PHOSPHOGLYCERATE MUTASES FROM MICROORGANISMS

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For

mum, dad and Julie.

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ABBREVIATIONS

1. Miscellaneous

A - adenine

ATP - adenosine-5'-triphosphate

bp - base pairs

BPG - bisphosphoglycerate

C - cytosine

C- - carboxy-

CAPS - 3-[cyclohexylamino]-1-propane sulphonic acid

CD - circular dichroism

cDNA - complementary deoxyribonucleic acid

CR - contact region

CTP - cytosine-5'-triphosphate

d- - deoxy-

DAB - 3,3'-diaminobenzidine

DCIC - 3,4-dichloroisocoumarin

DMSO - dimethyl sulphoxide

DNA - deoxyribonucleic acid

ds - double stranded

DTT - dithiothreitol

E-64 - trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane

EDTA - ethylenediaminetetraacetic acid

G - guanine

GTP - guanosine-5'-triphosphate

HEPES - N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]

HPLC - high pressure liquid chromatography

IPTG - isopropyl β-D-thiogalactopyranoside

kb - kilobase

K_m - Michaelis constant

M_r - relative molecular mass

NADH - nicotinamide adenine dinucleotide, reduced

NADPH - nicotinamide adenine dinucleotide phosphate, reduced

NMR - nuclear magnetic resonance

NTP - nucleotide triphosphates

OD - optical density

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEG - polyethyleneglycol

pfu - plaque forming unit

2-PGA - 2-phosphoglyceric acid

3-PGA - 3-phosphoglyceric acid

PMSF - phenylmethylsulfonyl fluoride

RNA - ribonucleic acid

rpm - revolutions per minute

SDS - sodium dodecyl sulphate

SDW - sterile distilled water

ss - single stranded

T - thymine

Tris - tris (hydroxymethyl) amino methane

TTP - thymidine-5'-triphosphate

UV - ultraviolet

X.gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

2. Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Glu	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
B	Asx	Aspartic acid or asparagine
Z	Glx	Glutamic acid of glutamine

3. Proteins

BSA - bovine serum albumin

F-2,6-BPase - fructose-2,6-bisphosphatase (E.C. 3.1.3.46)

GAP.DH - glyceraldehyde-3-phoshate dehydrogenase (E.C. 1.2.1.12)

PFK - 6-phosphofructo-1-kinase (E.C. 2.7.1.11)

PFK-2 - 6-phosphofructo-2-kinase (E.C. 2.7.1.105)

PGAM - phosphoglycerate mutase (E.C. 5.4.2.1)

PGK - phosphoglycerate kinase (E.C. 2.7.2.3)

PK - pyruvate kinase (E.C. 2.7.1.40)

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SYNOPSIS

Phosphoglycerate mutase catalyses the interconversion of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic/gluconeogenic pathways. There are two main types of phosphoglycerate mutase: 2,3-bisphosphoglycerate dependent and 2,3-bisphosphoglycerate independent. The enzyme from *Saccharomyces cerevisiae* has been extensively studied: the high resolution crystal structure of this tetrameric enzyme, subunit M$_r$ 27,000, is known (Winn *et al.*, 1981), the amino acid sequence has been determined (Fothergill and Harkins, 1982) and the gene encoding the enzyme has been isolated and sequenced (White and Fothergill-Gilmore, 1988).

Phosphoglycerate mutase from the fission yeast, *Schizosaccharomyces pombe*, has been purified and partially characterised (Price *et al.*, 1985; Johnson and Price, 1987). It is monomeric, of M$_r$ 23,000, and the sequences of a number of peptides produced by digestion of this enzyme have been determined (Fothergill and Dunbar, unpublished). Alignment of these sequenced peptides with the sequence of *S.cerevisiae* phosphoglycerate mutase shows 40% identity and the conservation of a number of residues which are known to be essential to the activity of the *S.cerevisiae* enzyme e.g. His-8, Arg-7, Ser-11, Thr-20 and Arg-59.

Attempts were made to isolate and sequence the gene encoding *S.pombe* phosphoglycerate mutase. The *S.cerevisiae* phosphoglycerate mutase gene

failed to detect gene sequence homologies in the *S.pombe* genome. An oligonucleotide, designed against part of the *S.pombe* phosphoglycerate mutase sequence (a stretch which was not homologous to the *S.cerevisiae* sequence) also failed to detect sequence homologies in the *S.pombe* genome. Thus under the conditions used, neither the *S.cerevisiae* gene nor the degenerate oligonucleotide appeared to be a suitable molecular probe to screen the *S.pombe* cDNA expression library in λgt11 (which was synthesised by V.Simanis).

A polyclonal antibody against *S.pombe* phosphoglycerate mutase was prepared and used to screen the *S.pombe* cDNA expression library. A number of small identical clones were isolated and sequenced. The cDNA inserts encoded 69 residues and part of this sequence was similar to part of the sequence of phosphoglycerate mutase from other sources. Part of the sequence was also similar to a stretch of fructose-2,6-bisphosphatase sequence (fructose-2,6-bisphosphatase appears to be divergently related to phosphoglycerate mutase, Pilkis *et al.*, 1987).

A purification scheme for phosphoglycerate mutase from the prokaryote, *Streptomyces coelicolor*, has been devised. The N-terminal sequence of this enzyme was determined and confirmed that the gene isolated and sequenced by Peter White, encoded phosphoglycerate mutase from *S.coelicolor*. The enzyme was shown to be a tetramer with a subunit M_r of 29,000. *S.coelicolor* phosphoglycerate mutase was also shown to be partially 2,3-bisphosphoglycerate dependent.

CHAPTER 1

INTRODUCTION

1.1 Glycolysis

Glycolysis is one of the central metabolic pathways (figure 1) which supplies the cell with energy and building blocks for biosynthetic pathways. Enzymes of the glycolytic pathway are found in every living organism, and in most cases, they are present at high concentrations. The structure, activity and evolution of the enzymes of glycolysis have been extensively studied. A number of glycolytic enzymes, from various sources, have been sequenced and the information available suggests that the glycolytic genes have been highly conserved during evolution (Fothergill-Gilmore, 1986). High resolution three-dimensional structure information gathered from X-ray crystallographic studies has revealed that the variety of domains expressed by the glycolytic proteins is limited to a core of β-pleated sheet surrounded by α-helices.

Developments in molecular biology have facilitated the isolation of many genes encoding glycolytic enzymes. As a result, many more protein sequences have become available. Overexpression and site-directed mutagenesis of these genes have made it possible to improve our understanding of the molecular basis of the reactions catalysed by the glycolytic enzymes.

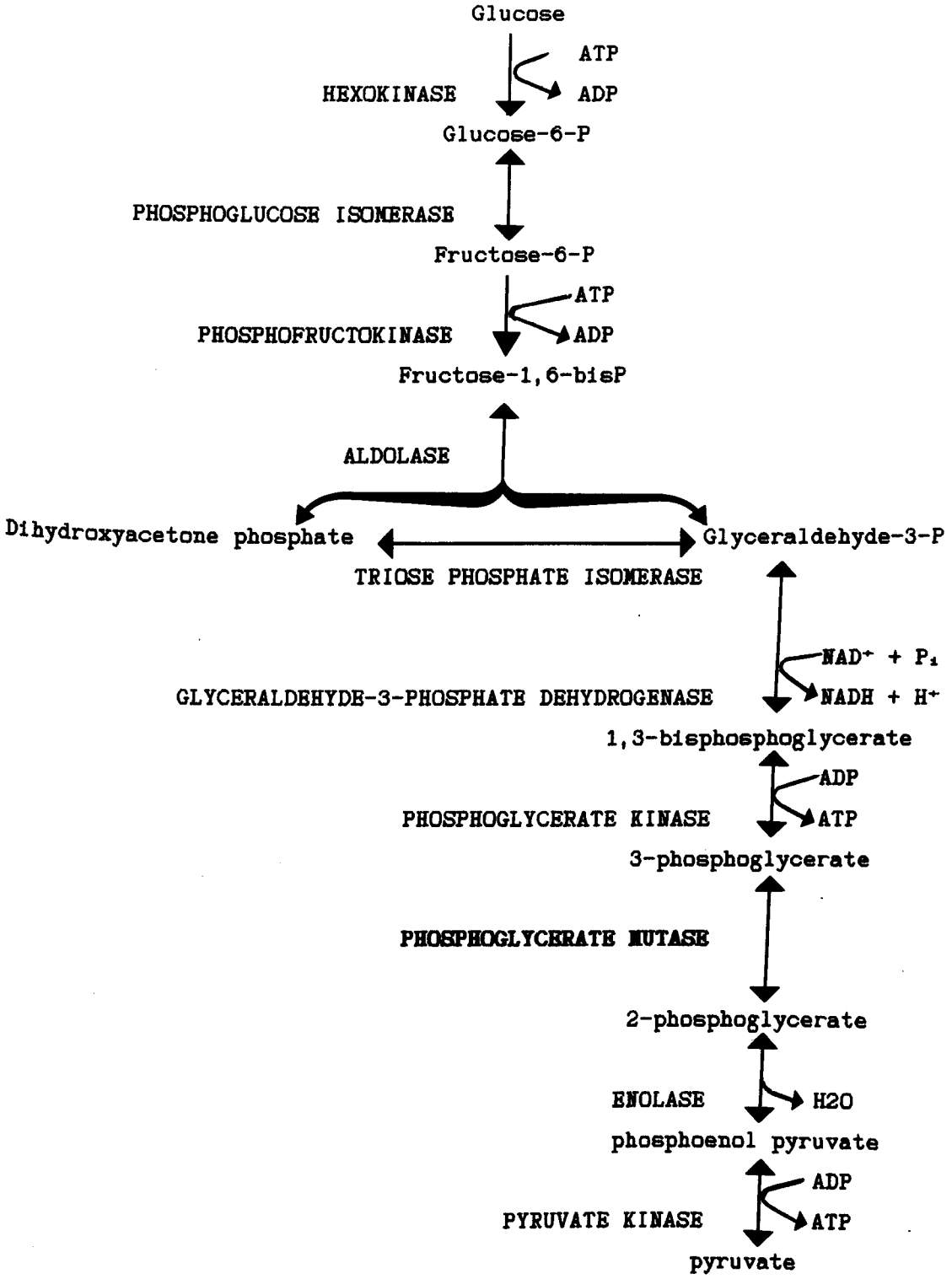

Figure 1 : The Glycolytic Pathway. The reaction catalysed by phosphoglycerate mutase is highlighted.

1.2 Phosphoglycerate mutases

The glycolytic enzyme phosphoglycerate mutase (E.C.5.4.2.1) catalyses the interconversion of 3- and 2-phosphoglycerates (Meyerhof and Kiessling, 1935). Phosphoglycerate mutase has been found in all organisms so far studied, with the possible exception of certain thermophilic archaebacteria (Budgen and Danson, 1986). There are broadly two distinct classes of phosphoglycerate mutase: those which are active in the absence of cofactor 2,3-bisphosphoglycerate (BPG), and those which depend on BPG for activity. BPG-independent enzymes have been found in plant tissues, filamentous fungi, certain algae and invertebrates, and strains of *Bacillus* (Carreras *et al.*, 1982; Price *et al.*, 1983). Phosphoglycerate mutase isolated from *Bacillus* species are BPG-independent, however, the enzyme also has a requirement for manganese (Watabe and Freese, 1979; Singh and Setlow, 1979). The BPG-dependent enzymes have been found in vertebrates, certain invertebrates (Carreras *et al.*, 1982) and fungi such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. BPG-dependent phosphoglycerate mutase has also been found in bacteria such as *E.coli* (D'Alessio and Josse, 1971), *Leuconostoc* spp. (Kawai *et al.*, 1981) and *Pseudomonas* AM1 (Hill and Atwood, 1976). The enzyme isolated from *Zymomonas mobilis*, an anaerobic bacteria, has been reported to show partial BPG-dependence (Pawluk *et al.*, 1986); 20% activity has been reported in the absence of or in the presence of extremely low concentrations of BPG.

1.3 Cofactor-independent phosphoglycerate mutases

Cofactor-independent phosphoglycerate mutases have been isolated from higher plants and filamentous fungi. Cofactor-independent mutases from such sources have proved difficult to purify which may be related to the fact that such enzymes appear to be structurally unstable (McAleese *et al.*, 1985; Johnson and Price, 1988). As a result, cofactor independent phosphoglycerate mutases have not been as well characterised as the BPG-dependent mutases. From the limited amount of information available, it can be concluded that cofactor-independent phosphoglycerate mutases are generally monomeric in nature, $M_r \approx 60,000$.

In certain higher plants, two isoenzyme forms of cofactor-independent phosphoglycerate mutase have been isolated, which correspond to cytosolic and plastid forms (Murphy and Leech, 1978; Journet and Douce, 1984; Mierny and Dennis, 1982; Botha and Dennis, 1986). The two isoenzyme forms isolated from the developing endosperm of castor oil seed have been partially characterised (Botha and Dennis, 1986): both exist as monomers, M_r 64,000 but the cytosolic form is more abundant, more stable and exhibits a higher affinity for substrate.

Phosphoglycerate mutase from *Bacillus* species also exists as a monomer of $M_r \approx 60,000$. However, unlike other cofactor-independent mutases, the activity of this enzyme has an absolute requirement for manganese (Singh and Setlow, 1979; Watabe and Freese, 1979).

1.4 Cofactor-dependent phosphoglycerate mutase

Cofactor-dependent phosphoglycerate mutase has been isolated from vertebrates, certain invertebrates, fungi and bacteria. Such enzymes have proved easier to purify and as a result, have been more extensively characterised, particularly the mutase from *Saccharomyces cerevisiae*. The X-ray structure of *S.cerevisiae* phosphoglycerate mutase has been determined to 0.28nm resolution (Winn *et al.*, 1981), and the amino acid sequence of the protein has been established (Fothergill and Harkins, 1982). More recently the gene encoding *S.cerevisiae* phophoglycerate mutase has been isolated and sequenced (Kawasaki and Fraenkel, 1982: White and Fothergill-Gilmore, 1988). The *S.cerevisiae* enzyme is tetrameric with subunit M_r 27,500. Phosphoglycerate mutase from vertebrates, *E.coli, Z.mobilis and L.dextrancium*, exist as dimers with subunit M_r 27,000-30,000. Sequence information is now available for the dimeric enzymes from human muscle (Shanske *et al.*, 1987) and human brain (Blouquit *et al.*, 1988). The monomeric phosphoglycerate mutase from the fission yeast, *Schizosaccharomyces pombe*, differs from all other cofactor-dependent mutases in that it has a subunit M_r 23,000. Peptides generated by digestion of *S.pombe* phosphoglycerate mutase with endoproteinases, Glu-C, Asp-A and clostripain, have been sequenced (Fothergill and Dunbar, unpublished). Approximately 90% (196) residues of the expected number (220) of amino acids have been sequenced.

In mammals, two genes encode phosphoglycerate mutase (Fundele *et al.*, 1981; Junien *et al.*, 1982): one encodes the B-type and the other encodes the M-

type isoenzyme of phosphoglyerate mutase. The B-type isoenzyme is found in most tissues, excluding skeletal muscle, whereas the M-type is particular to cardiac and skeletal muscle. MB heterodimers have been found to occur in tissues such as the heart, where both isoenzymes are expressed (Bartrons and Carreras, 1982). The three isoenzymes (B,M and MB) differ in their electrophoretic mobility, heat stability and their susceptibility to inactivation by sulphydryl group reagents. M-type phosphoglycerate mutase is completely inhibited by Hg^{2+} (Diedrrich *et al.*, 1970) and tetrathionate (Mezquita *et al.*, 1981), under conditions where the B-type remains fully active. Sequence alignment of the *S.cerevisiae* phosphoglycerate mutase, with the B-type and M-type isoenzymes offers an explanation. Residue 20, which is located in the active site of the *S.cerevisiae* enzyme, is a cysteine residue in the M-type but is a threonine and serine residue in the *S.cerevisiae* and B-type isoenzyme, respectively. Sequence alignment also reveals around 80% identity for the mammalian M- and B-type isoenzymes (Sakoda *et al.*, 1988). Kinetic studies (Berrocal and Carreras, 1988) reveal the isoenzymes share similar properties.

In mammalian erythrocytes, a gene closely related to M- and B-type isoenzymes, encodes bisphosphoglycerate mutase. The sequence of bisphosphoglycerate mutase is 50% similar to the M- and B-type isoenzymes and this enzyme can also form heterodimers with the isoenzymes. For these reasons, bisphosphoglycerate mutase is considered an isoenzyme, which is referred to as the E-type. Sequence information is available for three E-type isoenzymes: human, rabbit and mouse. The sequences show a high degree of conservation, with 90% identity.

1.5 Reactions catalysed by phosphoglycerate mutase

Cofactor-independent phosphoglycerate mutases only catalyse the interconversion of 3- and 2- phosphoglycerates, whereas cofactor-dependent phosphoglycerate mutases catalyse three reactions: the interconversion of 3- and 2- phosphoglycerates and the synthesis and the degradation of 2,3-bisphosphoglycerate, see figure 2. All three reactions are also catalysed by bisphosphoglycerate mutase. Bisphosphoglycerate mutase (E-type) and phosphoglycerate mutases (B-type, M-type and from *S. cerevisiae*) differ in the rates at which they catalyse these reactions. Phosphoglycerate mutase has a very high ratio of mutase:synthase activities whereas for bisphosphoglycerate mutase, the catalytic constants of the mutase and synthase reactions are relatively similar (Fothergill-Gilmore and Watson, 1989).

1.6 Structures of phosphoglycerate mutase

1.6.1 Primary structure

The amino acid sequence of phosphoglycerate mutase from a number of sources has been determined: *S. cerevisiae* (White and Fothergill-Gilmore, 1988),

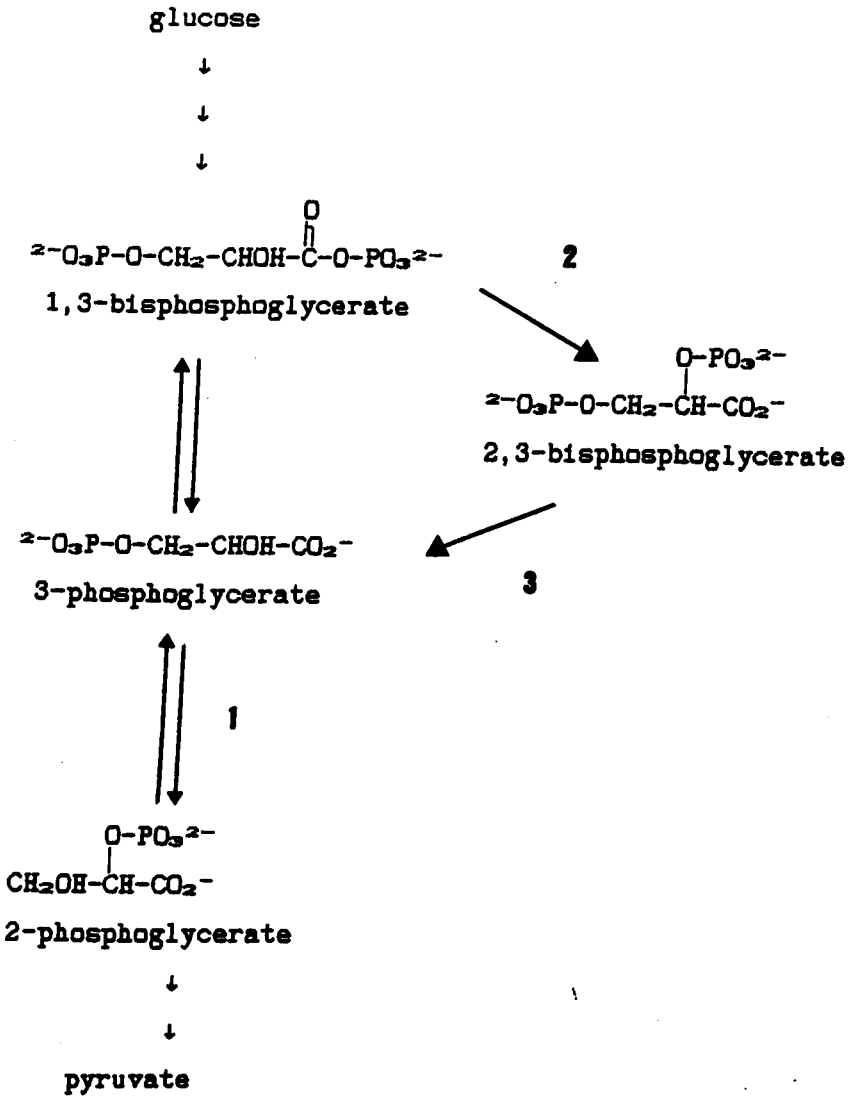

Figure 2: The Reactions Catalysed by Phosphoglycerate Mutases. The interconversion of 2- and 3-phosphoglycerates (reaction 1) is the major reaction catalysed by cofactor-dependent phosphoglycerate mutases. Cofactor-dependent mutases also catalyse reactions 2 and 3, but at relatively low rates. Bisphosphoglycerate mutase catalyses all three reactions but the major reaction is the interconversion of 1,3- and 2,3-bisphosphoglycerates (reaction 2). Cofactor-independent enzymes catalyse reaction 1.

human M-type (Shanske *et al.*, 1987), human B-type (Blouquit *et al.*, 1988) and *S.coelicolor* (White *et al.*, 1992). Sequence information is also available for bisphosphoglycerate mutase: human E-type (Joulin *et al.*, 1986), rabbit E-type (Yanagawa *et al.*, 1986) and mouse E-type (Leboulch *et al.*, 1988). Alignment of all of these sequences reveals a high degree of sequence identity (figure 3), which is indicative of a slow rate of evolution and typical of glycolytic genes (Fothergill-Gilmore, 1986).

The availability of the sequence information for a range of phosphoglycerate mutases and bisphosphoglycerate mutases has helped to probe the structure, mechanism and evolution of these enzymes, as will be discussed. No primary structure information is available for cofactor-independent phosphoglycerate mutase and so it is unclear whether cofactor-dependent and independent enzymes are evolutionarily related.

