differentiation. In addition, two phosphopolypeptides specific to the end, and later stages of differentiation were observed. Investigations in to protein kinase activities in cell-free extracts established that the conditions under which the experiments were carried out had to be extremely carefully controlled. On examining the effects of Mg^{2+} and Ca^{2+} on the pattern of phosphopolypeptides obtained from cell-free extracts it was found that both cations could influence the phosphopolypeptide patterns produced and that the effects were concentration-dependent in a way that supported the suggestion that there were multiple protein kinases active in the extracts. In particular, Ca^{2+} was found to prevent the phosphorylation of a Mr 47,000 phosphopolypeptide the phosphorylation of which was Mg^{2+}-dependent. The addition of chlorpromazine (a calmodulin antagonist) instead of Ca^{2+} produced the same effect leading to the suggestion that there may be a calmodulin-like molecule in Rm. vannielii that exerts its effect on protein kinases through an interaction with Mg^{2+} rather than Ca^{2+}. Other explanations were also put forward. As has been found in other studies on protein phosphorylation in prokaryotes, the resemblance between the proteins observed to be phosphorylated in vivo and in vitro was slight (see section 1.4). Examination of protein kinase activities through differentiation revealed that the

dark-incubated swarmer cell contained potentially active protein kinases despite there being no detectable phosphorylation of polypeptides *in vivo*. This suggests that protein kinases are present in the inhibited swarmer cell, but inactive. Examination of specific proteins for phosphopolypeptides by immunological techniques identified the β and β' subunits of a DNA-dependent RNA polymerase and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase as being phosphorylated. The large subunit of RuBisCO was identified on 1 dimensional polyacrylamide gels as the heavily-labelled Mr 54,500 phosphopolypeptide.

6.2 Conclusions

There are a number of inferences that can be drawn from this work. The first point addresses the question of the overall role of protein phosphorylation in *Rm. vannielii* and prokaryotes in general. To date, the protein kinases discovered and characterized in prokaryotes have all been highly specific, phosphorylating a single protein. This situation is radically different from that observed in eukaryotes, where (for example) protein kinase C is capable or phosphorylating a large number of polypeptides performing a central, coordinating, regulatory role (see section 1.2.2). In other words, as well a the specific regulatory protein kinases like those seen in prokaryotes, eukaryotes posses a number of kinases with broad specificity that perform a coordinating function. Is, then, the role of protein phosphorylation in prokaryotes much more limiting than that seen in eukaryotes ? As yet no broad specificity protein kinases have been discovered in prokaryotes, but I believe that this and other work contains evidence to suggest that the role for protein phosphorylation in prokaryotes may be more extensive than has yet

been demonstrated. Work carried out by Cortay et al. (1986b) demonstrated that as many as 10% of the polypeptides in E. coli were phosphorylated to some degree. It seems inconceivable that every one of these proteins should be matched with a specific kinase and phosphatase. The work described here showed that the dark-incubated swarmer cell contained no phosphopolypeptides, although it possessed potentially active protein kinases. During differentiation, substantial protein phosphorylation was observed. Why should the required state of the proteins in the inhibited swarmer cell be conferred exclusively by dephosphorylation ? One answer would be to suggest that as the initiation of differentiation would probably require wholesale changes in many enzyme activities within the cell, it would be efficient to have all these activities requiring coordination altered by a limited number of effectors. Thus the suggestion is that the environmental changes that bring about the onset of differentiation exert their effect by the activation of a small number of protein kinases, which then phosphorylate a larger number of proteins altering their activities to that required by the actively growing and dividing cell. If this theory is correct, then it may be possible to test it to some extent by examining the phosphorylation state of enzymes known to display different activities in the inhibited swarmer cell and the actively growing cell. Examples of such enzymes may be the DNA-dependent DNA-polymerase, the enzymes responsible for chlorophyll synthesis (only synthesized early in the swarmer cell maturation phase, Whittenbury & Dow (1977)) or the DNA-dependent RNA polymerase (differential sensitivity to inhibitors has been observed through differentiation, Whittenbury & Dow (1977)). Thus it could be established whether protein phosphorylation has a

central, coordinating, regulatory role in prokaryotes as well as eukaryotes. Though I believe that any such role is possibly not as extensive as that seen in eukaryotes.

A second point worthy of discussion is the potential of RuBisCO for bidirectional modification by a phosphorylation/dephosphorylation mechanism. In section 3.5.2 it was observed that the Mr 54,500 phosphopolypeptide (later demonstrated to be the RuBisCO large subunit) was subject to either bidirectional phosphorylation/dephosphorylation or to a high rate of protein turnover. It seems unlikely that RuBisCO, an enzyme required and synthesized in large quantities by the cell, should be rapidly turned over. Inactivation of the enzyme may, at times, be required but covalent modification (or some other form of allosteric modification) rather than wholesale degradation would seem to be a more efficient means of control. In addition, in vivo labelling experiments carried out in section 3.6 in the presence of a serine protease inhibitor (APBA) showed that the Mr 54,500 phosphopolypeptide was apparently not subject to proteolytic turnover by serine proteases. This does not, of course, preclude proteolytic turnover by other proteases, but it does add further substance to the claim for a bidirectional phosphorylation/dephosphorylation mechanism. If it were to be the case that RuBisCO was subject to bidirectional phosphorylation/dephosphorylation then it is very likely, given the abundance of RuBisCO in the cell, that the converter enzymes would be operating in the zero order region of their velocity curves. Thus the interconvertion of the phosphorylated and dephosphorylated forms of RuBisCO may be subject to zero order ultrasensitivity (see section 1.3), with all that implies for any regulatory function for the phosphorylation.