1.6.2 Tertiary structure

The high resolution three-dimensional structure of *S.cerevisiae* phosphoglycerate mutase has been determined by X-ray crytallographic studies (Winn *et al.*, 1981). These studies have shown that the enzyme is composed of four identical subunits, arranged with almost exact 222 symmetry. The α-carbon chain of each subunit folds into a single domain with a central core of β-sheet surrounded by α-helices (figure 4) in a manner similar to the nucleotide-binding domains of kinases and

Figure 3: Alignment of the known amino acid sequences of phosphoglycerate mutases: Sco=*S.coelicolor* PGAM; Sce=*S.cerevisiae PGAM*; Hre2=human BPGAM; Mre=mouse BPGAM; Rre=rabbit BPGAM; Hmu=human M-type BPGAM; Rmu=rabbit M-type PGAM; Pgms=*S.pombe* PGAM. All of the sequences, except *S.pombe* PGAM, were selected from the NBRF database of proteins. The *S.pombe* PGAM sequence is derived from the partial peptide sequence information (Dunbar and Fothergill, unpublished). All of the sequences, except *S.pombe* PGAM, were aligned using the GCG multiple sequence editing programme, LINEUP (Devereux *et al.*,1984). To align the *S.pombe* sequence against the multiple alignment, the GCG PROFILE programme (Gribskov *et al.*,1987) was used. PROFILE was then used to generate a consensus sequence of the multiple alignment, labelled 'consensus'.

```
           1                                                  50
     Sco   ADAPYKLILL RHGESEWNEK NLFTGWVDVN LTPKGEKEAT RGGELLKDAG
     Sce   ..MP.KLVLV RHGQSEWNEK NLFTGWVDVK LSAKGQQEAA RAGELLKEKK
    Hre2   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
     Mre   .MSKHKLIIL RHGEGQWNKE NRFCSWVDQK LNNDGLEEAR NCGRQLKALN
     Rre   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
     Hmu   .MATHRLVMV RHGETTWNQE NRFCGWFDAE LSEKGTEEAK RGAKAIKDAK
     Rmu   .MATHRLVMV RHGESSWNQE NRFCGWFDAE LSEKGAEEAK RGATAIKDAK
     Pgms  .AAPNLLVLT RHGESEWNKL NLFTGWKDPA LSETGIKEAK LGGERLKSRG
consensus  AMAPHKLVML RHGESEWNKE NWFCGWVDQK LSEKGMEEAK RGGKQLKDMN

           51                                                 100
     Sco   LLPDVVHTSV QKRAIRTAQL ALEAADRHWI PVHRHWRLNE RHYGALQGKD
     Sce   VYPDVLYTSK LSRAIQTANI ALEKADRLWI PVNRSWRLNE RHYGDLQGKD
    Hre2   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
     Mre   FEFDLVFTSI LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
     Rre   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
     Hmu   MEFDICYTSV LKRAIRTLWA ILDGTDQMWL PVVRTWRFNE RHYGGLTGFN
     Rmu   IEFDICYTSV LKRAIRTLWT ILDVTDQMWV PVVRTWRLNE RAYGGLTGLN
     Pgms  YKFDIAFTSA LNRANRT... .......... ........RQ RYYGDLQGLN
consensus  FEFDIVYTSV LNRAIRTARL ILEELDQEWV PVEWSWRLNE RHYGALIGLN

           101                                                150
     Sco   KAQTLAEFGE EQFMLWRRSY DTPPPALDRD AEYSQF..SD PRYAM.LPP.
     Sce   KAETLKKFGE EKFNTYRRSF DVPPPPIDAS SPFSQK..GD ERYKY.VDP.
    Hre2   REQMALNHGE EQVRLWRRSY NVTPPPIEES HPYYQEIYND RRYKVCDVPL
     Mre   REKMALNHGE EQVRLWRRSY NVTPPPIEES HPYFHEIYSD RRYKVCDVPL
     Rre   REKMALNHGE EQVRIWRRSY NVTPPPIEES HPYYHEIYSD RRYRVCDVPL
     Hmu   KAETAAKHGE EQVRSWRRSF DIPPPPMDEK HPYYNSISKE RRYA.GLKPG
     Rmu   KAETAAKHGE EQVKIWRRSF DTPPPPMDEK HNYYASISKD RRYA.GLKPE
     Pgms  KDDARKKWGA EQVQIWRRSY DIAPPNGESL KDTAERV... .......LPY
consensus  KAETAMKHGE EQVRIWRRSY DVPPPPIEES HPYYQEICSD RRYKVCLVPL

           151                                                200
     Sco   ELRPQTECLK DVVGRMLPYW FDAIVPDLLT GRTVLVAAHG NSLRALVKHL
     Sce   NVLPETESLA LVIDRLLPYW QDVIAKDLLS GKTVMIAAHG NSLRGLVKHL
    Hre2   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTILISAHG NSSRALLKHL
     Mre   DQLPRSESLK DVLERLLPYW KERIAPEILK GKSILISAHG NSSRALLKHL
     Rre   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTVLISAHG NSSRALLKHL
     Hmu   E.LPTCESLK DTIARALPFW NEEIVPQIKA GKRVLIAAHG NSLRGIVKHL
     Rmu   E.LPTCESLK DTIARALPFW NEEIAPKIKA GKRVLIAAHG NSLRGIVKHL
     Pgms  D.........P NLETERLEXL NSTIVAAILK GVKVLIAAHG NSLRALIMDL
consensus  DQLPRSESLK DVIERMLPYW NERIAPEILK GKTVLIAAHG NSLRALVKHL

           201                                                250
     Sco   DGISDADIAG LNIPTGIPLS YELNAEFKPL NPGGTYLDPD AAAAAIEAVK
     Sce   EGISDADIAK LNIPTGIPLV FELDENLKPS KP.SYYLDPE AAAAGAAAVA
    Hre2   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
     Mre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGNQE AIQAAIKKVD
     Rre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
     Hmu   EGMSDQAIME LNLPTGIPIV YELNKELKPT KPMQFLGDEE TVRKAMEAVA
     Rmu   EGMSDQAIME LNLPTGIPIV YELNQELKPT KPMRFLGDEE TVRKAMEAVA
     Pgms  EGLTGDQIVK RELATGVPIV YHLDKDGKYV SK.ELIDN.. ..........
consensus  EGISDEDIIN LNLPTGVPIV FELDENLKPV GPHQFFGDQE AIQAAIEAVA

     Sco   NQGKKK....
     Sce   NQGKK.....
    Hre2   DQGKVQ....
     Mre   DQGKVKQGKQ
     Rre   DQGKVKRAEK
     Hmu   AQGKAK....
     Rmu   AQGKAK....
consensus  DQGKVKQAEQ
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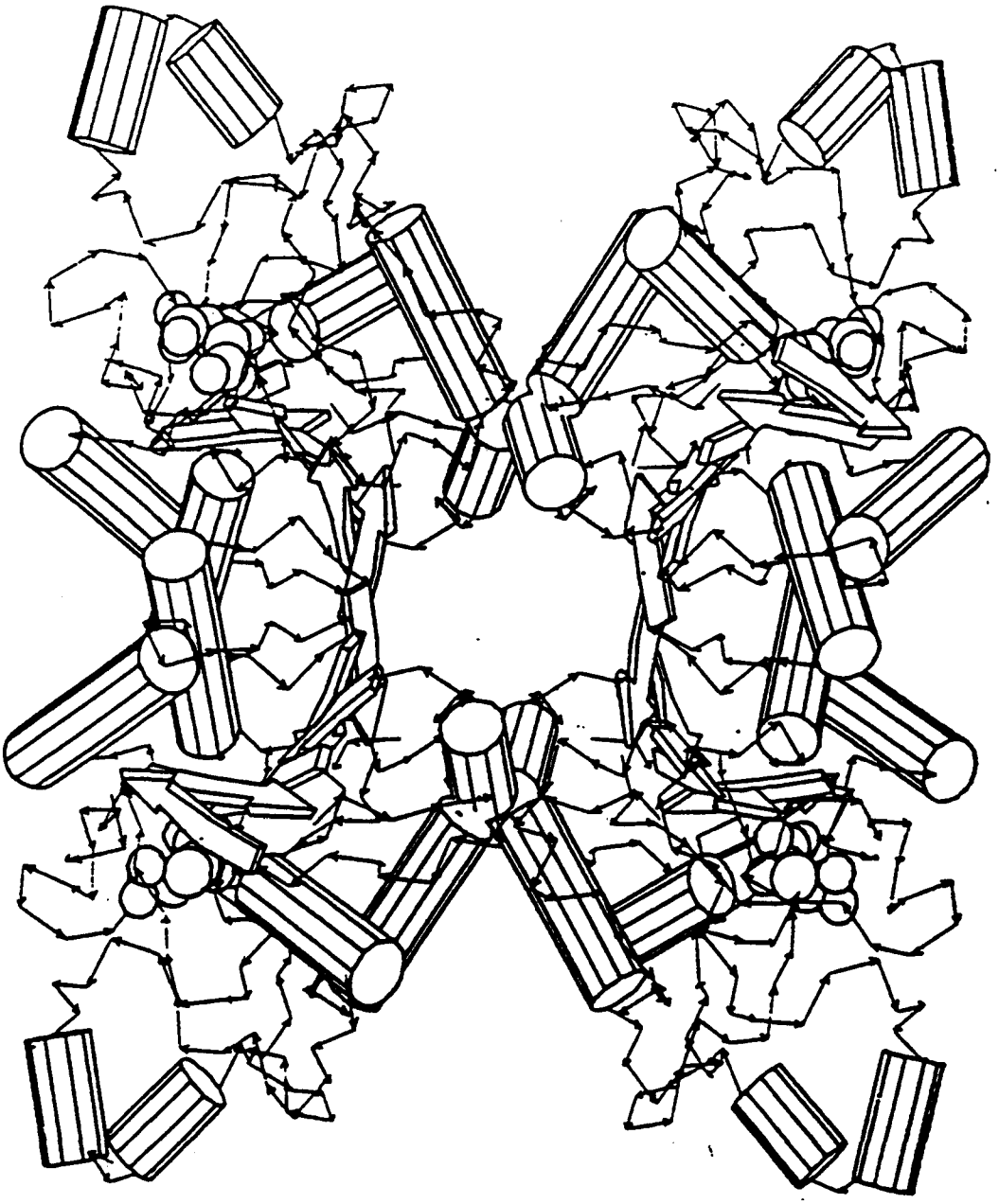


Figure 4: The tetrameric structure of *S.cerevisiae* phosphoglycerate mutase. The α-helices are represented by barrels and the β-strands by arrows. In each subunit, the polypeptide backbone folds into a single domain, consisting of a core of β-strands surrounded by α-helices. The active sites are shown by space filling models of 3-phosphoglycerate. This figure is reproduced from Fothergill-Gilmore and Watson, 1989, with the permission of the authors.

dehydrogenases. The location of the active site was determined by soaking crystals in 3-phosphoglycerate. The active site was found to lie at the bottom of a deep hollow formed by the residues of one subunit; in the tetramer the four sites are well separated. In the crystal structure of unliganded, native *S.cerevisiae* phosphoglycerate mutase, two sulphate ions bind in the position assumed to be occupied by the phospho-groups of bound ligands (figure 5). Under the conditions of crystallising the *S.cerevisiae* enzyme, the C-terminal fourteen residues were not observed in the electron density map, which is indicative of flexibility.

1.6.3 Active site

The active site of *S.cerevisiae* phosphoglycerate mutase is shown in figure 5. The most prominent features of the active site are the imidazole rings of His-8 and His-181, which lie parallel and are separated by a distance of only 0.4nm. His-8 has been shown to be phosphorylated during catalysis (Rose, 1970, 1971; Han and Rose, 1979). His-181 which does not appear to be phosphorylated may act as a proton donor/acceptor during phospho-transfer (see section 1.7). From available sequence information, it would appear that both histidines are conserved in all phosphoglycerate mutases. In the unligated, native form of the enzyme, one sulphate (S1) lies close to His-8. This probably represents the position of the transferable phospho-group of bound 2,3- or 1,3- bisphosphoglycerate. In this position, the transferable phospho-group would be able to form hydrogen bonds with Ser-11

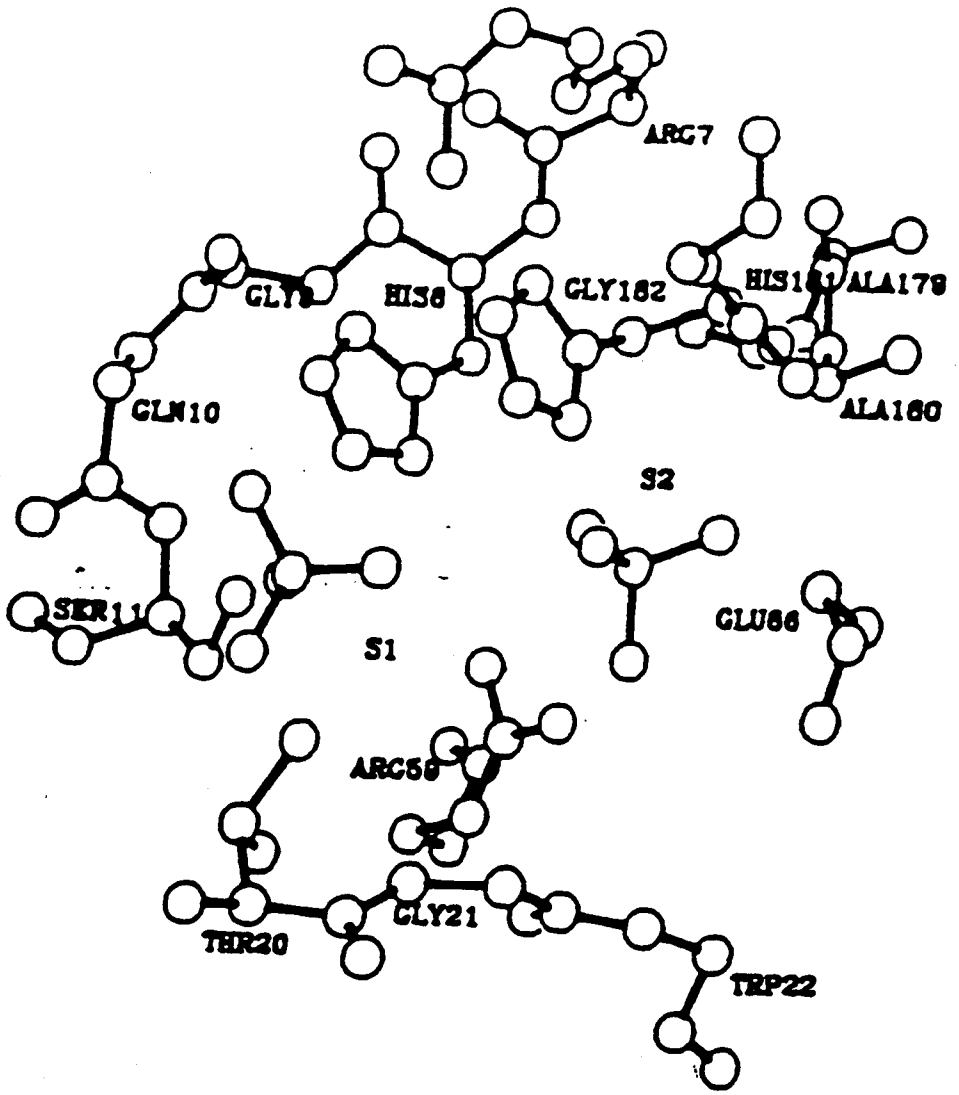


Figure 5: The active site of *S.cerevisiae* phosphoglycerate mutase. The side chains located in the active site are labelled. S1 and S2 indicate the positions of two sulphate ions in the crystal structure of unliganded enzyme (within the active site). This figure is reproduced from Fothergill-Gilmore and Watson, 1989, with the permission of the authors.

and Thr-20. The second active site sulphate (S2) is thought to interact with the positive charge dipole at the amino terminus of helix 7. This probably represents the position of the non-transferable phosphogroup of bound 2,3- or 1,3- bisphosphoglycerate. Arg-59, which is buried deep in the active site pocket, is positioned such that it could form a salt bridge with the carboxyl group of the bound substrate. Like His-181 and His-8, Arg-59 is conserved in all phosphoglycerate mutases sequenced so far.

Model building studies (Fothergill-Gilmore and Watson, 1989) indicate that the cofactor 2,3-bisphosphoglycerate can bind to the active site in two possible orientations (figure 6a): with the 3-phospho group close to His-8 and the 2-phospho group located next to the amino-terminus of helix 7. Alternatively, the positions of the 2- and 3- phospho groups may be reversed. These studies have also predicted how 2-phosphoglycerate and 3-phosphoglycerate bind to the phosphoenzyme, see figures 6b and 6c.

1.6.4 C-terminal tail

The C-terminal fourteen residues (also referred to as the C-terminal tail) of phosphoglycerate mutase are susceptible to proteolysis. Loss of these residues in the *S.cerevisiae* enzyme (Sasaki *et al.*, 1966; Winn *et al.*, 1981) and in the rabbit muscle enzyme (Price *et al.*, 1985) resulted in the loss of mutase activity with the retention of the overall structure of the enzyme. 'Tail-less' *S.cerevisiae* mutase possesses phosphatase activity

Figure 6: The proposed binding of cofactor and substrate at the active site of phosphoglycerate mutase. The positions of His-8 and His-181 are indicated, with the hatched atoms corresponding to the ring nitrogens.

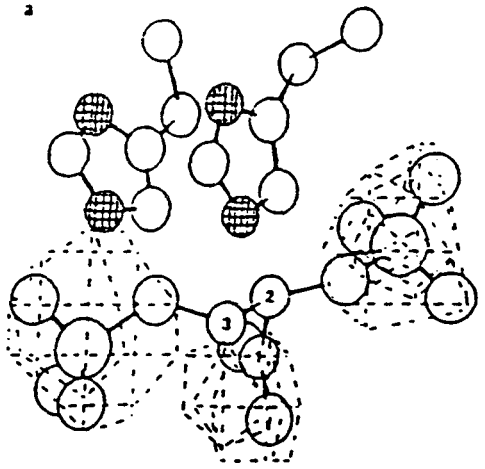
(a) 2,3-bisphosphoglycerate is shown bound with the 3-phospho group occupying the S1 or 'His-8' position and the 2-phospho group in the S-2 or helix dipole position (see figure 5). The carboxyl group is in a suitable position to interact with Arg-59. The dashed lines represent electron density corresponding to ions bound to the active site of the unligated enzyme.

(b) 2-phosphoglycerate is shown bound at the active site of the catalytically competent phosphoenzyme. The filled in atom represents the proton that is abstracted or donated by His-181 during a round of catalysis.

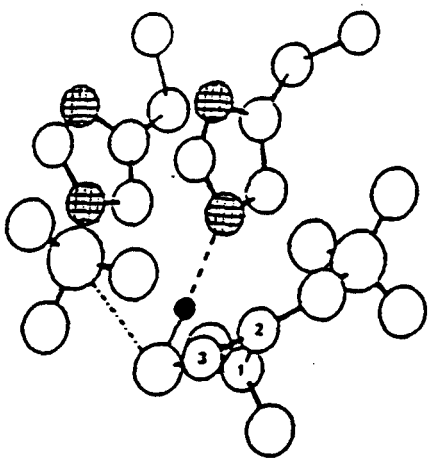
(c) 3-phosphoglycerate is shown bound at the active site.

This figure is reproduced from Fothergill-Gilmore and Watson, 1989, with the permission of the authors.

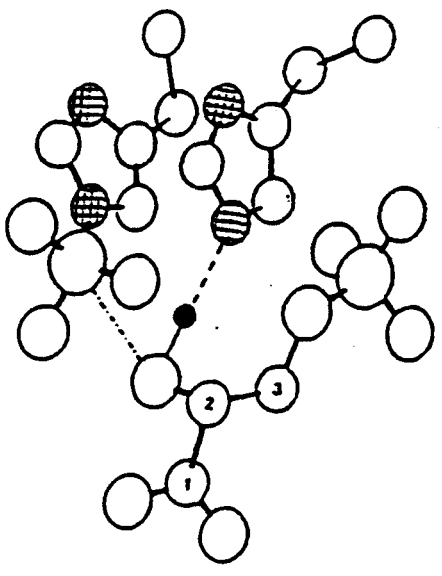
a



b



c



toward 2,3-bisphosphoglycerate, however, this proteolysed form of the enzyme is not as responsive to the phosphatase activator 2-phosphoglycollate as the native *S.cerevisiae* mutase (Sasaki *et al.*, 1971).

The inability to observe the C-terminal tail of *S.cerevisiae* phosphoglycerate mutase on the electron density map was indicative of a 'flexible tail'. This theory was corroborated by the amino acid sequence of the C-terminal region which is rich in residues with small side chains (-Ala-Ala-Ala-Gly-Ala-Ala-Ala-Val-Ala-Asn-Gln-Gly-Lys-Lys). The observation that 2,3-bisphosphoglycerate helps protect unphosphorylated rabbit muscle phosphoglycerate mutase from proteolysis, whereas 3-phosphoglycerate does not (Price *et al.*, 1985), implies that the tail can either adopt a tail-in mode, which requires the presence of two phosphogroups, or the tail can be exposed. Model building studies (Winn *et al.*, 1981) imply that the tail could adopt a conformation that could modulate access to the active site. Thus the tail could physically exclude water from the active site and in doing so, ensure phospho-transfer to the substrate rather than to water. These studies also suggest that the two consecutive lysines could approach the active site to provide a ligand or ligands for the transferable phospho-group thus preventing phosphatase activity. The lysine residues may also be involved in charge stablisation, to retain cofactor/intermediate 2,3-bisphosphoglycerate during the reaction sequence.

Analysis of the phosphoglycerate mutase and bisphosphoglycerate mutase sequences (figue 3) reveals conservation of a run of small side chain residues followed by two lysines at their C-termini.

1.6.5 Quaternary Structure

Cofactor-dependent phosphoglycerate mutases have been isolated from various sources. These enzymes have been shown to be monomeric, dimeric and tetrameric depending on the source, see table 1. At present, protein crystallography has only solved the detailed structure of the enzyme from *S.cerevisiae*. Nevertheless, circular dichroism studies suggest that the monomeric (Johnson and Price, 1987), the dimeric (Price *et al.*, 1985) and the tetrameric mutases (Herman *et al.*, 1983,1985; Johnson and Price, 1986; White *et al.*, 1992) all possess similar overall structure. The ability of all of the phosphoglycerate mutases to bind triazine dyes, such as Cibacron Blue and Procion Red, also indicates that they contain the characteristic β-sheet flanked by α-helices, the structural motif which is thought to be responsible for 'channelling' these dyes to the hydrophobically situated substrate binding site (Beissner *et al.*, 1979).

Subunit-subunit interface structural information is only available for *S.cerevisiae* phosphoglycerate mutase. However, the primary structures of all the other mutases are homologous to the *S.cerevisiae* enzyme, which permits attempts to explore the anatomy of the subunit contact regions with the aid of structure prediction and molecular modelling.

Table 1: Properties of phosphoglycerate mutases.*

Source	BPG-dependence	Number of subunits	Subunit M_r	Mn^{++} requirement	Vanadate inhibition
Rabbit muscle	+	2	30,000	-	+
S. cerevisiae	+	4	27,000	-	+
S. pombe	+	1	23,000	-	+
S. coelicolor	+	4	29,000	-	+
E. coli	+	nd	nd	nd	nd
Pseudomonas	+	1	32,000	-	+
Z. mobilis	+	2	26,000	-	+
Leuconostoc	+	nd	nd	nd	nd
Human erythrocyte	-	2	30,000	-	+/-
Castor oil (plant cytosol)	-	1	64,000	-	-
B. megatarium	-	1	61,000	+	nd

* see text for references

nd not determined

1.6.6 Subunit-subunit interfaces

Analysis of a range of dimeric and tetrameric proteins has led to the suggestion that there are four structural motifs which may be found at the interfaces of oligomeric proteins (Miller, 1989): (i) extended β-sheet (ii) helix-helix packing (iii) sheet sheet packing and (iv) loop interactions. From the structure of the tetrameric *S.cerevisiae* phosphoglycerate mutase (figure 4) it can be seen that there are two sets of intersubunit contacts: one set primarily involves loop interactions whereas the other set has much more extensive contacts involving extended β-sheet, helix-heix packing and loop interactions. In the simpler set of contacts, known as CR5, the side chains in a loop preceding helix 5 (residues 139-144) in one subunit fits between the side chains of helix 6 and the loop located between strands 6 and 7 of the other subunit. The other set of contacts, CR1/2, connects the central β-sheet core of the two subunits thus the main chain of strand 1 of the β-sheet, the residues of helix 2 and the connecting loop interact in an antiparallel fashion between the subunits.

The residues involved in CR1/2 are highly conserved in the dimeric and tetrameric mutases. This stretch of sequence (58-82) is not available for the monomeric enzyme from *S.pombe* (figure 3) which may reflect deletion of this part of the sequence or simply that this stretch has not been located and sequenced. Residues 139-144, which form the loop in CR5 of the *S.cerevisiae* enzyme, are not highly conserved which implies that this subunit interaction is no longer present in the dimeric forms of phosphoglycerate mutase.

1.6.7 Denaturation and Renaturation

Comparative studies of the denaturation and renaturation of phosphoglycerate mutase from *S.cerevisiae* (Herman *et al.*, 1983, 1985), rabbit muscle (Johnson and Price, 1985) and *S.pombe* (Johnson and Price, 1987) allow examination of the consequences of quaternary structure. The enzymes were denatured by guanidine hydrochloride and subsequently renatured by dilution of the denaturant. Changes in the structure of the enzymes was monitored by changes in activity, circular dichroism, cross-linking and susceptibility to proteolysis. The resultant data revealed that the oligomeric rabbit muscle and *S.cerevisiae* enzymes are more susceptible than the monomeric *S.pombe* enzyme to inactivation by low concentrations of guanidine hydrochloride (0-0.75M). Circular dichroism and fluorescence studies implied that the subunit-subunit interactions were important in maintaining the stability of the overall tertiary structure of the oligomeric enzymes toward denaturant.

Cross-linking of the *S.cerevisiae* enzyme with gluteraldehyde led to the proposal that subunit reassociation occurred as follows:

$$4 \text{ Monomers} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2 \text{ Dimers} \xrightarrow{k_2} \text{Tetramer}$$

where $k_1 = 6.26 \times 10^{3} M^{-1}s^{-1}$, $k_{-1} = 6 \times 10^{-3} s^{-1}$, $k_2 = 2.75 \times 10^{4} M^{-1}s^{-1}$ at 20°C, pH 7.5.

Thus, *S.cerevisiae* phosphoglycerate mutase renatures by refolding into monomers which associate to form dimers. The dimers then associate to form tetramers. The refolded tetramers are fully active and like the native enzyme, are not susceptible to proteolysis by trypsin, chymotrypsin and thermolysin. However, the intermediate monomers and dimers possess about 35% of the native enzyme activity and they are susceptible to proteolytic digest. This implies that the monomers, which are formed quickly (≈30sec) after the removal of denaturant, and the dimers have relatively 'loose' structures but on subsequent association to form tetramers, a species is formed which resembles the native enzyme in terms of activity and susceptibility to proteolysis. Therefore, it would appear that the subunit contacts are necessary to make the subunits more compact.

The monomeric enzyme from *S.pombe* was shown to regain activity rapidly, with no evidence of an intermediate which possessed a relatively 'loose' structure. Thus, it would appear that differences in the quaternary structure of these enzymes have resulted in differences in behaviour during denaturation and renaturation.

1.7 Proposed reaction mechanism of phosphoglycerate mutase

A reaction mechanism has been proposed (Fothergill-Gilmore and Watson, 1989) based on the structural and kinetic properties of these enzymes. The proposed reaction sequence for the mutase, phosphatase and synthetase reactions are summarised in figure 7. The residue numbering used here refers to phosphoglycerate mutase from *S.cerevisiae*, which has been particularly well characterised.

1.7.1 Mutase reaction

Priming of the mutase reaction by bisphosphoglycerate is required to permit a round of catalysis: 2,3 bisphosphoglycerate binds to the unphosphorylated form of the enzyme, labelled 'i' in figure 7, in one of two possible orientations ('d' or 'e'). In 'd', the 2-phospho group is in the transferable position, which lies close to His-8, and is thought to form hydrogen bonds with Ser-11, Thr-20 and one of the lysine residues located at the C-terminus. The 3-phospho group is interacting with the positive charge associated with the dipole at the N-terminus of helix-7. The alternative orientation for binding 2,3-bisphosphoglycerate to the unphosphorylated enzyme has the 2- and 3-phospho groups reversed. In each case, the carboxyl group forms a salt bridge with Arg-59.

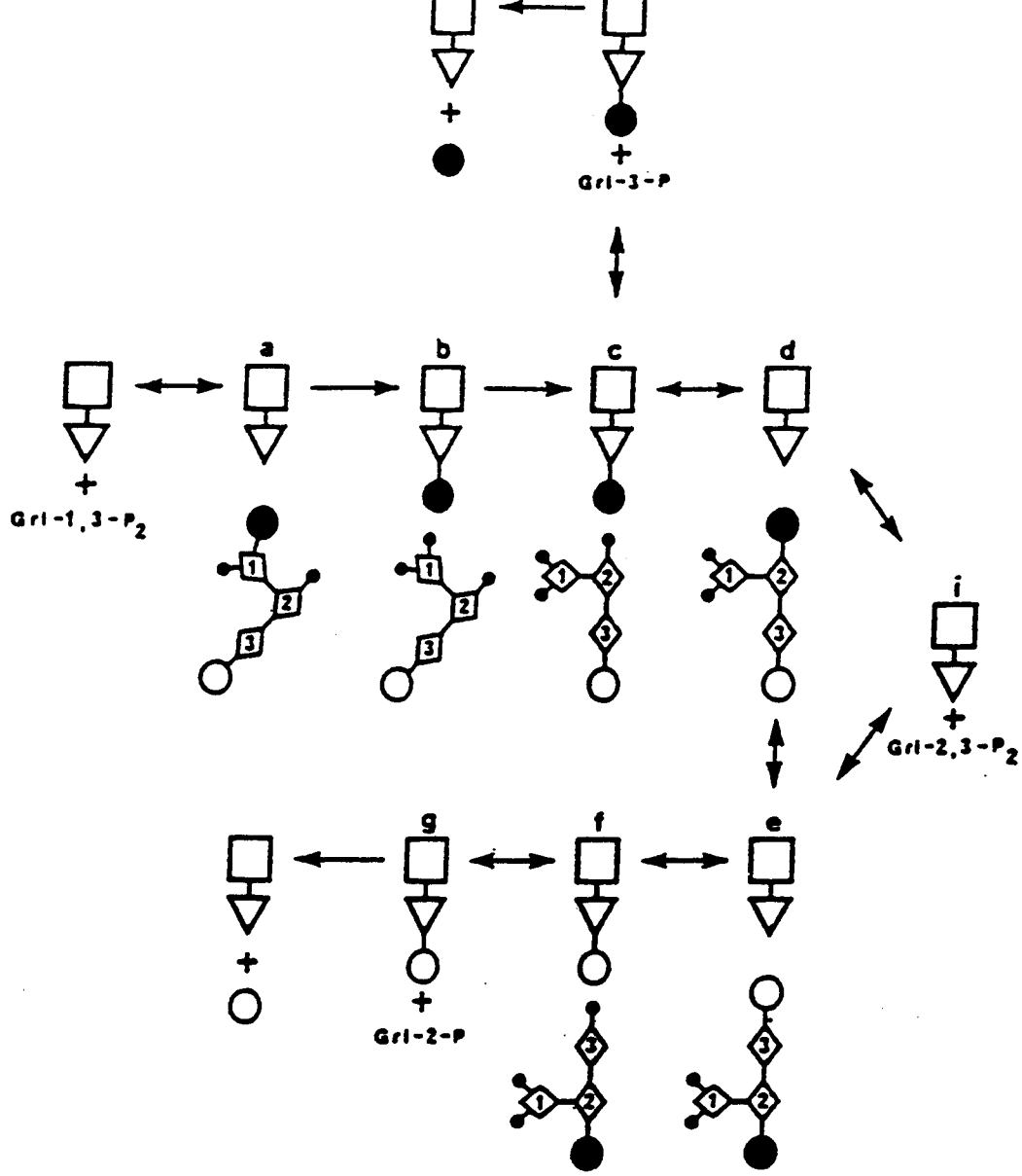


Figure 7: Proposed reaction sequence of cofactor dependent phosphoglycerate mutases. In each diagram the square represents the active site of the enzyme, with the triangle corresponding to His-8. The numerals 1,2 and 3 label the carbon atoms of glycerate. The large circles represent phosphogroups, and the small circles represent oxygen atoms of hydroxyl or carboxyl groups. Symbols (IUPAC-IUB Commission on Biochemical Nomenclature, 1978): Gri-2,3-P_2, 2,3-bisphosphoglycerate; Gri-1,3-P_2, 1,3-bisphosphoglycerate; Gri-3-P, 3-phosphoglycerate. This figure is reproduced from Fothergill-Gilmore and Watson, 1989, with the permission of the authors.

Transfer of the phosphogroup to His-8 would require the donation of a proton, presumably from His-181, which would produce a phosphorylated form of the enzyme with the concomitant release of a 2- or 3- phosphoglycerate. A round of catalysis would be initiated by the binding of 3-phosphoglycerate (for glycolysis) or 2-phosphoglycerate (for gluconeogenesis) to the active site of the phosphoenzyme. Phosphotransfer from the enzyme to the substrate would produce a 2,3-bisphosphoglycerate intermediate ('d' and 'e' in figure 7). Reorientation of this intermediate at the active site is required to allow the transfer of the substrate-associated phospho group to the enzyme. Modelling studies (Fothergill-Gilmore and Watson, 1989) imply that such a reorientation could be accommodated by the active site, assuming that the 2,3-bisphosphoglycerate could move away slightly from His-8 and His-181. This would result in the release of a monophosphorylated product and an enzyme primed for another round of catalysis ('h' and 'g' in figure 7).

1.7.2 Phosphatase reaction

The requirement for 2,3-bisphosphoglycerate to prime the mutase reaction arises due to the low rate of hydrolysis of the phosphoenzyme. The phosphoenzyme has been shown to have a half-life of 1-2 minutes (Britton *et al.*, 1972). The instability of the phosphoenzyme can be enhanced by the presence of anions, in particular the two-carbon substrate analogue, 2-phosphoglycollate (Rose and Liebowitz, 1972). It has been suggested that

this analogue binds to the active site, in a manner similar to the normal substrates, along with a water molecule (Fothergill-Gilmore and Watson, 1989). Thus, the phosphogroup of 2-phosphoglycollate is associated with the helix dipole, the carboxyl group of the analogue forms a salt bridge with Arg-59 and the water molecule occupies the phosphotransfer position located next to His-8. Such an arrangement would result in the C-terminal tail closing over the active site and the transfer of the phospho group from His-8 to water. This theory is supported by the observation that proteolysed phosphoglycerate mutase, which is lacking the C-terminal tail, retains a basal phosphatatase activity which can no longer be stimulated by 2-phosphoglycollate (Sasaki *et al.*, 1971).

1.7.3 Synthase reaction

The 2,3-bisphosphoglycerate synthase activity of phosphoglycerate mutase would be initiated by the binding of the substrate 1,3-bisphosphoglycerate to the active site of unphosphorylated enzyme ('a' in figure 7). It is likely that the substrate would bind so that the phospho group on carbon-1 occupies the the phospho transfer position, located next to His-8, and that the phospho group on carbon-3 associates with the helix dipole. With 1,3-bisphosphoglycerate in this orientation, Arg-59 would not be involved in substrate binding. Once phosphotransfer has taken place, ('b' in figure 7) the negatively charged carboxyl group of the bound 3-phosphoglycerate would be repelled phosphogroup on His-8 and would move to form a salt bridge with

Arg-59 ('c' in figure 7). This reorientation of 3-phosphoglycerate positions the 2' hydroxyl group so that it would be phosphorylated by the phospho group on His-8 to produce 2,3-bisphosphoglycerate ('d' in figure 7). Release of 2,3 bisphosphoglycerate completes the synthase reaction with the regeneration of unphosphorylated enzyme which is now ready to bind another molecule of 1,3-bisphosphoglycerate.

The synthase activity of phosphoglycerate mutase (from *S. cerevisiae*, rabbit B- and M-type) is extremely low, with a mutase:synthase activity ratio of 1000:1. However, the closely related bisphophoglycerate mutase (E-type) has a mutase:synthase activity ratio of 1:1. The K_m values for 1,3-bisphosphoglycerate is 5μM for the rabbit M-type isoenzyme (Laforet *et al.*, 1974) whereas the human E-type isoenzyme has a K_m value of 0.4μM (Pons and Carreras, 1985). Thus, the E-type isoenzyme has a higher affinity for the synthase substrate, 1,3-bisphophoglycerate. The K_m values for 2,3-bisphosphoglycerate is 0.5μM for chicken B- and pig M-type isoenzymes (Rose and Dube, 1978; Bartrons and Carreras, 1982) whereas the human E-type isoenzyme has a K_m value of 40μM (Rose, 1980). Therefore, the affinity for the synthase product, 2,3-bisphosphoglycerate, is lower for the bisphosphoglycerate mutase. The differences in the affinity constants for 1,3- and 2,3-bisphosphoglycerate for phosphoglycerate mutase and bisphosphoglycerate mutase appear to reflect the differences in mutase:synthase activities; the K_m ratio for 2,3-bisphosphoglycerate:1,3-bisphosphoglycerate is 1:10 for phosphoglycerate mutase and 100:1 for bisphosphoglycerate mutase.

1.8 Relationship with fructose-2,6-bisphophatase

The bifunctional enzyme 2-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (E.C. 2.7.1.105/3.1.3.46) catalyses the synthesis and degradation of fructose-2,6-bisphosphate (Pilkis *et al.*, 1983). Fructose-2,6-bisphosphate is a potent allosteric regulator of 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase and so levels of this metabolite regulate the flow of carbon through glycolysis and gluconeogenesis (Pilkis *et al.*, 1988). The synthesis of fructose-2,6-bisphosphate by 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (6-PF-2-K/Fru-2,6-BPase) occurs by the transfer of the γ-phosphate of ATP to the C-2 hydroxyl of fructose-6-phosphate, whereas degradation of fructose-2,6-bisphosphate by this enzyme produces fructose-6-phosphate and P_i via hydrolysis, see below:

$$\text{Fructose-6-P} + \text{ATP} \xrightarrow{\text{6-PF-2K}} \text{Fructose-2,6-P}_2 + \text{ADP}$$

$$\text{Fructose-2,6-P}_2 \xrightarrow{\text{Fru-2,6-BPase}} \text{Fructose-6-P} + P_i$$

1.8.1 Primary structure homology

The mammalian bifunctional 6-PF-2-K/Fru-2,6-BPase exists as a homodimer (subunit M_r 55,000) which catalyses the synthesis and degradation of fructose-2,6-bisphosphate at two discrete active sites: the N-terminal region of each subunit is responsible for the kinase activity whereas the phosphatase domain is located in the C-terminus (Pilkis *et al.*, 1987).

The genes encoding bovine, rat and human isoenzymes have been characterised. Alignment of the deduced amino acid sequences of all 6-PF-2-K/Fru-2,6-BPases exhibit a high degree of identity, see figure 8. A search of the Protein Identification Resource protein sequence database resulted in the proposal that the N-terminal sequence (residues 1-170 which correspond to the kinase domain) was similar to the functionally analagous domain of 6-PF-1-K. Likewise, it was revealed that the C-terminus or phosphatase domain (residues 250-450) of 6-PF-2-K/Fru-2,6-BPase shared sequence homology with *S.cervisiae* phosphoglycerate mutase (Bazan *et al.*, 1989).

Alignment of the sequences of phosphoglycerate mutases with the consensus sequence (figure 8) of the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase is given in figure 9. Thus it would appear that the phosphatase domain contains residues which have been shown to be important active site residues in *S.cerevisiae* mutase. For example (using the *S.cerevisiae* mutase

Figure 8: Alignment of 4 known amino acid sequences of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Bhppf2k=bovine heart 6PF2K/Fru-2,6-BPase; Blppf2k=bovine liver 6PF2K/Fru-2,6-BPase; Hlppf2k=human liver 6PF2K/Fru-2,6-BPase; Rlppf2k=rat liver 6PF2K/Fru-2,6-BPase. The alignment was generated by the GCG multiple alignment editing programme, LINEUP (Devereux *et al.*, 1984). The consensus pf2kcon sequence was generated using PROFILE (Gribskov *et al.*, 1987).

```
            1                                                        60
    Bhpf2k  ...SGNPASSSEQNNNSYET KASLRISEKKCSWASYMTNS PTMVIMVGLPARGKTYVSKK
    Blpf2k  SQEMGELTQTRLQKIWIPHN NGNSRLQRRRGSSIPQFTNS PTMVIMVGLPARGKTYISTK
    Hlpf2k  SPEMGELTQTRLQKIWIPHS SGSSRLQRRRGSSIPQFTNS PTMVIMVGLPARGKTYISTK
    Rlpf2k  SREMGELTQTRLQKIWIPHS SSSSVLQRRRGSSIPQFTNS PTMVIMVGLPARGKTYISTK
  consensus SQEMGELTQTRLQKIRIPHS SGSSRLQRRRGSSIPQFTNS PTMVIMVGLPARGKTYISTK

            61                                                       120
    Bhpf2k  LTRYLNWIGVPTKVFNLGVY RRQAVKSYKSYDFFRHDNEE AMKIRKQCALVALKDVKAYL
    Blpf2k  LTRYLNWIGTPTKVFNLGQY RR.EDVSYRNYEFFLPDNME ALLIRKQCALAALKDVHSYL
    Hlpf2k  LTRYLNWIGTPTKVFNLGQY RR.EAVSYKNYEFFLIDNME ALQIRKQCALAALKDVHNYL
    Rlpf2k  LTRYLNWIGTPTKVFNLGQY RR.EAVSYRNYEFFRPDNTE AQLIRKQCALAALKDVHKYL
  consensus LTRYLNWIGTPTKVFNLGQY RRQEAVSYKNYEFFWPDNME ALMIRKQCALAALKDVHNYL

            121                                                      180
    Bhpf2k  TEESGQIAVFDATNTTRFRR DLILNFAEENSFKVFFVESV CNDPDIIAANILEVKVSSPD
    Blpf2k  SHEEGRVAVFDATNTTRFRR SLILQFAKEHGYKVFFIESI CNDPDIIAENIRQVKLGSPD
    Hlpf2k  SHEEGHVAVFDATNTTRFRR SLILQFAKEHGYKVFFIESI CNDPGIIAENIRQVKLGSPD
    Rlpf2k  SREEGHVAVFDATNYTRFRR SLILQFAKEHGYKVFFIESI CNDPEIIAENIKQVKLGSPD
  consensus SHEEGHVAVFDATNTTRFRR SLILQFAKEHGYKVFFIESI CNDPDIIAENIRQVKLGSPD

            181                                                      240
    Bhpf2k  YPDRNRENVMDDFLKRIECY KVTYQPLDPDSHDKDLSFIK VINVGQRFLVNKVQDYVQSK
    Blpf2k  YIDCDREKVLEDFLKRIECY EVNYQPLD.DELDSHLSYIK IFDVGTRYMVNRVQDHVQSR
    Hlpf2k  YIDCDREKVLEDFLKRIECY EVNYQPLD.EELDSHLSYIK IFDVGTRYMVNRVQDHVQSR
    Rlpf2k  YIDCDQEKVLEDFLKRIECY EINYQPLD.EELDSHLSYIK IFDVGTRYMVNRVQDHVQSR
  consensus YIDCDREKVLEDFLKRIECY EVNYQPLDPDELDSHLSYIK IFDVGTRYMVNRVQDHVQSR

            241                                                      300
    Bhpf2k  IVYYLMNIHVHPRTIYLCRH GESELNLLGKIGGDSGVSVR GKQFAQALRNFLEEQEIADL
    Blpf2k  TVYYLMNIHVTPRSIYLCRH GESELNLRGRIGGDSGVSAR GKQYAYALANFIQSQGISSL
    Hlpf2k  TVYYLMNIHVTPRSIYLCRH GESELNIRGRIGGDSGVSVR GKQYAYALANFIQSQGISSL
    Rlpf2k  TAYYLMNIHVTPRSIYLCRH GESELNLRGRIGGDSGLSAR GKQYAYALANFIRSQGISSL
  consensus TVYYLMNIHVTPRSIYLCRH GESELNLWGRIGGDSGVSAR GKQYAYALANFIQSQGISSL

            301                                                      360
    Bhpf2k  KVWTSQLKRTIQTAESLGVT YEQWKILNEIDAGVCEEMTY AEIQEQYPDEFALRDEEKYL
    Blpf2k  KVGTSHMKRTIQTAEALGLP YEQWKALNEIDAGVCEEMTY EEIQEHYPEEFALRDQDKYR
    Hlpf2k  KVWTSRMKRTIQTAEALGVP YEQWKALNEIDAGVCEEMTY EEIQEHYPEEFALRDQDKYR
    Rlpf2k  KVWTSHMKRTIQTAEALGVP YEQWKALNEIDAGVCEEMTY EEIQEHYPEEFALRDQDKYR
  consensus KVRTSHMKRTIQTAEALGVP YEQWKALNEIDAGVCEEMTY EEIQEHYPEEFALRDQDKYW

            361                                                      420
    Bhpf2k  YRYPGGESYQDLVQRLEPVI MELERQGNVLVISHQAVMRC LLAYFLDKGADELPYLRCPL
    Blpf2k  YRYPKGESYEDLVQRLEPVI MELERQENVLVICHQAVMRC LLAYFLDKSSDELPYLKCPP
    Hlpf2k  YRYPKGESYEDLVQRLEPVI MELERQENVLVICHQAVMRC LLAYFLDKSSDELPYLKCPL
    Rlpf2k  YRYPKGESYEDLVQRLEPVI MELERQENVLVICHQAVMRC LLAYFLDKSSDELPYLKCPL
  consensus YRYPKGESYEDLVQRLEPVI MELERQENVLVICHQAVMRC LLAYFLDKSSDELPYLKCPL

            421                                                      480
    Bhpf2k  HTIFKLTPVAYGCKVETIKL NVEAVNTHRDKPTNNFPKSQ TPVRMRRNSFTPLSSSNTIR
    Blpf2k  STVLKLTPVAYGCKVESIYL NVEAVNTHREKPENVDITRE AEEALDTVPAHY........
    Hlpf2k  HTVLKLTPVAYGCKVESIYL NVEAVNTHREKPENVDITRE AEEALDTVPAHY........
    Rlpf2k  HTVLKLTPVAYGCRVESIYL NVEAVNTHRDKPENVDITRE AEEALDTVPAHY........
  consensus HTVLKLTPVAYGCKVESIYL NVEAVNTHRDKPENVDITRE AEEALDTVPAHYLSSSNTIR

            481                                                      533
    Bhpf2k  RPRNYSVGSRPLQPLSPLRA LDTQEGADQPKTQAETSRAA HRLPSPAPPTSPS
```

Figure 9: Alignment of phosphoglycerate mutases with the consensus sequence of the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase. The mutase sequences are: Sco=*S. coelicolor* PGAM; Sce=*S. cerevisiae PGAM*; Hre2=human BPGAM; Mre=mouse BPGAM; Rre=rabbit BPGAM; Hmu=human M-type BPGAM; Rmu=rabbit M-type PGAM; Pgms=*S. pombe* PGAM.' Consensus' is the PGAM consensus sequence (see figure 3) and 'pf2k consensus' is the consensus sequence of the phosphatase region of 6PF2K/Fru-2,6-BPase (see figure 8). The sequences have been aligned using LINEUP, Devereux *et al.*, 1984.

```
            1                                                  50
      Sco   ADAPYKLILL RHGESEWNEK NLFTGWVDVN LTPKGEKEAT RGGELLKDAG
      Sce   ..MP.KLVLV RHGQSEWNEK NLFTGWVDVK LSAKGQQEAA RAGELLKEKK
     Hre2   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
      Mre   .MSKHKLIIL RHGEGQWNKE NRFCSWVDQK LNNDGLEEAR NCGRQLKALN
      Rre   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
      Hmu   .MATHRLVMV RHGETTWNQE NRFCGWFDAE LSEKGTEEAK RGAKAIKDAK
      Rmu   .MATHRLVMV RHGESSWNQE NRFCGWFDAE LSEKGAEEAK RGATAIKDAK
      Pgms  .AAPNLLVLT RHGESEWNKL NLFTGWKDPA LSETGIKEAK LGGERLKSRG
 consensus  AMAPHKLVML RHGESEWNKE NWFCGWVDQK LSEKGMEEAK RGGKQLKDMN
   pf2kcon   VTPRSIYLC RHGESELNLW GRIGG..DSG VSARGKQYAY ALANFIQSQG

            51                                                 100
      Sco   LLPDVVHTSV QKRAIRTAQL ALEAADRHWI PVHRHWRLNE RHYGALQGKD
      Sce   VYPDVLYTSK LSRAIQTANI ALEKADRLWI PVNRSWRLNE RHYGDLQGKD
     Hre2   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Mre   FEFDLVFTSI LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Rre   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Hmu   MEFDICYTSV LKRAIRTLWA ILDGTDQMWL PVVRTWRFNE RHYGGLTGFN
      Rmu   IEFDICYTSV LKRAIRTLWT ILDVTDQMWV PVVRTWRLNE RAYGGLTGLN
      Pgms  YKFDIAFTSA LNRANRT... .......... ........RQ RYYGDLQGLN
 consensus  FEFDIVYTSV LNRAIRTARL ILEELDQEWV PVEWSWRLNE RHYGALIGLN
   pf2kcon  ISSLKVRTSH MKRTIQTAEA LG..     .V PYEQWKALNE IDAGV     .

            101                                                150
      Sco   KAQTLAEFGE EQFMLWRRSY DTPPPALDRD AEYSQF..SD PRYAM.LPP.
      Sce   KAETLKKFGE EKFNTYRRSF DVPPPPIDAS SPFSQK..GD ERYKY.VDP.
     Hre2   REQMALNHGE EQVRLWRRSY NVTPPPIEES HPYYQEIYND RRYKVCDVPL
      Mre   REKMALNHGE EQVRLWRRSY NVTPPPIEES HPYFHEIYSD RRYKVCDVPL
      Rre   REKMALNHGE EQVRIWRRSY NVTPPPIEES HPYYHEIYSD RRYRVCDVPL
      Hmu   KAETAAKHGE EQVRSWRRSF DIPPPPMDEK HPYYNSISKE RRYA.GLKPG
      Rmu   KAETAAKHGE EQVKIWRRSF DTPPPPMDEK HNYYASISKD RRYA.GLKPE
      Pgms  KDDARKKWGA EQVQIWRRSY DIAPPNGESL KDTAERV... ........LPY
 consensus  KAETAMKHGE EQVRIWRRSY DVPPPPIEES HPYYQEICSD RRYKVCLVPL
   pf2kcon  .......... .......... .....TYEEI QEHYPEEFAL RDQDKYWY..