6.3 Future Prospects

This project opens the way for considerable further investigation of the phenomenon of protein phosphorylation. Firstly, there are those investigations that continue and expand on the work described here. Regarding the general study of the phenomenology of protein phosphorylation in Rm. vannielii, it would first of all be useful to more completely categorize the phosphopolypeptides seen in vivo. This could be done through the analysis of phosphopolypeptides on two-dimensional gels as carried out for the phosphopolypeptides of E. coli by Cortay et al. (1986b). Utilizing the labelling regimes described in section 3.4 for the visualization of phosphoamino acids, it may be possible for enough activity to be accumulated in individual phosphopolypeptides to permit their individual phosphorylated amino acid to be identified. For phosphopolypeptides present in great abundance within the cell (e.g. the RuBisCO large subunit) such analysis should be relatively straightforward. It might then be interesting to examine and compare the phosphopolypeptide patterns obtained from Rm. vannielii grown under different growth regimes. E.g. between photoheterotrophic growth and photoautotrophic growth on H_2 and CO_2. Possibly more interestingly, the light-harvesting polypeptides could be examined for further evidence (to that presented in chapter 3) of phosphorylation and the effects of such phosphorylation could be investigated to determine whether it performs the role hypothesized by Allen & Holmes (1986). The role of phosphorylation of the β and β' subunits of the DNA-dependent RNA polymerase is worthy of further investigation, especially as regards any alterations in the template specificity of the

polymerase thus induced and any relationship to the stage of growth or current stage of the cell cycle.

One point as yet unconsidered is the role of vanadate in protein phosphorylation. Vanadate has been known as a potent inhibitor of the sodium pump for some time and is also known to be capable of inhibiting a variety of kinases and phosphatases (Macara, 1980) for which reason vanadate-free ATP was specified for *in vitro* kinase assays. A possible explanation for these phenomena can be proposed based on the interaction of vanadate with phenol and tyrosine described by Tracey & Gresser (1986). They describe how vanadate is capable of esterifying tyrosine in an extremely stable manner that mimics the esterification of tyrosine with phosphate. Thus the inhibition of kinases and phosphatases can be explained by the blocking of the tyrosine residue to be phosphorylated/dephosphorylated by a vanadate ion. More interestingly, it is possible that those systems known to be affected by vanadate may be demonstrating the effects of phosphorylation on a tyrosine residue e.g. the vanadate-sensitive proton-pumping ATP-hydrolase described by Scherer & Boger (1984). Thus it is possible that sensitivity to, or ability to bind vanadate may be an easy way to test systems for possible regulation by phosphorylation on a tyrosine residue.

Apart from these general points, there are clearly a number of specific experiments that may yield useful information and specific areas worthy of further investigation. One such area is the initiation of phosphorylation early in differentiation. The evidence presented here seems to support the hypothesis that protein kinases already present in the swarmer cell could be activated by environmental factors to phosphorylate key proteins in response to, or initiating, differentiation. It may be

worthwhile studying the pattern of phosphorylation obtained early in differentiation in the presence of protein synthesis inhibitors. If phosphorylation is still observed, then there would be stronger evidence for the presence of potentially active protein kinases in the inhibited swarmer cell. Studies into the nature of the Mr 55,000 phosphopolypeptide seen in the *in vitro* labelling experiments carried out with the highly purified cell free extracts (see sections 4.4.5 and 4.4.6) may prove interesting. As was reported, the Mr value determined for this phosphopolypeptide was different to the Mr values obtained for the phosphopolypeptides in the same region of the gel in earlier experiments carried out in less stringently defined reaction conditions. As a result, it cannot be certain that this phosphopolypeptide does not correspond with a phosphopolypeptide seen in *in vivo* labelling experiments (see section 4.4.2). The obvious phosphopolypeptide seen at this Mr *in vivo* is the RuBisCO large subunit, the most abundant phosphopolypeptide in the cell. Thus the obvious experiment is to attempt to precipitate the Mr 55,000 phosphopolypeptide from an *in vitro* protein kinase assay with anti-RuBisCO antiserum. If it should prove that the polypeptide being phosphorylated *in vitro* is the RuBisCO large subunit, then there is a ready-made assay for the RuBisCO kinase and phosphatase. This would be very important for the future investigation of the role of RuBisCO phosphorylation and its regulation in the cell. The discovery of the phosphorylation of the RuBisCO and the potential it offers for future investigation is probably the most significant result from this project. Possibly the most important experiments to be carried out in this area involve the separation and purification of the phosphorylated and non-phosphorylated forms of the enzyme. Using

these purified enzymes the specific activitiesof the two forms could be determined and the effects of phosphorylation on RuBisCO clearly identified. With regard to the purification of the two forms of the enzyme, one technique in particular may prove very effective. This technique is Fe^{3+} chelate chromatography (Andersson & Porath, 1986), by which phosphoproteins and phosphoamino acids can be bound to ferric ions immobilized on a column. The phosphoproteins can subsequently be eluted by the addition of PO_4^- or by a pH increase. Preliminary work involving the separation of the two forms of RuBisCO by this technique has already been carried out by Dr. N.H. Mann (with occasional assistance from myself) with considerable success.

In conclusion, I believe that this work may pave the way for a greater understanding of the extent of the role of protein phosphorylation in prokaryotes.

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