            151                                                200
      Sco   ELRPQTECLK DVVGRMLPYW FDAIVPDLLT GRTVLVAAHG NSLRALVKHL
      Sce   NVLPETESLA LVIDRLLPYW QDVIAKDLLS GKTVMIAAHG NSLRGLVKHL
     Hre2   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTILISAHG NSSRALLKHL
      Mre   DQLPRSESLK DVLERLLPYW KERIAPEILK GKSILISAHG NSSRALLKHL
      Rre   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTVLISAHG NSSRALLKHL
      Hmu   E.LPTCESLK DTIARALPFW NEEIVPQIKA GKRVLIAAHG NSLRGIVKHL
      Rmu   E.LPTCESLK DTIARALPFW NEEIAPKIKA GKRVLIAAHG NSLRGIVKHL
      Pgms  D........P NLETERLEXL NSTIVAAILK GVKVLIAAHG NSLRALIMDL
 consensus  DQLPRSESLK DVIERMLPYW NERIAPEILK GKTVLIAAHG NSLRALVKHL
   pf2kcon  .RYPKGESYE DLVQRLEPVI MELERQE      NVLVICHQ AVMRCLLATF

            201                                                250
      Sco   DGISDADIAG LNIPTGIPLS YELNAEFKPL NPGGTYLDPD AAAAAIEAVK
      Sce   EGISDADIAK LNIPTGIPLV FELDENLKPS KP.SYYLDPE AAAAGAAAVA
     Hre2   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
      Mre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGNQE AIQAAIKKVD
      Rre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
      Hmu   EGMSDQAIME LNLPTGIPIV YELNKELKPT KPMQFLGDEE TVRKAMEAVA
      Rmu   EGMSDQAIME LNLPTGIPIV YELNQELKPT KPMRFLGDEE TVRKAMEAVA
      Pgms  EGLTGDQIVK RELATGVPIV YHLDKDGKYV SK.ELIDN.. ..........
 consensus  EGISDEDIIN LNLPTGVPIV FELDENLKPV GPHQFFGDQE AIQAAIEAVA
   pf2kcon  LDKSSDELPY LKCPL HTVL KLTPVAYGCK VESIYL NVE AVNTHRDKPE

      Sco   NQGKKK....
      Sce   NQGKK.....
     Hre2   DQGKVQ....
      Mre   DQGKVKQGKQ
      Rre   DQGKVKRAEK
      Hmu   AQGKAK....
      Rmu   AQGKAK....
 consensus  .DQGKVKQAEQ
   pf2kcon  NVDITREAEE
```

numbering), His 8, His 181, Arg 59 and Ser 11 appear to be conserved in the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase.

1.8.2 Secondary structure homology

Alignment of the predicted secondary structure of the phosphatase domain of 6-PK-2-K/Fru-2,6-BPase with the known secondary structure of *S.cerevisiae* phosphoglycerate mutase (figure 10) has shown that these enzymes share similar secondary structural elements (Bazan *et al.*, 1988). The *S.cerevisiae* mutase and the predicted secondary structure of the rat phosphatase domain are composed of alternating α- and β-structures. These structures are known to fold into a central core of β-sheet surrounded by α-helices in the *S.cerevisiae* mutase. Modelling studies imply that the phosphatase domain may be similar to the *S.cerevisiae* phosphoglycerate mutase fold (Bazan *et al.*, 1989). Such models predict that His 258 and His 392 (rat 6-PF-2-K/Fru-2,6-BPase numbering) would be brought together in a spatial arrangement like the two active site histidines of *S.cerevisiae* mutase. This arrangement of histidines has also been observed in acid phosphatases.

Acid phosphatases catalyse phosphoryl transfer to water and some alcohol acceptors. Such enzymes proceed with the formation of a phosphohistidine like phosphoglycerate mutase and the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase. Secondary structural alignments of acid phosphatase with mutase

Figure 10: Structurally based alignment of phosphoglycerate mutases with the consensus sequence of the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase. The mutase sequences are: Sco=*S.coelicolor* PGAM; Sce=*S.cerevisiae PGAM*; Hre2=human BPGAM; Mre=mouse BPGAM; Rre=rabbit BPGAM; Hmu=human M-type BPGAM; Rmu=rabbit M-type PGAM; Pgms=*S.pombe* PGAM.' Consensus' is the PGAM consensus sequence (see figure 3) and 'pf2k consensus' is the consensus sequence of the phosphatase region of 6PF2K/Fru-2,6-BPase (see figure 8). The sequences have been aligned using LINEUP, Devereux *et al.*, 1984.

Conserved active site residues are italicized and underlined, other strongly conserved residues are simply underlined.

The secondary structural elements of *S.cerevisiae PGAM* are indicated: β-strands are labelled A-F and α-helices are labelled 1-5.

```
                1                                                  50
          Sco  ADAPYKLILL RHGESEWNEK NLFTGWVDVN LTPKGEKEAT RGGELLKDAG
          Sce  ..MP.KLVLV RHGQSEWNEK NLFTGWVDVK LSAKGQQEAA RAGELLKEKK
         Hre2  .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
          Mre  .MSKHKLIIL RHGEGQWNKE NRFCSWVDQK LNNDGLEEAR NCGRQLKALN
          Rre  .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
          Hmu  .MATHRLVMV RHGETTWNQE NRFCGWFDAE LSEKGTEEAK RGAKAIKDAK
          Rmu  .MATHRLVMV RHGESSWNQE NRFCGWFDAE LSEKGAEEAK RGATAIKDAK
         Pgms  .AAPNLLVLT RHGESEWNKL NLFTGWKDPA LSETGIKEAK LGGERLKSRG
    consensus  AMAPHKLVML RHGESEWNKE NWFCGWVDQK LSEKGMEEAK RGGKQLKDMN
     pf2kcon   VTPRSIYLC RHGESELNLW GRIGG..DSG VSARGKQYAY ALANFIQSQG
                        β-A                          α-1
               51                                                 100
          Sco  LLPDVVHTSV QKRAIRTAQL ALEAADRHWI PVHRHWRLNE RHYGALQGKD
          Sce  VYPDVLYTSK LSRAIQTANI ALEKADRLWI PVNRSWRLNE RHYGDLQGKD
         Hre2  FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
          Mre  FEFDLVFTSI LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
          Rre  FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
          Hmu  MEFDICYTSV LKRAIRTLWA ILDGTDQMWL PVVRTWRFNE RHYGGLTGFN
          Rmu  IEFDICYTSV LKRAIRTLWT ILDVTDQMWV PVVRTWRLNE RAYGGLTGLN
         Pgms  YKFDIAFTSA LNRANRT... .......... .........RQ RYYGDLQGLN
    consensus  FEFDIVYTSV LNRAIRTARL ILEELDQEWV PVEWSWRLNE RHYGALIGLN
     pf2kcon   ISSLKVRTSH MKRTIQTAEA LG..    .V PYEQWKALNE IDAGV
                β-B          α-2                 β-C
               101                                                150
          Sco  KAQTLAEFGE EQFMLWRRSY DTPPPALDRD AEYSQF..SD PRYAM.LPP.
          Sce  KAETLKKFGE EKFNTYRRSF DVPPPPIDAS SPFSQK..GD ERYKY.VDP.
         Hre2  REQMALNHGE EQVRLWRRSY NVTPPPIEES HPYYQEIYND RRYKVCDVPL
          Mre  REKMALNHGE EQVRLWRRSY NVTPPPIEES HPYFHEIYSD RRYKVCDVPL
          Rre  REKMALNHGE EQVRIWRRSY NVTPPPIEES HPYYHEIYSD RRYRVCDVPL
          Hmu  KAETAAKHGE EQVRSWRRSF DIPPPPMDEK HPYYNSISKE RRYA.GLKPG
          Rmu  KAETAAKHGE EQVKIWRRSF DTPPPPMDEK HNYYASISKD RRYA.GLKPE
         Pgms  KDDARKKWGA EQVQIWRRSY DIAPPNGESL KDTAERV... ........LPY
    consensus  KAETAMKHGE EQVRIWRRSY DVPPPPIEES HPYYQEICSD RRYKVCLVPL
     pf2kcon   .......... .......... .....TYEEI QEHYPEEFAL RDQDKYWY..
                          α-3
               151                                                200
          Sco  ELRPQTECLK DVVGRMLPYW FDAIVPDLLT GRTVLVAAHG NSLRALVKHL
          Sce  NVLPETESLA LVIDRLLPYW QDVIAKDLLS GKTVMIAAHG NSLRGLVKHL
         Hre2  DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTILISAHG NSSRALLKHL
          Mre  DQLPRSESLK DVLERLLPYW KERIAPEILK GKSILISAHG NSSRALLKHL
          Rre  DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTVLISAHG NSSRALLKHL
          Hmu  E.LPTCESLK DTIARALPFW NEEIVPQIKA GKRVLIAAHG NSLRGIVKHL
          Rmu  E.LPTCESLK DTIARALPFW NEEIAPKIKA GKRVLIAAHG NSLRGIVKHL
         Pgms  D........P NLETERLEXL NSTIVAAILK GVKVLIAAHG NSLRALIMDL
    consensus  DQLPRSESLK DVIERMLPYW NERIAPEILK GKTVLIAAHG NSLRALVKHL
     pf2kcon   .RYPKGESYE DLVQRLEPVI MELERQE     NVLVICHQ AVMRCLLATF
                          α-4                    β-D      α-5
               201                                                250
          Sco  DGISDADIAG LNIPTGIPLS YELNAEFKPL NPGGTYLDPD AAAAAIEAVK
          Sce  EGISDADIAK LNIPTGIPLV FELDENLKPS KPSYYL DPE AAAAGAAAVA
         Hre2  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
          Mre  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGNQE AIQAAIKKVD
          Rre  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
          Hmu  EGMSDQAIME LNLPTGIPIV YELNKELKPT KPMQFLGDEE TVRKAMEAVA
          Rmu  EGMSDQAIME LNLPTGIPIV YELNQELKPT KPMRFLGDEE TVRKAMEAVA
         Pgms  EGLTGDQIVK RELATGVPIV YHLDKDGKYV SK.ELIDN.. ..........
    consensus  EGISDEDIIN LNLPTGVPIV FELDENLKPV GPHQFFGDQE AIQAAIEAVA
     pf2kcon   LDKSSDELPY LKCPL HTVL KLTPVAYGCK VESIYL NVE AVNTHRDKPE
                                β-E          β-F

          Sco  NQGKKK....
          Sce  NQGKK.....
         Hre2  DQGKVQ....
          Mre  DQGKVKQGKQ
          Rre  DQGKVKRAEK
          Hmu  AQGKAK....
          Rmu  AQGKAK....
    consensus  DQGKVKQAEQ
     pf2kcon   NVDITREAEE
```

and the phosphatase domain (figure 11) imply that the core α- and β-structural elements are evolutionarily related (Bazan *et al.*, 1989).

1.8.3 Functional homology

The structural similarity of phosphoglycerate mutase and 6-PF-2-K/Fru-2,6-BPase is reflected in functional homology (Tauler *et al.*, 1987):

(a) 6-PF-2-K/Fru-2,6-BPase is able to catalyse the hydrolysis of 1,3-bisphosphoglycerate

(b) incubation of 6-PF-2K/Fru-2,6BPase with the 1-[^{32}P] 1,3-bisphosphoglycerate results in the formation of a labelled phospho-enzyme intermediate

(c) this labelled intermediate results from the formation of a phosphohistidine

(d) this phosphohistidine is the same residue phosphorylated on incubation 6-PF-2-K/Fru-2,6-BPase with fructose-2,6-bisphosphate.

However, 6-PF-2K/Fru-2,6-BPase cannot catalyse the mutase, synthase or 2,3-bisphosphoglycerate phosphatase activities of phosphoglycerate mutase. Conversely, phosphoglycerate mutase is not phosphorylated by fructose-2,6-bisphosphate and so fails to act as a phosphatase toward it.

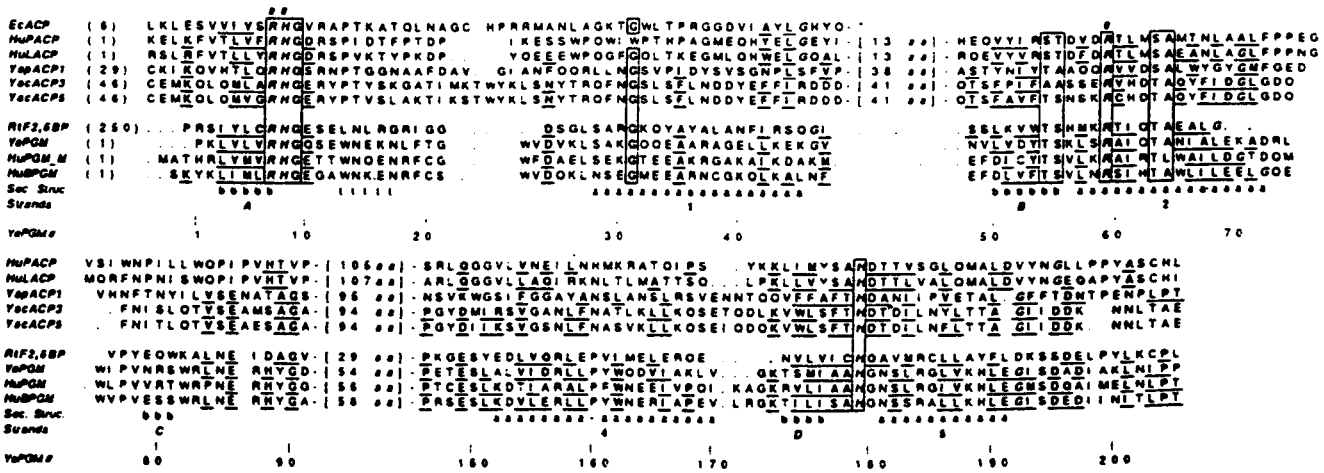


Figure 11: Alignment of acid Pase sequences with the rat Fru-2,6-BPase domain and representative mutases. The *S. cerevisiae* (YscACP3,5), *S. pombe* (YspACP1), human lysosomal (HuLACP) and prostatic (HuPACP) acid Pases, and the N-terminal segment of *E. coli* acid Pase (EcACP) are arrayed over the aligned rat Fru-2,6-BPase, *S. cerevisiae* and human M-type PGAMs, and the human BPGAM sequences. The *S. cerevisiae* numbering is followed. Identical residues are boxed and denoted # whereas chemically conserved residues are underlined and in boldface letters. The secondary structural elements of *S. cerevisiae* PGAM are indicated below the alignment. Gaps in the alignment are indicated by dots. This figure is reproduced from Bazan *et al.*, 1989.

1.9 Evolution of phosphoglycerate mutases

The glycolytic enzymes are among the most highly conserved proteins known (Fothergill-Gilmore, 1986). Therefore, sequence alignments of cofactor-dependent phosphoglycerate mutases (figure 3) reveals a high degree of identity, which is consistent with a very slow evolutionary rate. From the sequence information, it would appear that the human B- and M-isoenzymes evolved by gene duplication followed by divergence. Sequence similarity and the ability to catalyse the synthase, mutase and phosphatase reaction implies that bisphosphoglycerate mutase is closely related to cofactor-dependent phosphoglycerate mutase and so bisphosphoglycerate mutase is considered an isoenzyme (E-form) of the cofactor-dependent enzyme. As the M- and B-isoenzymes are more similar to one another than to the E-isoenzyme, it has been suggested that the M- and B-isoenzymes diverged later than the gene duplication event which resulted in the E-isoenzyme (Fothergill-Gilmore and Watson, 1989).

The quaternary structure of cofactor-dependent phosphoglycerate mutase differs depending on the organism from which it is isolated, see table 1. The evolutionary significance of the distribution of the different structures remains unclear. Tetrameric forms have been found in several fungi, the invertebrate *F. hepatica* and more recently in the bacterium *S.coelicolor*. Dimeric forms have been located in vertebrates and the bacterium *Z. mobilis*. Monomeric forms have been isolated from the fission yeast *S. pombe* and from the bacteria *B. megaterium* and *Pseudomonas* AM1. As

monomeric phosphoglycerate mutases have not been isolated from higher organisms, it has been suggested that the monomeric enzyme represents the ancestral form (Fothergill-Gilmore and Watson, 1989).

Cofactor-independent phosphoglycerate mutase also exists as a monomer, with subunit size twice that of the cofactor-dependent enzymes. However, the evolutionary relationship between cofactor-dependent and cofactor-independent remains unclear due to the lack of sequence information for the cofactor-independent enzymes. The phylogenic distribution of cofactor-dependent and cofactor-independent may reveal the evolutionary relationship between the two classes of enzyme. Cofactor-independent mutases have been found in plants, filamentous fungi, certain invertebrates and the Gram positive *Bacillus* bacteria (Price *et al.*, 1983; Carreras *et al.*, 1982; Singh and Setlow, 1979; Watabe and Freese, 1979). Cofactor-dependent enzymes are found in vertebrates, certain invertebrates (Carreras *et al.*, 1982), fungi such as *S. cerevisiae* and *S. pombe* (Price *et al.*, 1983) and Gram negative bacteria such as *E. coli* (D'Alessio and Josse, 1971). Phosphoglycerate mutase from *Zymomonas mobilis* (Pawluck *et al.*, 1986) and *Streptomyces coelicolor* (White *et al.*, 1992) appears to be partially cofactor-independent, with 20% activity retained in the absence of bisphosphoglycerate (conditions which would render the *S. cerevisiae* enzyme inactive).

The complex distribution of cofactor-dependent and cofactor-independent enzymes suggest that the genes encoding these enzymes were present early in evolution and have been inherited in a haphazard fashion.

The structural and functional homology between the phosphatase domain of 6-PF-2-K/Fru-2,6-BPase and cofactor-dependent phosphoglycerate mutase (section 1.8) supports the theory that these enzymes are divergently related (Tauler *et al.*, 1987). Likewise, the phosphohistidine enzymes, including the acid phosphatases (Bazan *at al.*, 1989) and *S. cerevisiae* phosphatase (Cohen *et al.*, 1978) appear to be evolutionarily related to phosphoglycerate mutase. It has been suggested that the phosphatase and mutase families are related and have diverged from a common ancestor (Bazan *et al.*, 1989).

CHAPTER 2

INTRODUCTION TO ATTEMPTED ISOLATION OF THE GENE ENCODING PHOSPHOGLYCERATE MUTASE FROM *SCHIZOSACCHAROMYCES POMBE*

2.1 Aim of the Project

The initial aim of this project was to isolate the gene encoding phophoglycerate mutase from the fission yeast *Schizosaccharomyces pombe*. It was envisaged that site directed mutagenesis and the development of an overexpression system would produce large quantities of native and mutant forms of *S.pombe* PGAM. A combination of site directed mutagenesis and biophysical techniques, including high resolution NMR, would then be used to study the structure and mechanism of *S.pombe* phosphoglycerate mutase.

2.2 Reasons for studying phosphoglycerate mutase from S.pombe

High resolution NMR has been used to probe the structure and mechanism of the well characterised PGAM from *S.cerevisiae*. *S.cerevisiae* PGAM is a tetramer with an overall molecular weight of 108,000 with the result that NMR spectra are densely crowded with resonance lines: line broadening would be recorded due to rotational tumbling and line narrowing would be recorded due to the flexible tail of the enzyme. Thus the spectra obtained would only give structural information regarding the flexible tail of *S.cerevisiae* PGAM. If stable functional monomers of PGAM from *S.cerevisiae* could be isolated, this would be an ideal system for NMR studies from PGAM. In the absence of such a system, we must turn to the small monomeric PGAM from the fission yeast *S.pombe*.

It has been shown that *S.pombe* PGAM, like other BPG-dependent enzymes, binds to Cibacron-Blue Sepharose (Price and Stevens, 1983). During this study, it was also noted that the subunit molecular weight of PGAM from

S. pombe was unusually low. Further studies by Price *et al.*, 1985, confirmed this and indicated that PGAM from *S. pombe* existed as a monomer of molecular weight 23,000.

Alignment of the sequences of peptides isolated from *S. pombe* PGAM (Fothergill and Dunbar, unpublished) with the complete sequence of *S. cerevisiae* PGAM (White & Fothergill-Gilmore, 1988) reveals around 50% of the residues are identical, see figure 12.

Evidence for structural similarity between the enzymes from *S. pombe* and *S. cerevisiae* has been provided by chemical modification studies, inhibition studies and preliminary circular dichroism work. The initial observation that the enzymes bind Cibacron-Blue (Price and Stevens, 1983) implies that the enzymes contain the characteristic β-sheet flanked by α-helices, which is thought to be the structural basis for chanelling dyes such as Cibacron-Blue to the hydrophobically situated substrate binding site (Beissner *et al.*, 1979). Chemical modification studies have been used to indicate which amino acid residues may be involved in the catalytic mechanism. Histidine-specific and arginine-specific reagents led to the inactivation of *S. cerevisiae* PGAM, indicative of the presence of these groups at the active site (Carreras *et al.*, 1982(b); Borders and Wilson, 1976). The catalytic activity of *S. cerevisiae* PGAM is not affected by cysteine-specific reagents, suggesting that the activity, unlike the rabbit muscle enzyme, involves no cysteine residues (Carreras *et al.*, 1982(c); Price *et al.*,1985(a)). Similar modifications of the *S. pombe* enzyme (Price *et al.*, 1985) imply the presence of a histidine residue is required for activity

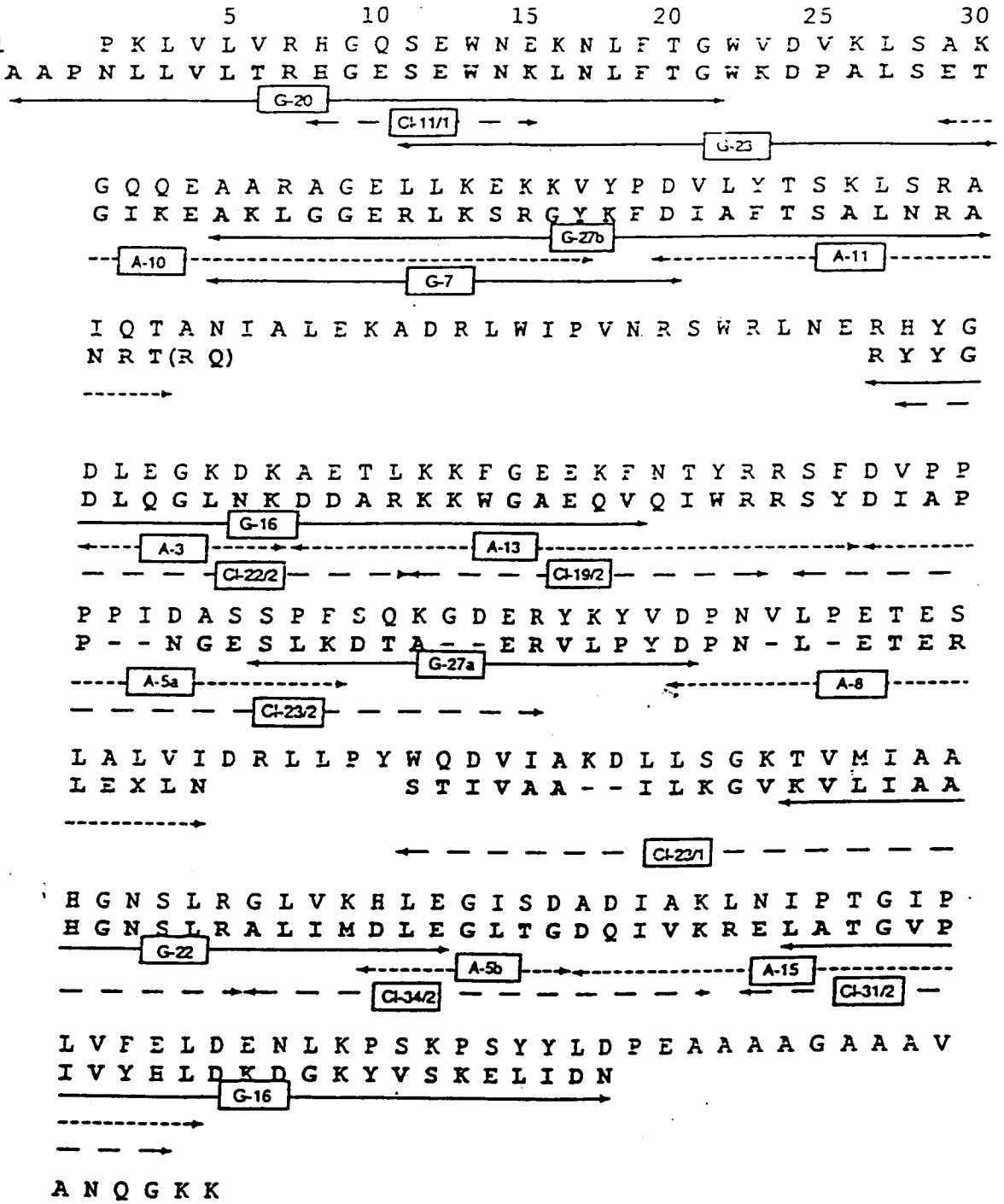


Figure 12: Alignment of *S.cerevisiae* PGAM amino acid sequence (White and Fothergill-Gilmore, 1989) with the partial peptide sequence of *S.pombe* PGAM (Dunbar *et al.*, unpublished). The upper sequence is that of the *S.cerevisiae* enzyme whereas the lower, bold sequence is that of the *S.pombe* enzyme. The solid lines represent Glu-C peptides, large hatched lines represent clostripain generated peptides whereas the small dashed lines indicate Asp-N peptides.

and that the *S.pombe* enzyme has no cysteine residues involved in the catalytic activity or in the maintenance of the 3-D stucture.

Cofactor dependent enzymes, including *S.cerevisiae* PGAM, are inhibited by micro-molar concentrations of vanadate (Carreras *et al.*, 1980). Vanadate inhibition studies have implied that PGAM from *S.pombe* is BPG dependent. Preliminary C.D. experiments have indicated that the far U.V spectrum of the *S.pombe* enzyme was similar to that determined for the *S.cerevisiae* enzyme suggesting that the two enzymes have similar overall secondary structure (Hermann *et al.*, 1983 and Johnson and Price, 1987).

Thus, from the data oulined in this section, it would appear that PGAM from *S.pombe* and *S.cerevisiae* are similar and bearing in mind the small monomeric nature of the *S.pombe* enzyme, the *S.pombe* mutase lends itself to high resolution NMR studies.

2.3 Information available at the outset of the project

As *S.pombe* has proved to be the system of choice for many workers to study the problems of eukaryotic cell and molecular biology, techniques involving the manipulation of this yeast have been developed e.g. isolation of DNA and RNA, development of *S.pombe* plasmids, methods of transformation and integration. Thus, the tools for the genetic manipulation of *S.pombe* were available.

The gene encoding PGAM from *S.cerevisiae* had been isolated by complementation (Kawasaki and Fraenkel, 1982) and sequenced (White and Fothergill-Gilmore, 1988). Malcolm White kindly donated a plasmid encoding *S.cerevisiae* PGAM for use as a potential molecular probe to isolate the *S.pombe* gene.

At the outset of the project, a number of peptides had been isolated from *S.pombe* PGAM which, once sequenced, accounted for 50% of the entire sequence (assuming the entire sequence consists of 220 residues, as judged by the molecular weight of the enzyme). As the project neared completion, more peptides had been isolated and sequenced, and so around 90% of the entire sequence was available, see figure 12. This information not only suggested the potential use of the *S.cerevisiae* PGAM gene as a molecular probe but also made possible the ability to design degenerate oligonucleotides which may also act as molecular probes for the *S.pombe* gene encoding PGAM.

The purification method for *S.pombe* PGAM (Price *et al.*, 1985) permitted the production of an antibody probe to screen an *S.pombe* expression library. Such a library was supplied by Dr Paul Nurse (the library was constructed by V. Simanis, see section 3.13.1).

Presented with a number of potential means to isolate the gene encoding *S.pombe* PGAM, a suitable strategy was devised.

2.4 Strategy of Project

Given the information set out in the previous section, initial attempts to isolate the *S.pombe* PGAM gene would be focussed on the use of the *S.cerevisiae* PGAM gene and degenerate oligonucleotides as molecular probes. Prior to screening the λgt11 library, it would be necessary to determine the hybridisation conditions of these molecular probes by use of Southern analysis of genomic DNA isolated from *S.pombe*.

An alternative method to screen the λgt11 library would also be investigated i.e. production of polyclonal antibody against *S.pombe* PGAM. Prior to use of the antibody to screen the library, it would be necessary to characterise the antibody e.g. ensure the antibody cross-reacts with *S.pombe* PGAM and no other *S.pombe* proteins and ensure no cross-reactivity with bacteria expressing sequences of the vector (i.e. Y1090 infected with λgt11 carrying no insert).

Use of molecular and antibody probes would result in the isolation, characterisation and eventual sequencing of selected clones.

CHAPTER 3

METHODS AND MATERIALS

3.1 Materials

All biochemical reagents were supplied by Sigma Chemical Company, Poole, Dorset, unless specified below.

3.1.1 Strains

E.coli: NM522 supE, thi, hsd5, (lac-proAB), [F', proAB, lacI^{q}ZM15]

Y1088 lacU169, proC::Tn5, tonA2, hsoR, supE, supF, metB, trpR, F^-, λ^-

Y1090 lacU169, lon, araD139, strA, supF, trpC22::Tn10, F^-, λ

E.coli strains NM522, Y1090 and Y1088 were supplied by Pharmacia.

S.cerevisiae - gift of the Distillers Company Ltd., Menstrie, Scotland.

S.pombe(CMI 39917) - Commonwealth Mycological Institute, Kew, England.

S.coelicolor(JI 3456) - provided by Prof. D.A. Hopwood, John Innes Institute.

3.1.2 Vectors

S.pombe cDNA library in λgt11 - Dr V. Simanis, ICRF laboratories, PO Box 123, Lincoln's Field, London.

pIBI 30 and M13K07 - IBI Ltd., 36 Clifton Rd., Cambridge.

S. pombe cDNA library in 2μm URA3 - J.D. Fikes, Institute of Technology, Cambridge, Massachusetts.

3.1.3 Growth Media

Bacto-tryptone (NZ-amine) and Bacto-agar - Difco labs, Central Avenue, East Molesly, Surrey.

Yeast extract and casamino acids - Oxoid Ltd., Basingstoke, Hampshire.

Ampicillin and kanamycin - Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, Northumberland.

3.1.4 Radiochemicals

Amersham plc, Lincoln Place, Aylesbury, Buckinghamshire, supplied all of the following radiochemicals:

Deoxyadenosine 5'(α-^{32}P)triphosphate, triethylammonium salt, stablised in aqueous solution, 3000Ci/mmol.

Deoxyadenosine 5'-(α-^{35}S)thiotriphosphate, triethylammonium salt, stablised aqueous solution, >400Ci/mmol.

Adenosine 5'-(γ-^{32}P)triphosphate, triethylammonium salt, stablised aqueous solution, 3,000Ci/mmol.

3.1.5 Oligonucleotides

Three oligonucleotides, designed from the *S.pombe* PGAM peptide sequence, were ordered at different stages of this project. Figure 13 illustrates which part of the sequence these oligos encode. The oligos were supplied by:

oligo 84-92 - Medprobe, P.O. Box 2640 St. Hanshaugen N-0131 Oslo 1, Norway.

96-105 - Oswel DNA service, Department of Chemistry, University of Edinburgh.

107-114 - Dr V. Math, Department of Biochemistry, University of Glasgow.

The λgt11 primers ('405' and '406') and oligo dT were supplied by Biotechnology Unit, Department of Biochemistry and Molecular Biology, University of Leeds.

3.1.6 Enzymes for DNA manipulation

Northumbria Biologicals Limited, South Nelson Industrial Estate, Cramlington, Northumberland supplied the following enzymes: Klenow fragment, T_4 polynucleotide kinase, BamH I, Bgl II, EcoR I, Hind III, Kpn I, Nlu I and Sal I.

Boeringher Mannheim, Bell Lane, Lewes, East Sussex, supplied the following enzymes: T_4 DNA ligase, RNase A and Proteinase K.

Figure 13: Alignment of known PGAM sequences showing the regions to which oligonucleotides(84-92, 96-105 and 107-114) were designed. The region of protein sequence to which these oligos were designed are underlined. Both 84-92 and 107-114 were designed against a region of PGAM consensus sequence, whereas 96-105 is designed from the *S.pombe* partial protein sequence (Dunbar and Fothergill, unpublished). Labelling as in figure 3.

```
            1                                                   50
      Sco   ADAPYKLILL RHGESEWNEK NLFTGWVDVN LTPKGEKEAT RGGELLKDAG
      Sce   ..MP.KLVLV RHGQSEWNEK NLFTGWVDVK LSAKGQQEAA RAGELLKEKK
     Hre2   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
      Mre   .MSKHKLIIL RHGEGQWNKE NRFCSWVDQK LNNDGLEEAR NCGRQLKALN
      Rre   .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
      Hmu   .MATHRLVMV RHGETTWNQE NRFCGWFDAE LSEKGTEEAK RGAKAIKDAK
      Rmu   .MATHRLVMV RHGESSWNQE NRFCGWFDAE LSEKGAEEAK RGATAIKDAK
      Pgms  .AAPNLLVLT RHGESEWNKL NLFTGWKDPA LSETGIKEAK LGGERLKSRG
 consensus  AMAPHKLVML RHGESEWNKE NWFCGWVDQK LSEKGMEEAK RGGKQLKDMN

            51                                  oligo 84-92   100
      Sco   LLPDVVHTSV QKRAIRTAQL ALEAADRHWI PVHRHWRLNE RHYGALQGKD
      Sce   VYPDVLYTSK LSRAIQTANI ALEKADRLWI PVNRSWRLNE RHYGDLQGKD
     Hre2   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Mre   FEFDLVFTSI LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Rre   FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
      Hmu   MEFDICYTSV LKRAIRTLWA ILDGTDQMWL PVVRTWRFNE RHYGGLTGFN
      Rmu   IEFDICYTSV LKRAIRTLWT ILDVTDQMWV PVVRTWRLNE RAYGGLTGLN
      Pgms  YKFDIAFTSA LNRANRT... .......... .........RQ RYYGDLQGLN
 consensus  FEFDIVYTSV LNRAIRTARL ILEELDQEWV PVEWSWRLNE RHYGALIGLN

      oligo 96-105 oligo107-114                                 150
      Sco   KAQTLAEFGE EQFMLWRRSY DTPPPALDRD AEYSQF..SD PRYAM.LPP.
      Sce   KAETLKKFGE EKFNTYRRSF DVPPPPIDAS SPFSQK..GD ERYKY.VDP.
     Hre2   REQMALNHGE EQVRLWRRSY NVTPPPIEES HPYYQEIYND RRYKVCDVPL
      Mre   REKMALNHGE EQVRLWRRSY NVTPPPIEES HPYFHEIYSD RRYKVCDVPL
      Rre   REKMALNHGE EQVRIWRRSY NVTPPPIEES HPYYHEIYSD RRYRVCDVPL
      Hmu   KAETAAKHGE EQVRSWRRSF DIPPPPMDEK HPYYNSISKE RRYA.GLKPG
      Rmu   KAETAAKHGE EQVKIWRRSF DTPPPPMDEK HNYYASISKD RRYA.GLKPE
      Pgms  KDDARKKWGA EQVQIWRRSY DIAPPNGESL KDTAERV... .......LPY
 consensus  KAETAMKHGE EQVRIWRRSY DVPPPPIEES HPYYQEICSD RRYKVCLVPL

            151                                                 200
      Sco   ELRPQTECLK DVVGRMLPYW FDAIVPDLLT GRTVLVAAHG NSLRALVKHL
      Sce   NVLPETESLA LVIDRLLPYW QDVIAKDLLS GKTVMIAAHG NSLRGLVKHL
     Hre2   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTILISAHG NSSRALLKHL
      Mre   DQLPRSESLK DVLERLLPYW KERIAPEILK GKSILISAHG NSSRALLKHL
      Rre   DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTVLISAHG NSSRALLKHL
      Hmu   E.LPTCESLK DTIARALPFW NEEIVPQIKA GKRVLIAAHG NSLRGIVKHL
      Rmu   E.LPTCESLK DTIARALPFW NEEIAPKIKA GKRVLIAAHG NSLRGIVKHL
      Pgms  D........P NLETERLEXL NSTIVAAILK GVKVLIAAHG NSLRALIMDL
 consensus  DQLPRSESLK DVIERMLPYW NERIAPEILK GKTVLIAAHG NSLRALVKHL

            201                                                 250
      Sco   DGISDADIAG LNIPTGIPLS YELNAEFKPL NPGGTYLDPD AAAAAIEAVK
      Sce   EGISDADIAK LNIPTGIPLV FELDENLKPS KP.SYYLDPE AAAAGAAAVA
     Hre2   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
      Mre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGNQE AIQAAIKKVD
      Rre   EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
      Hmu   EGMSDQAIME LNLPTGIPIV YELNKELKPT KPMQFLGDEE TVRKAMEAVA
      Rmu   EGMSDQAIME LNLPTGIPIV YELNQELKPT KPMRFLGDEE TVRKAMEAVA
      Pgms  EGLTGDQIVK RELATGVPIV YHLDKDGKYV SK.ELIDN.. ..........
 consensus  EGISDEDIIN LNLPTGVPIV FELDENLKPV GPHQFFGDQE AIQAAIEAVA


      Sco   NQGKKK....
      Sce   NQGKK.....
     Hre2   DQGKVQ....
      Mre   DQGKVKQGKQ
      Rre   DQGKVKRAEK
      Hmu   AQGKAK....
      Rmu   AQGKAK....
 consensus  DQGKVKQAEQ
```

3.1.7 Protein purification and characterisation

Boeringer Mannheim, Bell Lane, Lewes, East Sussex, supplied the following biochemical reagents: enolase, glycerate-3-phosphate (grade I), glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phoshoglycerate kinase, phosphoglycerate mutase (rabbit muscle).

Aspartate aminotransferase (mitochondrial, pig heart) was a kind gift of Doris Duncan, University of Stirling.

The C_{18} μBondapack column and S-300 were supplied by Pharmacia,

Immobilon-P was supplied by Millipore.

3.1.8 Antibody production and characterisation

Northeast Biomedical Laboratories Ltd., PO Box 187, Uxbridge, Middlesex supplied the peroxidase-conjugated goat anti-rabbit IgG

3.1.9 PCR

Taq polymerase and NTPs were supplied by Perkin-Elmer Ltd.

3.1.10 Miscellaneous

Amersham plc, Lincoln Place, Ayelsbury, Buckinghamshire, supplied Hybond-N and Hyperfilm MP X-ray film

Cambridge Bioscience, Newton House, Devonshire Rd., Cambridge, supplied the Sequenase™ DNA sequencing kit.

Novo Enzymes Ltd., 4 St. Georges Yard, Castle Street, Farnham, Surrey, supplied Novozym™ 234.

Pharmacia supplied the oligodeoxyribonucleotide hexamers.

3.2 General Techniques

Many of the methods outlined in this chapter are standard experimental techniques. These general techniques are listed below alongside the reference from which they were obtained.

Methods in Enzymology, Volume 152, 'A Guide to Molecular Cloning Techniques'. 1988.
Analysis of DNA by Southern hybridisation. Preparation of plasmids and phage.

'DNA Cloning, volume 1'. Edited by Glover, D.M.. IRL Press, 1985.
Screening cDNA library in λgt11 and generation of fusion proteins

'Antibodies-a laboratory manual' Edited by Harlow, E. and Lane, D.. Cold Spring Harbor, 1988
Preparation of polyclonal antibodies

'Step-by-Step Protocols for DNA Sequencing with Sequenase^R, Version 2.0. 5th edition.' United States Biochemical, 1991.
DNA sequencing

'PCR Protocols: A Guide to Methods and Applications.' Edited by Gelfund, D.H., Shinsky, J.J. and White, P.J.. Academic Press Inc., 1990.
Characterisation of λgt11 clones and attempted isolation of gene encoding PGAM from *S. pombe*.

'Protein Sequencing: A Practical Approach.' Edited by Findlay, J.B.C. and Geisow, M.J.. IRL Press, 1989.
Amino acid analysis. Peptide generation and characterisation.

3.3 Analysis of *S. pombe* DNA by Southern Hybridisation

3.3.1 Isolation of *S. pombe* Genomic DNA

High molecular weight genomic DNA was isolated from *S. pombe* using a method outlined by Peter Fantes (personal communication). 200ml liquid cultures of *S. pombe* were grown to late log phase as outlined in section 3.4.1. The cells were harvested by centrifugation at 20,000g for 5min, at 4°C and were resuspended in 50ml SED (1M sorbitol, 25mM disodium EDTA, pH 8.0 and 6.7mg/ml DTT). Once the cells were thoroughly resuspended in 50ml SED, they were subjected to centrifugation for 5min at top speed in a bench top centifuge. The cells were then resuspended in 5ml SCE (1M sorbitol, 0.1M sodium citrate, 10mM disodium EDTA, pH 5.8) plus 10μl mercaptoethanol.

After resuspension, protoplast formation was started by the addition of 5ml SCE, containing 50mg Novozym™234 and this mixture was incubated at 30°C. Using a light microscope, protoplast formation was monitored by checking susceptibility to lysis in the presence of an equal volume of 2% SDS. Once > 80% of the cells were SDS sensitive, the protoplasts were harvested by centrifugation for 5min at half speed in a bench top centrifuge. The protoplasts were gently resuspended in 4ml 0.15M NaCl/0.1M disodium EDTA, pH 8.0 and to this, 20µl Proteinase K (10mg/ml) and 250µl 15% SDS were added. This mixture was incubated at 45°C for 1-2 hours, followed by a 15min incubation at 70°C and then placed onto ice. 1/10 volume 5M potassium acetate was added and stored on ice for a further 30min. Cell debris was removed by centrifugation at 20,000g for 10min at 4°C. The supernatant from this step was collected and treated with 40µl RNAase (10mg/ml, DNAase free) at 37°C for 1 hour. Following the RNAase treatment, the supernatant was cooled to room temperature and deproteinised by adding 5ml chloroform/isoamyl alcohol (24:1). The aqueous phase was carefully removed following centifugation (top speed, bench centrifuge) and subjected to ethanol precipitation. The precipitate collected by centrifugation 30,000g, 10min, 4°C) was dried and redissolved in 4ml TE (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0), overnight. The resuspended DNA was precipitated by adding 400µl 3M sodium acetate, pH 7.0 and 2.4ml isopropanol. The precipitate was collected by centrifugation (30,000g, 10min, 4°C) and rinsed with 70% ethanol. The purified DNA was then dried and redissolved in 1ml TE. A 200ml liquid culture of *S.pombe* yielded around 200µg genomic DNA.

3.3.2 Digestion of *S. pombe* Genomic DNA with Restiction Enzymes and Separation of Restriction Fragments by Agarose Gel Electrophoresis

S.pombe genomic DNA was digested with a range of restriction endonucleases e.g. BamH I, Bgl II, EcoR I, Hind III and Sal I. A typical restriction reaction contained 30µg *S.pombe* genomic DNA and 10 units of restriction enzyme. The digests were electrophoresed on a 0.8% agarose gels (20cm x 20cm) at a current of 60mA, overnight at 4°C. After electrophoresis, the gel was stained with ethidium bromide and then photographed.

3.3.3 Transfer of Restricted *S.pombe* Genomic DNA from Agarose Gels onto Hybond-N

Following electophoresis, unused areas of gel were excised. The restricted DNA was denatured by incubating the gel in several volumes of denaturing solution (1.5M NaCl, 0.5M NaOH) for 1 hour at room temperature. The denaturing solution was then poured off and the gel dried with paper tissues. The gel was then equilibrated in several volumes of neutralising solution (1.5M NaCl, 0.5M Tris-HCl, pH 7.2, 1mM EDTA) for 2 hours and the blotting apparatus was set up as follows. A sponge was placed in a plastic tray, half-filled with transfer buffer, 10 x SSC. (SSC was used during blotting and washing and 1 x SSC was composed of 150mM NaCl, 15mM Na_3 citrate). The sponge was covered by a piece of Whatman 3MM filter, presoaked in transfer buffer. Each end of the 3MM paper dips into the transfer buffer to act as a wick. The gel was placed on top of the wick, open wells face down, and trapped air bubbles removed. A window of parafilm

was placed around the edge of the gel to direct capillary flow through the gel and prevent 'short-circuits' betweeen the wick and the paper towels. A piece of Hybond-N, cut to fit the gel, was placed on top of the gel taking care to avoid air bubbles. Two sheets of 3MM paper, presoaked in transfer buffer, were placed on top of the Hybond-N. Capillary flow was maintained by placing a stack of paper towels, measuring 5cm in height, on top of the 3MM paper. A perspex plate was placed on the stack of paper towels and finally a 1kg weight was placed on the perspex plate. The blot was left at room temperature, overnight, to allow moblised DNA to transfer onto the Hybond-N, then the apparatus was dismantled and the filter air dried for 1 hour. The dried filter was wrapped in Saran wrap and placed DNA side down onto a transilluminator for 3-5min to fix the DNA. The filter was now ready for hybridisation.

3.3.4 Radiolabelling of *S.cerevisiae* PGAM gene by Random Priming

The *S. cerevisiae* PGAM gene was isolated by complementation (Kawasaki and Fraenkel, 1982) in the plasmid YEP 13.GPM. The PGAM gene was later characterised by M.F. White (White and Fothergill-Gilmore, 1988), who located the PGAM gene on a 1.3Kb SalI-HindIII fragment on YEP 13.GPM. The probe was isolated by M.F. White as follows: the Sal I - Hind III fragment encoding PGAM was excised from a 1% agarose gel. The excised band was weighed in a microfuge tube and SDW (sterile distilled water) was added in the ratio 1.5ml SDW to 1g gel. The capped tube was placed in a boiling waterbath for 7min and then equilibrated at 37°C for at least 10min prior to labelling. Isolated fragments were stored at -20°C and reboiled for 3min, equilibrated at 37°C for 10min prior to labelling.

The SalI-HindIII fragment of YEP 13.GPM encoding the *S. cerevisiae* PGAM prepared by M.F. White was random primed to generate a probe of very high specific activity. The fragment was boiled for 3min and then equilibrated at 37°C for 10 min prior to the addition of the following reagents:

18µl SDW
5µl OLB, see below
2µl BSA (10mg/ml)
20µl Sal I - Hind III fragment (20ng)
3µl (α-^{32}P)dATP (10µCi/µl)
2µl Klenow (1.5 units/µl)

The reaction mixture was incubated overnight at room temperature. 5 min prior to hybridisation, the probe was denatured by boiling for 5 min.
OLB is composed of solutions A,B and C mixed in the ratio 2:5:3, respectively.

Solution A : 625µl 2M Tris-HCl, pH8
25µl 5M $MgCl_2$
19µl 2-mercaptoethanol
5µl 0.1M dCTP
5µl 0.1M dGTP
5µl 0.1M dTTP
350µl SDW

Solution B : 2M HEPES buffer titrated to pH6.6 with NaOH

Solution C : Oligodeoxyribonucleotide hexamers, evenly suspended in 3M Tris-HCl, 0.2mM EDTA pH7.0 at 90 A_{260}units/µl

3.3.5 Radiolabelling of oligonucleotide probe

The oligonucleotide 100-107, see figure 13, was labelled using (γ-^{32}P)ATP under the following conditions:

20pmol	oligonucleotide
2µl	(10x) polynucleotide kinase buffer
20pmol	(γ-^{32}P)ATP (specific activity=3,000Ci/mmol)
8units	polynucleotide kinase

The final volume was adjusted to 20µl with SDW and the labelling mix was incubated at 37°C for 1 hour. The labelled oligonucleotide was purified by gel filtration on a 1ml Sephadex G-50 column, equilibrated with TE. The labelling mix was applied to the column with xylene cyanol FF and bromophenol blue. The column was washed with a few volumes of TE, resulting in the coelution of the labelled oligonucleotide with bromophenol blue.

3.3.6 Hybridisation and washing conditions

Hybond-N filters from Southern blotting were soaked in 30ml pre-hybridisation solution (6 x SSC, 50µg/ml heparin, 0.1% Na Pyrophosphate, 0.2% SDS) in a heat sealed bag from which most of the air was expelled. The filter was incubated in the pre-hybridisation solution for at least two hours at room temperature. 5ml of pre-hybridisation solution was removed from the bag and mixed with the labelled probe. The probe solution was added to the bag which was then resealed and the contents of the bag were thoroughly mixed. The filters were incubated overnight at room temperature, with gentle shaking, to allow hybridisation. Following the overnight incubation, the hybridisation solution was carefully removed and disposed

of in a designated sink. The filters were carefully removed from the bag and transferred to a plastic box containing 6 x SSC, 0.1% SDS. The filters were washed in this solution for 10min at room temperature. More stringent washes were achieved by lowering the ionic strength e.g. 4 x SSC, 2 x SSC, 0.5 x SSC. Once 0.5 x SSC washes were used, the temperature was increased in a stepwise fashion. The blot was scanned for activity after each wash and rewashed if necessary. Finally the blot, whilst still moist, was wrapped in Saran wrap and autoradiographed.

3.4 Purification of phosphoglycerate mutase from *Schizosaccharomyces pombe*

3.4.1 Growth of *Schizosaccharomyces pombe*

S. pombe was routinely maintained on slopes of malt agar containing 5% glucose. These slopes were incubated at 25°C. *S. pombe* was grown in a liquid culture medium containing, per litre, 20g yeast extract, 2g $(NH_4)_2SO_4$, 25g KH_2PO_4 and 20g glucose, in an orbital incubator (150 r.p.m.) at 30°C. Initially, a 10ml culture was grown overnight, which was then used to innoculate 5 x 200ml batches, which were grown to early stationary phase (48-60 hours).

3.4.2 Cell lysis and ammonium sulphate fractionation

All the following procedures were conducted at 4°C. The cells were harvested by centifugation at 20,000g, for 5 minutes, and were resuspended in an equal volume of extraction buffer containing 10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.1% v/v Triton X-100. Proteinase inhibitors, see section 3.4.3, were added to the extraction buffer immediatley before cell lysis. This suspension was then transferred to a 200ml glass beaker, to which acid washed sand was added until no surface liquid was visible. The cells were then lysed over a period of 5 min using a motorised homogeniser, fitted with a teflon pestle. 20ml extraction buffer were added to the homogenate and mixed with a glass rod. The sand was washed a further 4-5 times with 20ml extraction buffer, and on each occasion the supernatant was collected. Cell debris was removed by centrifugation at 100,000g for 1 hour. The

supernatant was then collected and subjected to ammonium sulphate fractionation. The fraction precipitating between 50-70% saturation was collected and resuspended in a small volume (around 2ml) 10mM Tris-HCl, pH 8.0, and dialysed overnight against this buffer.

3.4.3 Proteinase inhibitors

A general proteinase inhibitor cocktail solution was added to *S. pombe* and *S. cerevisiae* lysates.

COMPOUND	STOCK SOLUTION	WORKING CONC	PROTEINASE CLASS
1,10 Phenanthroline	0.1M in DMSO	0.1mM	Metallo-
3,4 DCIC	5M in DMSO	0.1mM	Serine-
E-64	1mM in H_2O	0.02mM	Cysteine-

3.4.4 Affinity chromotography

After dialysis, the 50-70% ammonium sulphate fraction was applied to a column (12cm x 0.8cm^2) of Reactive Blue 2-Sepharose CL-6B. The column was then washed with several column volumes of 10mM Tris-HCl, pH 8.0 to remove all unbound material. One column volume of 1mM NADH was then applied to the column, followed by several volumes of buffer in an attempt to remove dehydrogenases. Phosphoglycerate mutase activity was then eluted with a column volume of 4mM 2,3 bisphospoglycerate, followed by several volumes of

buffer. Phosphoglycerate mutase activity emerged from the column as a single peak in a volume of 8-10ml.

To regenerate the column, the Reactive Blue 2-Sepharose was removed and mixed with 50ml 5M NaCl for 30min. The column was then repacked and washed with buffer until the conductivity of the column effluent returned to a value equal to that of the 10mM Tris-HCl, pH8.0 buffer.

3.5 Purification of phoshoglycerate mutase from *Saccharomyces cerevisiae*

Cell lysis, ammonium sulphate fractionation and affinity chromatography were conducted in a manner identical to that for *S.pombe*, as outlined in section 3.1.

3.6 Phosphoglycerate mutase assay

Phoshphoglycerate mutase activity was assayed spectrophotometrically at 240nm, 30°C, using the enolase coupled procedure, described by Rodwell et al. (1957).

$$\text{glycerate-3-phosphate} \xrightleftharpoons{\text{PGAM}} \text{glycerate-2-phosphate}$$

$$\text{glycerate-2-phosphate} \xrightleftharpoons{\text{Enolase}} \text{phosphoenol pyruvate}$$

Phosphoglycerate mutase activity was coupled to enolase, resulting in the formation of phosphoenol pyruvate, which absorbs at 240nm. The standard assay mix contained a final volume of 1ml:

Tris-HCl, pH 7.0	30mM
Glycerate-3-phosphate	10mM
2,3-Bisphosphoglycerate	0.3mM
$MgSO_4$	3mM
Enolase	10µg(0.4 units)

The reaction was initiated by the addition of phosphoglycerate mutase (50µl, max) and the rate of increase in absorbance at 240nm, at 30°C, was monitored. One Enzyme Unit of phosphoglycerate mutase activity was defined as producing an increase in absorbance of 0.1 min^{-1}. To ensure the enolase system did not become the rate limiting step, the phosphoglycerate mutase added was limited such that the change in A_{240} was less than 0.15 min^{-1}.

3.7 Phosphoglycerate Kinase Assay

Phosphoglycerate kinase activity was estimated by coupling the reaction with glyceraldehyde-3-phosphate dehydrogenase and monitoring the change in A_{340} at 25°C.

$$\text{ATP} + \text{glycerate-3-P} \xrightleftharpoons{\text{PGK}} \text{glycerate-1,3-P}_2 + \text{ADP}$$

$$\text{glycerate-1,3-P} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{GAP.DH}} \text{glyceraldehyde-3-P} + \text{NAD}^+ + \text{Pi}$$

A standard 1ml reaction mix contained:

Triethanolamine buffer, pH 7.6	82.3mM
ATP	1.1mM
Glycerate-3-P	6.2mM
NADH	0.2mM
EDTA	0.9mM
$MgSO_4$	2mM
Glyceraldehyde-3-P Dehydrogenase	2.7U/ml

3.8 Protein Concentration Estimation

Protein concentration estimations were made using the method outlined by Sedmak and Grossberg, 1977. The protein assay was conducted by adding 1ml of protein solution to 1ml of 0.06% w/v Coomassie Brilliant Blue G-250, in 3% perchloric acid . The absorbance of the protein-reagent mixture was measured at 620nm and 465nm, against an absorbance blank of distilled water. The 620/465 absorbance ratio was calculated for each protein-reagent mixture. Likewise, the 620/465 absorbance ratio was calculated for a protein blank (i.e. 1 ml distilled water and 1ml Coomassie reagent) and this value was subtracted from the 620/465 absorbance ratio of each of the protein-reagent mixtures.

A standard curve was prepared for each batch of Coomassie reagent, using known concentrations of bovine serum albumin, over the range 0-40μgml^{-1} of

protein. Thus, 620/465 absorbance ratios, less 620/465 absorbance ratio of the protein blank, were plotted against protein concentration.

3.9 S-300 Gel Filtration

A gel filtration column was prepared using Sephacryl-S300, which was equilibrated with 0.1M sodium phosphate, pH 7.4. The column measured 40cm x 6.2cm^2, with a flow rate of approximately 1mlmin^{-1}. Samples applied to the column were made up to a final volume of 1ml, and the eluant was collected in 5ml fractions, which were weighed and assayed for protein concentration and/or enzyme activity. The void volume of the column was determined using dextran blue.

3.10 SDS-PAGE

Polyacrylamide gel electrophoresis, in the presence of SDS, was conducted using the method outlined by Laemmli (1970). Protein samples were prepared by mixing an equal volume of boiling mix (10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0.005% w/v bromophenol blue). This mixture was boiled for 2 min prior to loading onto a gel.

Details of buffers, solutions and conditions for the preparation and running of polyacrylamide slab gels are given in Appendix I.

3.11 HPLC

Reverse phase HPLC was used as a final purification step in the preparation of *S.pombe* PGAM for amino acid analysis and sequencing. An Altex system was used with a C_{18} μBondapak column (Waters) and typically a gradient of 30% to 90% solution B was run over a period of 40min, where solution A was 0.1% TFA in SDW and solution B was 0.1% TFA in acetonitrile, with a flow rate of 1ml/min. The column effluent was monitored at a wavelength of 220nm and the absorbance recorded on a chart recorder on the range 0-0.2A, running at 2mm/min.

3.12 Amino Acid Analysis

Prior to hydrolysis of *S.pombe* PGAM, the enzyme was freeze dried and pyridylethylated as follows. Two samples of 50pmol *S.pombe* PGAM were spotted onto glass fibre dics, dried and placed at the top of stoppered tubes containing:

100μl pyridine
100μl SDW
20μl 4-vinylpyridine
20μl tributylphosphine

The tube was thoroughly purged with argon before and after the addition of the disc. The tube was then quickly sealed and immersed in a water bath at 60°C for 2 hours. The samples were dried and then hydrolysed in the

presence of 6M HCl. One sample was hydrolysed for 22 hours and the other for 44 hours.

The pyridylethylated, hydrolysed samples were appled to an Applied Biosystems 420A Amino Acid Analyser. The analyser was operated by Andy Cronshaw, University of Edinburgh.

3.13 Cyanogen Bromide Digestion of PGAM from *S.pombe*

S.pombe PGAM was digested with cyanogen bromide in a vapour phase reaction. 300pmol of *S.pombe* PGAM was spotted onto a glass fibre disc and dried. 500µl formic acid was pipetted into the bottom of a stoppered flask and then one crystal of CNBr was added. The tube was purged with argon and then the disc was placed at the top of the flask whilst continuing to purge with argon. Finally the flask was stoppered and incubated at 30°C for 4-5 hours in the dark.

3.14 Preparation of Antibodies

3.14.1 Preparation of *S.pombe* PGAM for Injection

Antisera against *S.pombe* phosphoglycerate mutase were obtained from two female New Zealand white rabbits. A preparation of *S.pombe* PGAM, containing 200μg of protein, was subjected to polyacrylamide electrophoresis in a preparative 12% acrylamide slab gel as shown in figure 14. As the PGAM appeared to run anomalously on the slab gel, a section believed to be the PGAM band, was excised. This excised section was homogenised and run alongside a small sample of the PGAM preparation and molecular weight markers, on a 12% acrylamide gel. Figure 15 suggests that the band excised from the slab gel corresponds to pure *S.pombe* PGAM. Thus, the remaining PGAM band was excised from the slab gel and washed in several volumes of PBS for 30 min. The band was then frozen at -20°C. Following this, the band was dried with Whatman filter paper, homogenised and then resuspended in 2-3ml PBS. Storing this suspension on ice, it was then subjected to sonication (at an amplitude of 12microns for 10 x 2min). To this, 1.5 volumes of Freund's complete adjuvant was added and the mixture shaken vigorously for 6-8 hours at 4°C, to create a water in oil emulsion. This emulsion was injected subcutaneously into several sites on the side of each rabbit. A series of successive injections were given at three week intervals; however, Freund's incomplete adjuvant was used to create the emulsion in these cases.

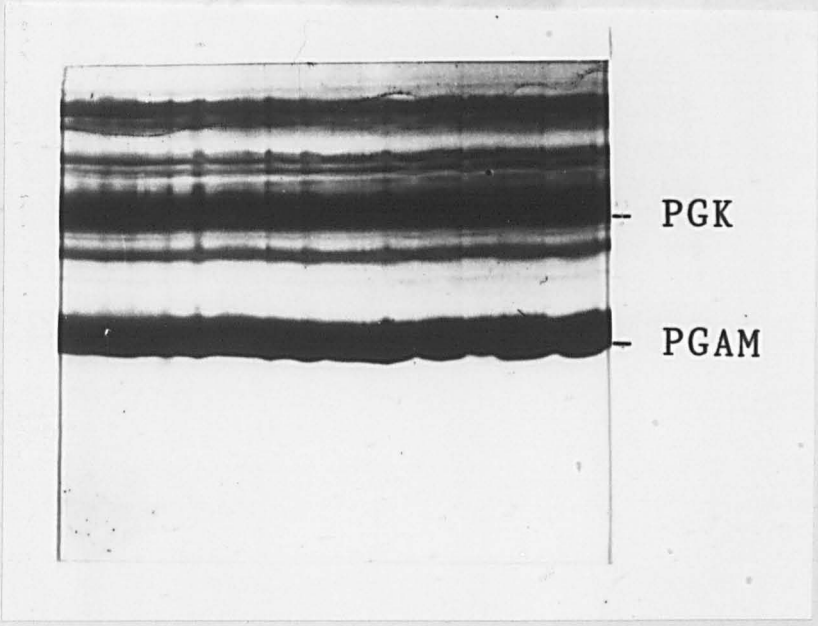


Figure 14: Preparative SDS-PAGE of partially purified *S.pombe* phosphoglycerate mutase. The band of lowest electrophoretic mobility (PGAM) was excised from the gel and prepared for injection to raise antibodies.

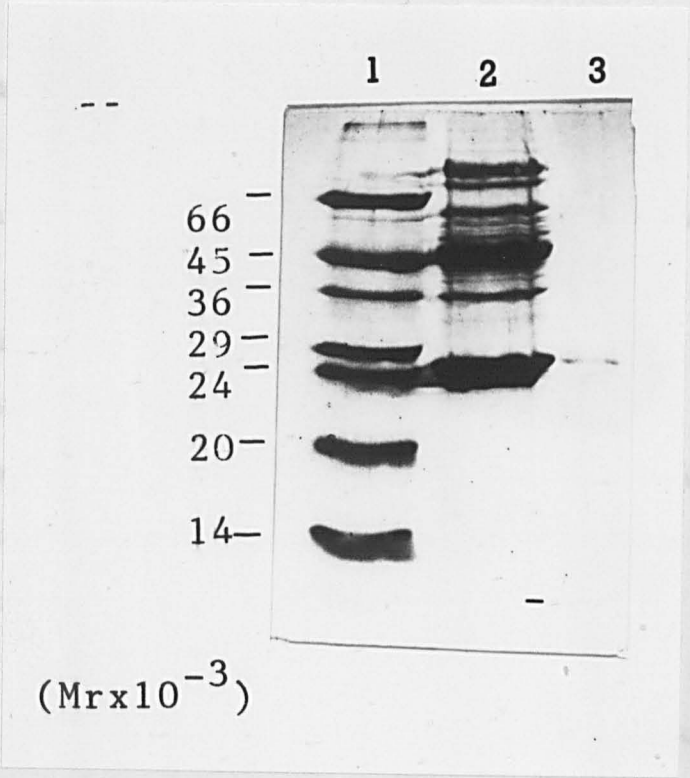


Figure 15: SDS-PAGE (12%) of phosphoglycerate mutase band excised from preparative gel (figure 14).

Lane	Sample
1	Molecular weight markers
2	Partially purified *S.pombe* phosphoglycerate mutase
3	*S.pombe* phosphoglycerate mutase band excised from the preparative gel (figure 14)

3.14.2 Test bleeds and serum preparation

Prior to any injections, non-immune blood samples were taken from the rabbits. Using a clean razor, the marginal ear vein was cut and 4ml of blood was collected in a falcon tube. The blood was incubated at 37°C for 30min, to allow the blood to clot. The clot was then separated from the sides of the tube before incubating at 4°C, overnight. The serum was then separated from the clot by centrifugation (top speed, bench centrifuge). The serum was then aliquoted into 100µl batches and stored at -70°C.

This procedure was repeated 2 weeks after each injection to monitor the production of *S. pombe* PGAM cross reactivity.

3.15 Western Blotting

3.15.1 SDS-PAGE and transfer onto nitrocellulose

SDS-PAGE gels were prepared and run as outlined in section 3.8. The gels were run in duplicate: one for Coomassie staining and the other for Western blotting followed by immunostaining. The molecular weight marker system used on these analytical gels was the prestained molecular weight standard supplied by Sigma, containing: α-macroglobulin, β-galactosidase, phosphofructokinase, fumarase,

lactate dehydrogenase and triose phosphate isomerase. These standards not only acted as molecular weight references but also gave an indication of how well transfer had taken place from the gel onto nitrocellulose on immunoblotting.

The amount of protein loaded onto the analytical gels depended on its purity; 1-5μg of purified protein and 20-50μg if a crude extract was being analysed. Once SDS-PAGE was complete, the gels were washed briefly in transfer buffer (20mM Tris-HCl, 190mM glycine, 20% methanol). Using the Bio-Rad Trans-Blot™ cell, proteins were transferred from the gels onto nitrocellulose over a period of 90 min, at 300mA in transfer buffer.

Following transfer, non-specific protein binding was blocked by incubating the nitrocellulose filters in PBS, containing 0.2% gelatin and 0.1% Triton X-100, overnight.

3.15.2 Immunostaining

Following incubation in blocking agent, the blots were washed in PBS (20mM NaH_2PO_4, pH 7.4, 140mM NaCl) for 2min prior to a one hour incubation in freshly prepared diluted anti-serum. All anti-sera were diluted in PBS containing 0.2% (w/v) gelatin and 0.1% (v/v) Triton X-100 (buffer A). Blots were then washed three times in buffer A, each wash lasting 5 min. This was followed by a one hour incubation with peroxidase-conjugated goat anti-rabbit Ig-G. The blots were then washed 3 x 5 min in PBS prior to a brief wash in 50mM Tris-HCl, pH 7.4.

The blots were then incubated in 50mM Tris-HCl, pH 7.4, containing 0.5mg/ml diaminobenzidine tetrahydrochloride (DAB) for a few minutes. Following this hydrogen peroxide was added; 1µl of 30% H_2O_2 per ml of DAB solution.

Once immunoreactive proteins were visualised by a colour reaction with DAB, the reaction was stopped by the addition of SDS (1ml of 20% SDS per 100ml DAB solution). The blots were finally washed in several volumes of distilled water and air dried at room temperature on Whatman filter paper.

3.16 Screening λgt 11 *S.pombe* expression library constructed in λgt 11, with anti-PGAM serum

3.16.1 Source of λgt11 library

The *S.pombe* cDNA library in λgt11 was provided by Dr Viesturs Simanis, Imperial Cancer Research Laboratories, P.O. Box 123, Lincoln's Inn Field, London. This library was constructed using RNA extracted from wild type cells (972h$^-$) grown on minimal medium, EMM2 (Mitchinson, 1970). The cDNA was primed by oligo dT and made using the RNaseH/pol1 second strand method. Pre-amplification, the bank contained 2 x 10^6 clones of which 93% were recombinant. After amplification via Y1088, It was 84% recombinant. The average insert size was around 650bp, with the largest clone measuring around 3kb (V. Simanis, personal communication).

3.16.2 Plating library for primary screen

The library was screened using the *E. coli* host strain Y1090, which was periodically streaked out on 2YT plates containing 50µg/ml ampicillin to ensure no loss of the transposon pMC9. A single colony of Y1090 was used to inoculate 5ml NZ (per litre: 10g Bacto-tryptone, 5g yeast extract, 1g casamino acids, 5g NaCl, 2g $MgSO_4.7H_2O$), containing 0.2% maltose and 100µg/ml ampicillin. This culture was grown in an orbital incubator (200r.p.m.) at 37°C, overnight. 200µl of this overnight culture was used for each 90mm petri-dish, on which the primary screen would be carried out. Six 90mm petri-dishes were screened initially, each containing 25,000 pfu, which were prepared as follows; 200µl of the Y1090 overnight culture was incubated with diluted library stock (25µl of 1:1000 dilution) at 37°C for 15 minutes. Following this incubation, 3.5ml molten top NZ (NZ with 7g agar/l) 50°C was added and this entire mixture was poured onto a 90mm petri-dish containing 10ml set bottom NZ (NZ with 15g agar/l). Once the top NZ had set, the plates were incubated at 42°C until plaques could be observed (3-4 hours), taking care to prevent plaques reaching confluence.

3.16.3 Blotting

Nitrocellulose filters were cut to fit the 90mm petri-dishes and then autoclaved at 1.5kg/cm² for 20min. The filters were then impregnated with IPTG by soaking in 700µl 18mM IPTG and then air dried in the sterile hood. The dried filters were then placed on the plates containing 25,000 pfu, as prepared in section 3.16.2. The filters were numbered and their orientation on the plates marked.

The plates, covered with the filters were then incubated at 37°C for 3 hours, thus allowing the expression of fusion proteins and their transfer onto the nitrocellulose filters. The filters were then carefully removed from the NZ plates and non-specific protein binding was blocked by incubating with PBS containing 0.2% gelatin, overnight at room temperature.

3.16.4 Immunostaining

Immunostaining of the filters was carried out as outlined in section 3.15.2, however the incubation period with the anti-PGAM serum was extended to 4 hours. Following the addition of DAB and H_2O_2, as soon as immunoreactive plaques appeared, the reaction was stopped by the addition of SDS, thus minimising background staining and hence the identification of false positives.

Positive immunoreactive plaques identified on the nitrocellulose filters were located on the NZ plates and removed as a plug of agar, using the wide end of a pasteur pipette. These plugs were taken up in 500µl SM (100mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl, pH 7.5, 0.01% gelatin) and a few drops of chloroform.

3.16.5 Secondary Screen

The titre of each of the plugs selected in the primary screen was determined. Using the method outlined in section 3.16.2, 1,000 pfu were plated out from each plug. The plates were then blotted and the resultant filters were immunostained as described for the primary screen (sections 3.16.3 and 3.16.4). Positive

immunoreactive plaques were identified and a single positive plaque was removed from each plate as an agar plug. These plugs were taken up in 500µl SM and a few drops of chloroform.

3.16.6 Tertiary Screen

The titre of each of the plugs selected from the secondary screen was determined. Using the method outlined in section 3.16.2, 100 pfu were plated out from each plug. The plates were then blotted and the resultant filters were immunostained as described for the primary screen (sections 3.16.3 and 3.16.4). Positive immunoreactive plaques were identified and single 'positives' and were removed from each plate. This final selection demanded removing the plaque cleanly, with no contaminating 'negatives', using a yellow tip. Each plaque was taken up in 500µl SM and a few drops of chloroform.

3.16.7 Preparation of High Titre Phage Stock from Positive Immunoreactive Plaques

25,000 pfu were plated out (see section 3.16.2) from the immunoreactive plaques identified in the tertiary screen. When the plaques appeared, 5ml SM and a few drops of chloroform were added to the plates. The plates were then sealed with parafilm and shaken gently overnight at 4°C. The surface liquid was then removed and stored at 4°C.

3.17 Characterisation of λgt11 immunoreactive clones

3.17.1 Analysis of Fusion Proteins

Fusion proteins synthesized by immunoreactive λgt11 clones were prepared via C600 cells. 5ml C600 cells were grown overnight in the presence of 0.2% maltose and 10mM $MgCl_2$ at 37°C in an orbital incubator (200rpm). The volumes quoted from now on refer to the quantities required for one λgt11 clone. Inoculated 1ml LB (per litre: 10g bacto-tryptone, 5g yeast extract, 10g NaCl), containing 0.2% maltose and 10mM $MgCl_2$ with a 1:20 dilution of the overnight culture of C600 cells. The culture was grown at 37°C in an orbital incubator (200rpm) until A_{450} was 1.3-1.6. The cells were harvested from 1ml culture in a microfuge tube in a microfuge, top speed for 5min. The media was discarded and the bacteria resuspended in 300µl LB and 10mM $MgCl_2$. 100µl of the high titre phage stock (see section 3.16.7) was added to the resuspended bacteria and incubated at 37°C for 15min, prior to transfer to an orbital incubator (200rpm) at 37°C, overnight. The cells

were then harvested in a microfuge, top speed for 5min at room temperature. The cells were resuspended in 100µl boiling mix (10% w/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0.005% bromophenol blue). This mixture was boiled for 2min prior to loading onto a gel. The fusion proteins were then analysed by SDS-PAGE and Western blotting. SDS-PAGE was carried out in duplicate on 7% SDS polyacrylamide gels for Coomassie staining and Western blotting: 5µl/lane of fusion protein samples were loaded onto the gels for Coomassie staining and 1µl/lane for Western blotting.

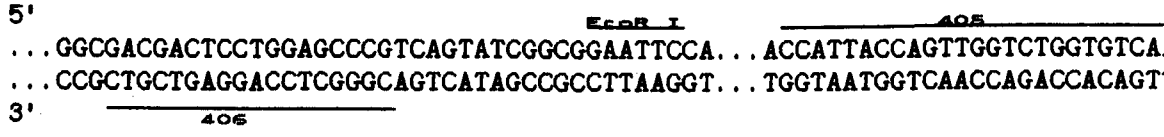
3.17.2 Isolation of λgt11 DNA from Immunoreactive Clones via C600 cells

DNA from the immunoreactive λgt11 clones was prepared via C600 cells. 100µl of an overnight culture of C600 cells was incubated with 300µl of high titre phage stock at 37°C for 20min. The infected C600 cells were then transferred to 50ml LB in a 250ml conical flask and grown overnight at 42°C in an orbital incubator (200rpm). 500µl chloroform was then added to the culture and incubated for a further 5min in the orbital incubator, with shaking. Cell debris was removed by centrifugation at 20,000g for 10min at 4°C. Phage DNA was collected from the supernatant by centrifugation at 100,000g for 1 hour at 4°C. The pellet of phage DNA was resuspended in 250µl SM and RNase treated (50µg/ml, 37°C for 30min). Following RNase treatment, 15µl 0.5M EDTA and 30µl 5M NaCl were added prior to a phenol-chloroform extraction step. The upper phase of this extraction was subjected to a chloroform extraction and then the DNA was precipitated at -80°C with two volumes of ethanol. The precipitated DNA was collected by centrifugation and then resuspended in 100µl SDW. The DNA was precipitated once again by the addition of 100µl 13% PEG/1M NaCl followed by a 30min

incubation on ice. The DNA precipitate was collected by centrifugation and the pellet was washed in 70% ethanol, dried and finally resuspended in 50µl TE. 5µl of this recombinant DNA was required for restriction analysis.

3.17.3 PCR to characterise immunoreactive clones

PCR was performed using DNA isolated from immunoreactive λgt11 clones (section 3.5.2). The purified DNA served as a template for PCR and the reactions were primed with the primers 405 (λgt11 primer,reverse,24mer) and 406 (λgt11 primer, forward, 15mer). These primers were complementary to the β-galactosidase portion of the λgt11 template., see figure 16.



406 Lambda gt11 Primer (forward) 5'd(GACTCCTGGAGCCCG)3'

405 Lambda gt11 Primer (reverse) 5'd(TTGACACCAGACCAACTGGTAATG)3'

Figure 16: Lambda gt11 Primers. These primers are complementary to the β-galactosidase portion of the gt11 template.

Reactions were carried out in 100µl:

60µl template (1ng)

10µl 8mM NTPs

10µl 10µM 405

10µl 10µM 406

10µl 10x Taq polymerase buffer

10µl Taq polymerase

After 30 cycles of denaturation (94°C, 1min), annealing (50°C, 1min) and polymerisation (72°C, 2min), the reaction products were separated by electrophoresis on 1% agarose gels and visualised by ethidium bromide staining. PCRs were set up for each of the immunoreactive λgt11 clones, wild type λgt11 and I_1. I_1 was λgt11 clone known to contain a 1.1Kb fragment, and was a gift from Stewart Gillespie, University of Stirling. Three additional reactions were set up for I_1: containing no 405, containing no 406 and without 405 and 406.

3.17.4 Subcloning and Sequencing

To facilitate sequence analysis of the inserts in the immunoreactive λgt11 clones, KpnI-SacI fragments were subcloned into pIBI30. The KpnI-SacI fragments carrying cDNA inserts were isolated by agarose gel electrophoresis (0.8% agarose) and ligated to KpnI-SacI treated, dephosphosphorylated pIBI30.

Preparation of dephosphorylated KpnI-SacI digested pIBI30

10μg pIBI30 was digested with KpnI and SacI, using Onephorall as the digestion buffer. The final volume was made up to 30μl and the restriction reaction was incubated at 37°C for 2 hours. To this 30μl, 10μl BRL buffer 3, 1μl alkaline phosphatase and 59μl SDW were added to make up a final volume of 100μl, and incubated at 50°C for 2 hours. The volume was then increased to 400μl by addition of SDW. The digested, dephosphorylated pIBI30 was then subjected to: phenol/chloroform extraction, chloroform extraction, ethanol precipitation and finally resuspended in 20μl TE. Only 1μl (200ng) of this prepared vector was required per ligation reaction.

Ligation and Transformation

90μl 10mM Tris-HCl, pH7.4 was added to the LMP-agarose containing the KpnI-SacI fragment, which carries the cDNA insert. To this, 200ng KpnI-SacI treated, dephosphorylated pIBI30 was added and the entire ligation mixture was incubated at 70°C for 15min. Following this, the ligation reaction was initiated by the addition of 2units BRL T_4 DNA ligase, 15μl 10mM ATP and 30μl 5 x T_4 ligase buffer. Ligation was carried out at room temperature over 2 hours.

300μl competent NM522 cells (prepared using the calcium chloride method outlined in Maniantis, 1982) were added to the entire ligation mix and stored on ice for 30min. The transformation mix was subjected to heat shock (42°C for 2min) and then 5ml LB added. The transformed cells were incubated at 37°C for 1 hour, harvested by centrifugation (2,000rpm, 10min,

4°C) and resuspended in 60µl LB. The resuspended cells were divided into a 10µl and 50µl aliquot, which were spread onto X-gal plates (LB agar containing 50µg/ml X-gal, 50µg/ml IPTG and 100µg/ml ampicillin). The plates were incubated overnight at 37°C. Recombinant and non-recombinant transformants were distinguished by the presence of the chromogenic substrate X-gal. Transformants containing non-recombinant pIBI30 were visualised as blue colonies whereas transformants containing recombinant DNA produce white colonies. The blue colour arises from the fact that the NM522 cells encode the N-terminal region of β-galactosidase which complements the C-terminal portion encoded by pIBI30 to produce an active β-galactosidase which hydrolyses X-gal to produce the blue dye. Recombinant transformants contain a disrupted C-terminal region of the β-galactosidase and so they fail to produce a functional β-galactosidase.

Analysis of Recombinants by Plasmid Isolation and Restriction

Individual colonies were selected from transformation plates and used to innoculate 5ml LB containing 100µg/ml ampicillin. The cells were grown overnight at 37°C and subjected to the plasmid miniprep method outlined in Maniantis. Aliquots of the plasmid preps were digested with a range of restriction enzymes and the resultant restriction fragments were analysed by agarose gel electrophoresis.

Production of Single Stranded DNA for Sequencing

Single stranded DNA was produced from the recombinant pIBI30 when superinfected with M13K07 helper phage. A starter culture of 1ml TY,

containing 100μg/ml ampicillin, was innoculated with NM522 infected with the recombinant pIBI30 subclones and grown at 37°C for 6 hours in an orbital incubator (200rpm). 20μl of this starter culture was used to innoculate 1.5ml TY containing 100μg/ml ampicillin, 75μg/ml kanamycin and 2μl M13K07 phage supernatant. This culture was grown overnight at 37°C in an orbital incubator (200rpm). Single stranded phagemid DNA was purified from the supernatant collected following centrifugation in a microcentrifuge, top speed, 5min. The supernatant was transferred to a fresh microfuge tube to which 200μl 20% PEG/2.5M NaCl was added. This was vortexed and incubated at room temperature for 15min prior to centrifugation (microfuge, top speed, 5min). The supernatant was discarded and all traces of PEG were removed using a drawn out pasteur pipette. The DNA pellet was resuspended in 100μl TE, phenol extracted, ethanol preciptated and finally, resuspended in 10μl TE. This provided enough material for one sequencing reaction (1pmol ssDNA). The 'Sanger' or 'dideoxy' method of DNA sequencing was used, as supplied in kit form (Sequenase™ DNA sequencing kit). Sequencing gels were run on BRL80 sequencing system then fixed, dried down on 3MM paper and autoradiographed overnight at room temperature, using Hyperfilm MP.

3.17.5 Analysis of DNA Sequence Information

All reading frames of the DNA sequence obtained from the subclone P4pIBI30 were considered. The reverse sequence was also checked in all three frames, see figure 17. An analysis of this information identified an open reading frame of 68 amino acids, see figure 18.

```
   I  F  N  I  K  Y  T  V  N  A  L  F  S  K  I  A  *  L  T  H
  N  F  Q  Y  *  I  Y  G  E  C  S  L  F  *  D  R  L  I  D  S
 E  F  S  I  L  N  I  R  *  M  L  S  F  L  R  S  L  N  *  L
5'GAATTTTCAATATTAAATATACGGTGAATGCTCTCTTTTCTAAGATCGCTTAATTGACTC
          10        20        30        40        50        60
3'CTTAAAAGTTATAATTTATATGCCACTTACGAGAGAAAAGATTCTAGCGAATTAACTGAG
  F  K  *  Y  *  I  Y  P  S  H  E  R  K  *  S  R  K  I  S  E
   I  K  L  I  L  Y  V  T  F  A  R  K  E  L  I  A  *  N  V  *
    N  E  I  N  F  I  R  H  I  S  E  K  R  L  D  S  L  Q  S  M

   F  F  *  P  F  S  K  W  M  K  S  L  E  S  I  L  I  *  F  Q
  S  F  L  T  F  Q  Q  V  D  E  V  A  R  I  H  L  D  L  I  P
 I  L  S  N  L  S  A  S  G  *  S  R  S  N  P  S  *  F  D  S
ATCCTTTCTAACCTTTCAGCAAGTGGATGAAGTCGCTCGAATCCATCTTGATTTGATTCC
        70        80        90       100       110       120
TAGGAAAGATTGGAAAGTCGTTCACCTACTTCAGCGAGCTTAGGTAGAACTAAACTAAGG
D  K  R  V  K  *  C  T  S  S  T  A  R  I  W  R  S  K  I  G
 G  K  *  G  K  L  L  H  I  F  D  S  S  D  M  K  I  Q  N  W
  R  E  L  R  E  A  L  P  H  L  R  E  F  G  D  Q  N  S  E  L

  R  S  I  Y  K  I  F  S  F  F  P  E
 K  I  Y  L  *  N  L  *  F  L  P  G
K  D  L  F  I  K  S  L  V  S  S  R
AAAGATCTATTTATAAAATCTTTAGTTTCTTCCCGGAA 3'
      130       140       150
TTTCTAGATAAATATTTTAGAAATCAAAGAAGGGCCTT 5'
F  I  *  K  Y  F  R  *  N  R  G  P
 L  D  I  *  L  I  K  L  K  K  G  S
  S  R  N  I  F  D  K  T  E  E  R  F
```

Figure 17: All possible reading frames of the sequence obtained from P4pIBI30. * indicates stop codon.

```
  1   F   R   E   E   T   K   D   F   I   N   R   S   L   E   S   N
  1 TTC CGC GAA GAA ACT AAA GAT TTT ATA AAT AGA TCT TTG GAA TCA AAT

 17   Q   D   G   F   E   R   L   H   P   L   A   E   R   L   E   K
 49 CAA GAT GGA TTC GAG CGA CTT CAT CCA CTT GCT GAA AGG TTA GAA AGG

 33   M   S   Q   L   S   D   L   R   K   E   S   I   H   R   I   F
 97 ATG AGT CAA TTA AGC GAT CTT AGA AAA GAG AGC ATT CAC CGT ATA TTT   1

 49   N   I   E   N
145 AAT ATT GAA AAT TC                                             1
```

Figure 18: Open reading frame of P4pIBI30 identified from figure 17.

A search of the Darsbury protein sequence database, using the programme Hits;1, was used to reveal which primary structures the sequence of P4pIBI30 resembled.

Secondary structure predictions using a variety of methods e.g Burgess & Sheraga, Chou & Fasman and Nagano were carried out by Lindsay Sawyer, Department of Biochemistry, University of Edinburgh. For each sequence, a number of secondary structure predictions were made (for each method) and then a consensus secondary structure was determined. The data obtained for P4pIBI30 was compared to the predicted secondary structure of *S.cerviaiae* PGAM, human M-type PGAM, human B-type PGAM, human BPGAM, mouse BPGAM, rabbit BPGAM and rat F-2,6-BPase.

3.18 Attempted Isolation of Gene Encoding PGAM from *S.pombe* Using PCR

PCR was performed using an *S.pombe* cDNA library as a template and degenerate synthetic oligonucleotides as primers, in an attempt to isolate the gene encoding PGAM from *S.pombe*.

3.18.1 Templates

PCR was performed using either the *S.pombe* cDNA library in λgt11 provided by V.Simanis, see section 3.16.1, or the *S.pombe* cDNA library in the 2μm URA3 plasmid, provided by J.D.Fikes (Dept. of Biology, Massachusetts Institute of Technology, Cambridge, Massachussetts), see Fikes *et al.*, 1990, for details of this library.

3.18.2 Primers

One of the primers used in PCR was an oligonucleotide designed from the partial amino acid sequence of *S.pombe* PGAM, see figure 13. The other primer was a poly-T oligonucleotide which should anneal to the poly-A stretch of cDNA inserts.

3.18.3 Reaction Conditions

Reactions containing 10ng of each library were primed with degenerate synthetic oligonucleotides in a total volume of 100µl:

0.5µl Taq polymerase
10µl 10 x Taq buffer
10µl 10µM primer 1 (either oligo 82-90 or 107-114, see fig. 13)
10µl 10µM primer 2 (oligo dT)
10µl 8mM NTP
10ng *S.pombe* cDNA library (either in λgt11 or 2µm URA3 plasmid)
59µl SDW

After 30 cycles of denaturation (94°C, 1min), annealing (50°C, 1min) and extension (72°C, 2min), the reaction products were extracted following chloroform treatment. The products were then separated by electrophoresis on a 1% agarose gel and visualised by ethidium bromide staining.

Three additional PCR mixtures were set up for each reaction: containing either no primer 1, no primer 2 and without primer 1 and primer 2.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Analysis of *S.pombe* DNA by Southern Hybridisation

4.1.1 Isolation of good yields of high molecular weight *S.pombe* DNA

Initial attempts to clone the PGAM gene from the fission yeast *S.pombe* were made using the *S.cerevisiae* gene as a molecular probe. Prior to screening the *S.pombe* expression library, it was necessary to determine the hybridisation conditions under which the *S.cerevisiae* gene would specifically bind to the *S.pombe* gene. To do this, digests of *S.pombe* genomic DNA were prepared for Southern analysis. Isolation of *S.pombe* genomic DNA was hampered by poor protoplast formation. Both lyticase (Sigma) and Protoplast Forming Enzyme (Boehringer Mannheim) failed to yield efficient prptoplast formation which led to poor yields of high molecular weight *S.pombe* DNA. Lyticase and Protoplast Forming Enzyme have proved useful for the formation of *S.cerevisiae* spheroplasts and so there would appear to be a difference in cell wall sensitivity to these enzymes between the two yeasts. This difference could be assigned to structural differences in the cell wall. The cell wall architecture of *S.pombe* has been studied by immunocytochemical techniques (Horisberger and Rouvet-Vauthey, 1984). It was found that the cell wall contained β-glucan and α-galactomannan. High levels of glucanase, mannanase and proteinase were found to exist in Novozym 234, Hamlyn *et al.*, 1981, and so it was tested for its ability to form *S.pombe* protoplasts. Absence of cell walls and osmotic sensitivity revealed that around 80% of the total protoplast formation was obtained within 30min.

4.1.2 *S.cerevisiae* gene as a molecular probe

Having maximised *S.pombe* protoplast formation, genomic DNA was successfully prepared (200µg DNA from 200ml liquid culture of *S.pombe*). 30µg genomic DNA was required per restriction reaction for Southern analysis. Once enough genomic DNA was prepared, a number of restriction reactions, with a range of endonucleases, were carried out. The restriction fragments were separated by electrophoresis on a 0.8% agarose gel, containing ethidium bromide. The restricted DNA was transferred onto Hybond-N by Southern Blotting and the probed with the end-labelled *S.cerevisiae* PGAM gene. Non-stringent washing conditions (6xSSC, room temperature) gave non-specific hybridisation, with a general smear of activity on the resticted *S.pombe* DNA and on the λHindIII markers (figure 19a). This pattern was observed until the stringency was reduced to 0.5xSSC, at room temperature, whereupon non-specific binding to *S.pombe* DNA was reduced to produce a single faint band in the lane corresponding to the BglI digest (figure 19b). This band measured 3Kb in length, however, the washing conditions were still rather relaxed as non-specific binding to λHindIII remained. Maintaining 0.5xSSC but increasing the temperature to 30°C, resulted in removal of any activity from the filter with the exception of hybridisation to the positive control. Thus, from the results it would appear that under the conditions described, the *S.cervisiae* PGAM gene is of no use as a molecular probe for the PGAM gene from *S.pombe*. This is consistent with a more extensive study carried out by Seehaus *et al.*, 1985, where a range of *S.cerevisiae* glycolytic genes, including PGAM, were used to probe chromosomal DNA from different yeast species, including *S.pombe*. From this study, it was

Figure 19: Attempted localisation of the *S.pombe* phosphoglycerate mutase gene using the *S.cerevisiae* phosphoglycerate mutase gene as a heterologous probe. The Southern blot of restricted *S.pombe* chromosomal DNA was probed with the phosphoglycerate mutase coding sequence from *S.cerevisiae* (excised from YEp13.GPM, as outlined in 3.1.4). Digest. transfer and hybridisation conditions as in text.

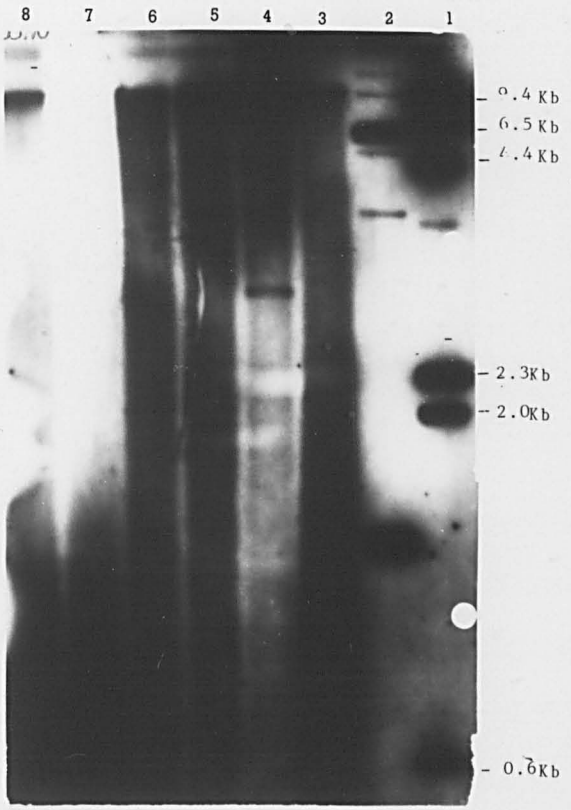
(A) 6 x SSC, room temperature wash

(B) 0.5 x SSC, room temperature wash

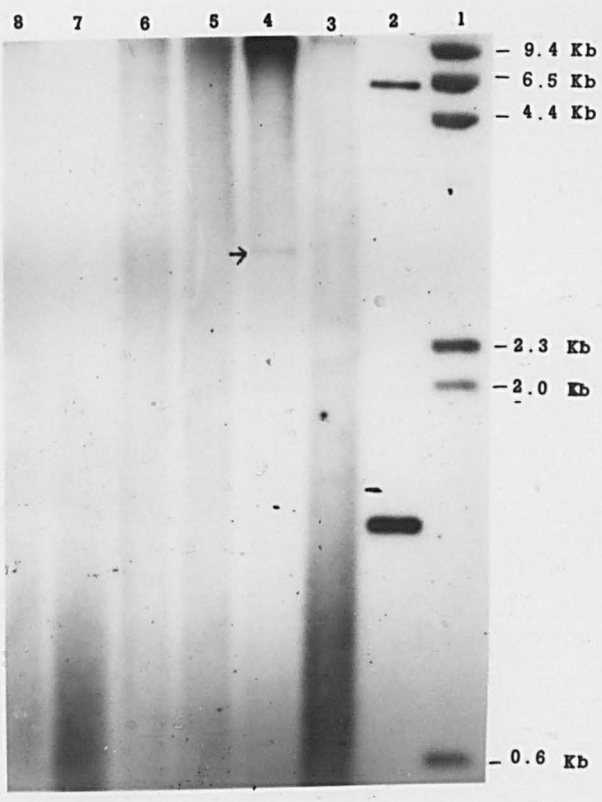
Lane	Sample
1	λ HindIII molecular weight markers
2	pvtU GLY 245* cut with BamH1 and HindIII
3	*S.pombe* chromosomal DNA digested with BamHI
4	*S.pombe* chromosomal DNA digested with BglI
5	*S.pombe* chromosomal DNA digested with EcoRI
6	*S.pombe* chromosomal DNA digested with SalI
7	*S.pombe* chromosomal DNA digested with Sau3A
8	Undigested *S.pombe* chromosomal DNA

* pvtU GLY 245 is a plasmid carrying the *S.cerevisiae* phosphoglycerate mutase gene (a gift from Malcolm White).

A.


8
7
6
5
4
3
2
1
9.4 Kb
6.5 Kb
4.4 Kb
2.3Kb
2.0Kb
0.6Kb

B.


8
7
6
5
4
3
2
1
9.4 Kb
6.5 Kb
4.4 Kb
2.3 Kb
2.0 Kb
0.6 Kb

concluded that only the PFK2 (the gene encoding the β-subunit of PFK) and the PK genes from *S.cerevisiae* gave poor hybridisation intensities when probing *S.pombe* DNA. PGI, PFK1 (encoding the α-subunit of PFK), PGK, PDC and PGAM genes failed to hybridise to *S.pombe* DNA under the stringent washing conditions imposed (0.4xSCP, 50°C), which were required for the yeast taxonomy studies. Despite these results, it was hoped that less stringent conditions might allow the *S.cerevisiae* PGAM gene to be used as a probe for the purpose of isolating the gene encoding PGAM from *S.pombe*. However, the Southern analysis results forced the development of alternative molecular probes.

4.1.3 Degenerate oligonucleotide as a molecular probe

A degenerate oligonucleotide was designed from the partial protein sequence of *S.pombe* PGAM, on the basis of minimal degeneracy and codon bias. Biased codon usage is a widespread phenomenon with patterns varying among species (Sharp *et al.*, 1988). Also, preferred codons seem to vary with the level of gene expression within species. Codon selection has been studied in both *S.pombe* and *S.cerevisiae* where it has been found that codon usage in these yeasts are very similar (Sharp and Wright, unpublished). With respect to codon usage, *S.pombe* and *S.cerevisiae* each contain two groups of genes: one group contains highly expressed genes, the other contains low to moderately expressed genes. Highly expressed genes in *S.pombe* and *S.cerevisiae* use a small subset of the genetic code whereas genes which are not highly expressed tend to use codons at more nearly equal frequencies, see appendix III.

Analysis of codon usage in highly expressed *S.pombe* genes e.g. histone 2A, histone 2B, alcohol dehydrogenase, actin and α-tubulin, was carried out using the 'codon frequency' programme, which is one of the range of programmes offered on the University of Wisconsin Genetics Computer Group (UWGCG). The results obtained were in agreement with those presented in appendix III and were used to design an oligonucleotide, measuring 23 nucleotides, which encodes the *S.pombe* PGAM sequence DDARKKWG. This stretch of sequence was particular to PGAM from *S.pombe*, showing no similarilty to the correspnding region in *S.cerevisiae* PGAM, see figure 13. The probe had 5 degeneracies.

	D	D	A	R	K	K	W	G	
5'	GA(T/C)	GA(T/C)	GC(T/C)	CGT	AA(G/A)	AA(G/A)	TGG	GG	3'

Digests of total DNA from *S.pombe* were transferred onto Hybond-N. The labelled oligonucleotide was hybridised under 6xSSC at room temperature. Minimal stringency was used during initial washes (6xSSC, room temperature), however, it was clear at this early stage that no signals were present. Possible problems including quantity and quality of *S.pombe* DNA, inefficient transfer onto Hybond-N and poor labelling of the oligonucleotide were considered. The amount of high molecular weight *S.pombe* genomic DNA per digestion was increased to 50μg and the labelling mixture was applied to G-50 gel filtration to separate the labelled DNA from the non-incorporated (γ-^{32}P)ATP. Despite these changes the oligonucleotide failed to be of any use as a molecular probe for any *S.pombe* DNA sequence.

These disappointing results meant that a new approach had to be adopted. Of the options available, it seemed obvious to purify *S.pombe* PGAM for two reasons: (1) it would provide material for further amino acid sequencing (to check the information gathered by Fothergill and Dunbar, unpublished and to fill in the 'gaps') and (2) for the preparation of a polyclonal antibody against *S.pombe* PGAM which could be used to screen the *S.pombe* expression library.

4.2 Purification of PGAM from *S.pombe*

PGAM was routinely purified from *S.pombe* cells which were grown to late log phase. The purification strategy used was a slight variation to the method outlined by Price *et al.*, 1985. The cells were lysed using a motorised homogeniser and sand to maximize cell lysis and minimize the time period over which the cells were lysed. The lysate was then centrifuged at 100,000g for 1 hour to remove cell debris prior to ammonium sulphate fractionation. The fraction which precipitated at 50%-70% saturation contained the PGAM activity. The precipitate was dissolved in 2ml 10mM Tris-HCl, pH 8.0, dialysed overnight and applied to a Cibacron-Blue Sepharose column. Following a pulse of one column volume of 1mM NADH in 10mM Tris-HCl, pH 8.0, PGAM was eluted with a pulse of 4mM BPG in 10mM Tris-HCl, pH 8.0. The purification of *S.pombe* PGAM is summarised in table 2.

Table 2 : Purification scheme for phosphoglycerate mutase from *S. pombe* (from 10g wet weight cells)

STEP	TOTAL PROTEIN(mg)	TOTAL ACTIVITY(units)	SPECIFIC ACTIVITY(U/mg)	YIELD (%)
Crude Extract	40	513	13	100
50-70% ppt.	12	437	36	85
Cibacron-Blue	0.092	102	1108	20

The simplicity of this purification scheme comes at the price of a rather low yield: a small percentage of PGAM activity fails to bind to the Cibacron-Blue, around 100µg was specifically eluted by 4mM BPG and the remaining activity stays bound to the column. Raising the BPG concentration removes some of this tightly bound PGAM and increasing NaCl concentrations removes the rest, however, both methods result in the elution of numerous other proteins, in particular phosphoglycerate kinase as determined by activity and SDS-PAGE. Enzyme preparations were 80-95% homogeneous, as judged by SDS-PAGE (fig. 20) and HPLC (fig 21). A 85-fold purification resulted in a specific activity of 1108 units/mg.

4.3 HPLC, Amino Acid Analysis and Attempted Sequencing of *S. pombe* PGAM

HPLC was used as a final purification step to obtain homogeneous *S. pombe* PGAM. Attempts to determine the amino-terminal amino acid sequence of the

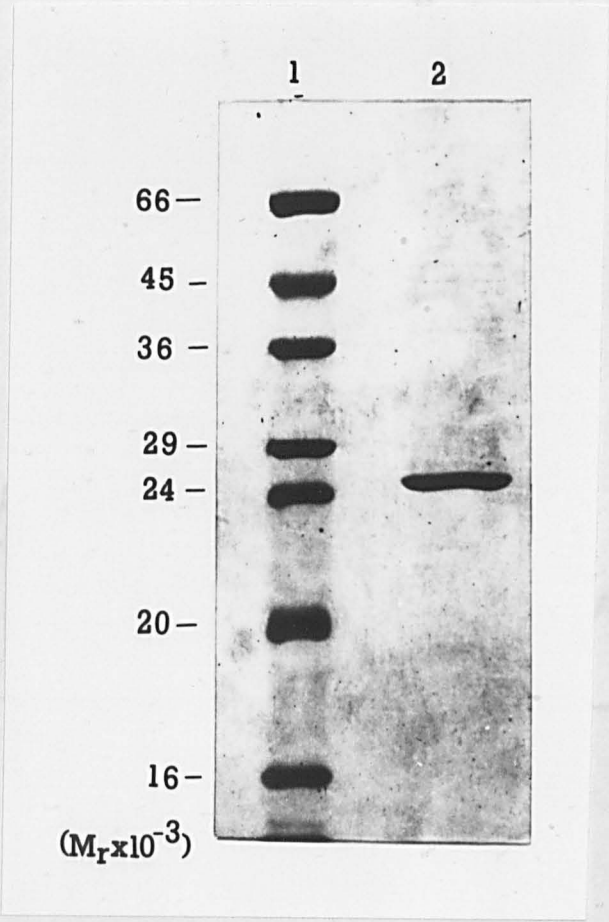


Figure 20: SDS-PAGE of phosphoglycerate mutase isolated from *S. pombe* by the one step purification method outlined in section 4.2. Coomassie staining was used to visualise the protein bands.

Lane	Sample
1	Molecular weight markers
2	*S. pombe* phosphoglycerate mutase

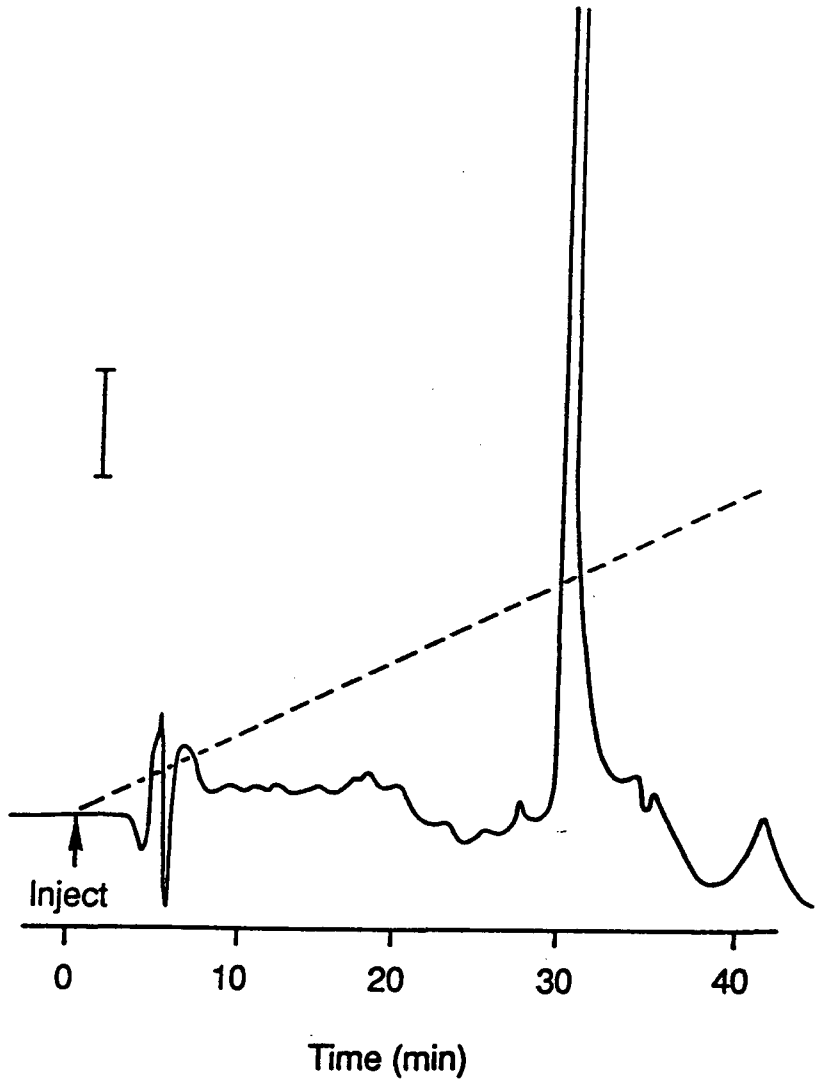


Figure 21: HPLC analysis of purified *S. pombe* phosphoglycerate mutase. 0.5 nmoles of purified protein were applied to the following system.

Column: C_{18} μBondapak column (Waters)

Gradient: 30%→90% acetonitrile, containing 0.1% TFA, over a period of 40 minutes.

The vertical bar represents an A_{220} of 0.01.

enzyme failed to yield any results, which is indicative of an N-terminal blocked sequence, and so the enzyme must be cleaved to give any sequence information. The amino acid composition was determined to help decide which method of cleavage should be adopted. *S. pombe* PGAM was subjected to a 22 hour and 44 hour acid hydrolyisis. The amino acid composition of the *S. pombe* enzyme is given in table 3. From this information, it was concluded that the presence of one methionine residue could permit the simple cleavage of the enzyme by cyanogen bromide, using the vapour phase method. This method requires the presence of one methionine residue and a blocked N-terminal sequence such that when the enzyme is cleaved into two fragments, only one is sequenced i.e. there is no need to separate fragments prior to sequencing. Indeed, only one sequence was produced when the CNBr treated *S. pombe* PGAM was applied to the Applied Biosystems 447a Protein Microsequencer, which was limited to 10 cycles. The sequence was obtained from 300pmol enzyme, with an initial yield of 10% and step yield of 88%. The low initial yield implied either an overestimation of the amount of protein cleaved or inefficient cleavage of the enzyme. The sequence obtained was SLEGLTGLQI, which is very similar to a portion of sequence on the C-terminal side of a methionine residue located on one of the *S. pombe* PGAM peptides studied by Fothergill and Dunbar, unpublished, see figure 12. To improve the initial yield, preliminary attempts were made to digest *S. pombe* PGAM with CNBr in the liquid phase. HPLC analysis of the digested enzyme revealed that non-specific acid hydrolysis of the enzyme had taken place resulting in the production of numerous, inseparable fragments. These results were rather discouraging; much time and effort would have to be expended on devising a successful sequencing strategy and so it was decided

to investigate the possiblitiy of an alternative probe which would not require *S.pombe* sequence information.

Table 3: Amino acid composition of HPLC purified *S.pombe* phosphoglycerate mutase*. *S.pombe* phosphoglycerate mutase was analysed as outlined in section 3.12.

AMINO ACID	NUMBER OF RESIDUES	MOLE (%)
Ala	22.8	11.1
Asx	21.8	10.6
Cys	0.8	0.4
Glx	20.3	9.9
Phe	4.9	2.4
Gly	14.6	7.1
His	6.4	3.1
Ile	7.3	3.5
Lys	14.0	6.8
Leu	25.2	12.2
Met	1.5	0.7
Pro	14.6	7.1
Arg	12.9	6.3
Ser	6.5	3.2
Thr	9.8	4.8
Val	10.6	5.2
Trp	4.8	2.3
Tyr	6.5	3.2

* Molecular weight of *S.pombe* PGAM = 23,000
Average residue molecular weight = 111.8
Total number of residues = 205

4.4 Preparation and Characteristion of Polyclonal Antibody

A polyclonal antibody to *S.pombe* PGAM was raised in rabbits as outlined in section 3.12. The behaviour of the antibody was observed on Western blots of *S.pombe* crude extracts and purified *S.pombe* phosphoglycerate mutase. PGAM cross-reactivity was observed with the serum collected from the initial test-bleed and subsequent test bleeds, following each booster injection. After the third booster, an apparently highly specific antibody was obtained. The signal-to-noise ratio was determined by incubating Western blots of *S.pombe* crude extracts with serial dilutions of the anti-PGAM serum. 1:500 to 1:1000 dilutions offered maximum detection of the *S.pombe* PGAM and minimal cross-reactivity with other *S.pombe* proteins, see figure 22. At this concentration, the antibody also failed to cross-react with a crude extract of Y1090, the host cells required for the λgt11 expression system. It also failed to cross react with Y1090 infected with wild type λgt11 (i.e. no insert). Thus the antibody failed to cross-react with any proteins in Y1090 and any proteins encoded by wild type λgt11. Taking all the facts together, the antibody meets all the criteria to permit its use to screen the λgt11 cDNA library for the *S.pombe* PGAM gene.

Whilst conducting the preliminary experiments to test the quality of the polyclonal antibody to *S.pombe* PGAM, cross-reactivity with *S.cerevisiae* crude extract, purified *S.cerevisiae* PGAM and rabbit muscle PGAM was analysed by Western blotting (figure 23). Western blot analysis demonstrated that the antiserum shows cross-reactivity with rabbit muscle PGAM but not with *S.cerevisiae* PGAM. This suggests that rabbit muscle PGAM

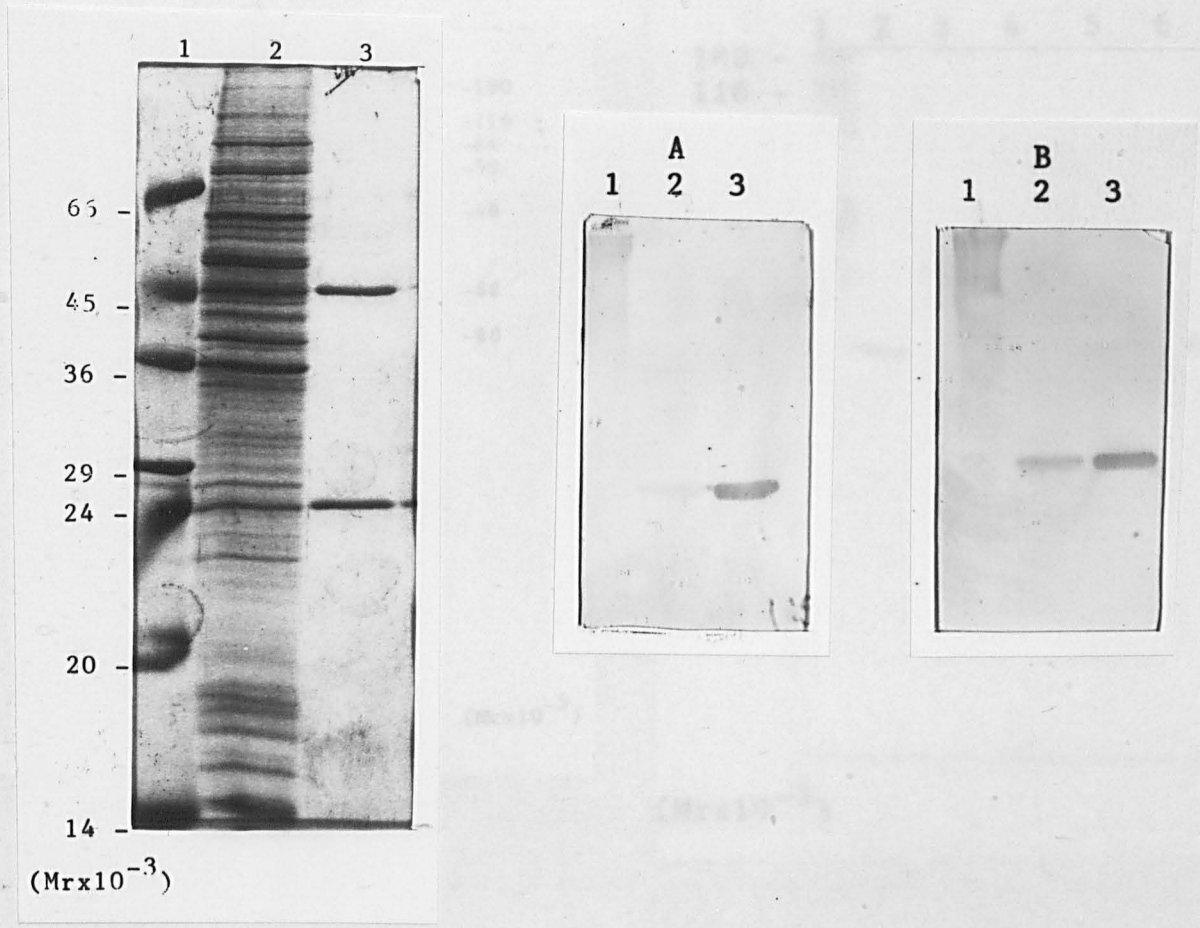


Figure 22: Western analysis of crude extracts and partially purified phosphoglycerate mutase from *S. pombe* using the polyclonal antibody raised against *S. pombe* phosphoglycerate mutase.

I - Coomassie stained SDS-PAGE

II - Western blot using (A) 1:500 serum dilution

(B) 1:1000 serum dilution

Lane	Sample
1	Molecular weight markers
2	*S. pombe* crude extract
3	Partially purified *S. pombe* phosphoglycerate mutase

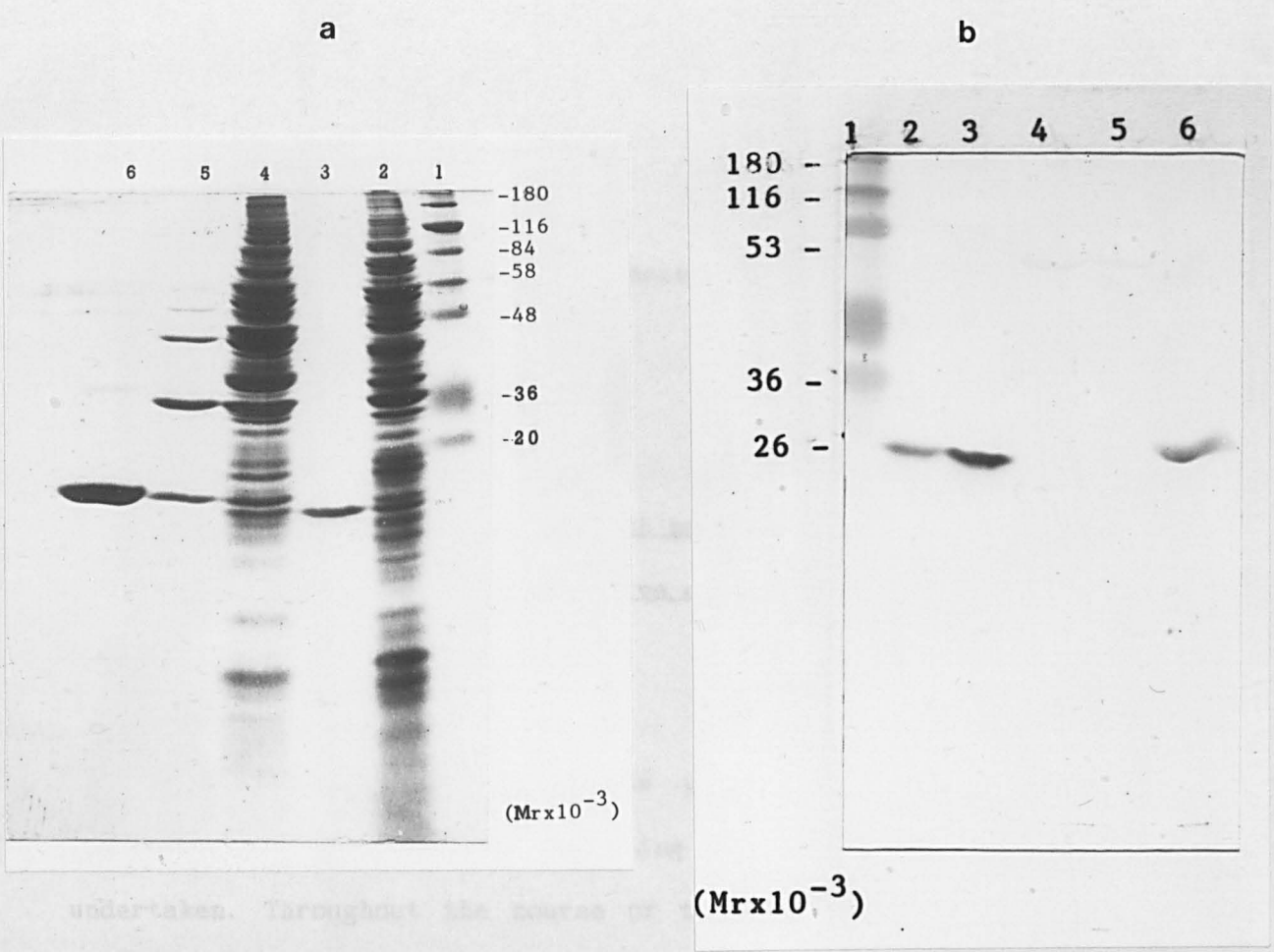


Figure 23: Western analysis of phosphoglycerate mutase from *S.pombe*, *S.cerevisiae* and rabbit muscle, using the polyclonal antibody raised against *S.pombe* phosphoglycerate mutase.

(a) Coomassie stained (12%) SDS-PAGE

(b) Western blot of (a)

Lane	Sample
1	Molecular weight marker
2	*S.pombe* crude extract
3	*S.pombe* phosphoglycerate mutase (Mr 23,000)
4	*S.cerevisiae* crude extract
5	*S.cerevisiae* phosphoglycerate mutase (subunit Mr 27,000)
6	Rabbit muscle phosphoglycerate mutase (subunit Mr 30,000)

subunits possess some antigenic determinants in common with those of *S. pombe* PGAM.

4.5 Screening expression library with polyclonal antibody and characterisation of immunoreactive clones

4.5.1 Screening

Having checked the quality of the polyclonal antibody raised against *S. pombe* PGAM, immunological screening of the *S. pombe* cDNA library was undertaken. Throughout the course of the screening procedure, the method for detecting bound antibody was use of a peroxidase coupled secondary antibody. Screening results in the generation of a series of nitrocellulose filters reproducing the pattern of plaques on the plate. Following immunostaining, the exact location of a single plaque producing a signal can be determined thus reducing the work involved in plaque purification. More importantly, this method reduces the chances of identifying false positive signals.

The *S. pombe* genome measures 14,000Kb, and so a copy of the PGAM gene should be located in a screen of 10^5 recombinants. A typical cell contains ≈1pg mRNA, equivalent to about 10^6 molecules, transcribed from about 15,000 different genes. Since the gene encoding PGAM is expected to be highly expressed, at ≈20+ mRNA copies per cell, ≈20+ PGAM mRNA copies should be located in 10^6 recombinants. From a primary screen of 6 x 10^5 recombinants, a number of putative PGAM clones were identified, eight of which were taken

through three rounds of screening with 1:500 dilution of polyclonal antibody.

Having identified eight putative PGAM clones, it was necessary to validate the identity of these clones using the following methods:

1. studying restriction digests of recombinant phage DNA from the immunoreactive clones
2. PCR, using immunoreactive clones as templates and universal primers
3. Western blot analysis of fusion proteins
4. DNA sequencing.

4.5.2 Isolation and restriction pattern of DNA via C600 cells

Phage DNA was prepared from each of the immunoreactive clones and from λgt11 wild type, containing no insert. As the library was constructed by inserting DNA at the unique EcoRI site of the λgt11, attempts were made to digest the recombinant and non-recombinant phage DNA with EcoRI. This restriction reaction failed to release the insert. The inability to excise cDNA inserts from λgt11 using EcoRI is a common problem, S. Gillespie and M. Leaver, personal communication. It seems that the λgt11 adopts a conformation which makes the EcoRI site inaccessible. The λgt11 restriction map, see figure 24, shows that a KpnI-SacI digestion should release the fragment of interest. From the KpnI-SacI restriction pattern, it appears that all the immunoreactive clones contain an insert measuring around 250 base pairs.

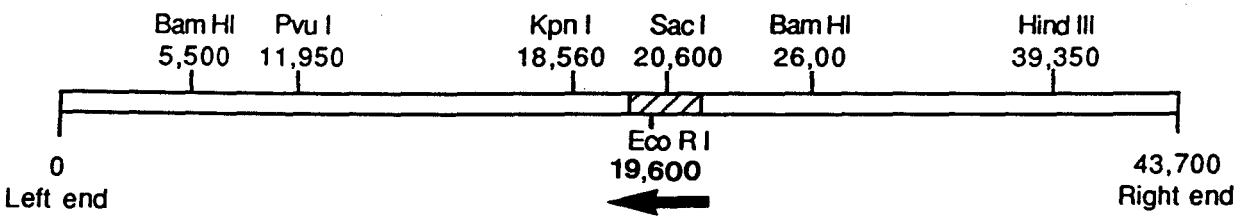


Figure 24: Restriction map of λgt11. Restriction endonuclease cleavage sites are numbered in base pairs from the left end. The transcriptional orientation of lacZ is indicated by the arrow. cDNA inserts are cloned into the unique EcoRI site located in lacZ.

4.5.3 PCR

A PCR experiment was conducted to check the presence of an insert in each of the immunoreactive clones and to estimate the size of the insert present in each of these clones. Recombinant DNA isolated from the immunoreactive clones served as a template and the oligonucleotides 405 and 406 were primers for PCR (see section 3.17.5). 405 and 406 are complementary to the DNA on either side of the unique EcoRI site in λgt11. At the end of 30 cycles, each PCR reaction yielded multiple copies of the insert present in each of the immunoreactive clones. The reaction products, analysed by agarose gel electrophoresis, implied that each of the immunoreactive clones contains an insert measuring 250 base pairs. This was in good agreement with the KpnI-MluI restriction pattern obtained with the recombinant DNA.

4.5.4 SDS-PAGE and Western Blot analysis of fusion proteins

The *E.coli* strain C600 was infected with high titre phage stocks prepared from immunoreactive plaques. Infected cells were grown overnight to allow fusion protein production. The cells were then lysed and prepared for SDS-PAGE by the addition of boiling mix. The cell lysates, containing fusion proteins, were analysed on duplicate 7% SDS-PAGE gels. One was stained with Coomassie Brilliant Blue, see figure 25 , and the other gel was subjected to Western blotting followed by immunostaining with the polyclonal antibody against *S.pombe* PGAM, see figure 26. C600 cells infected with non-recombinant DNA produced β-galactosidase with a subunit Mr of 120,000, which failed to cross-react with the polyclonal antibody. C600 cells infected with the recombinant phage, produced β-galactosidase fusion

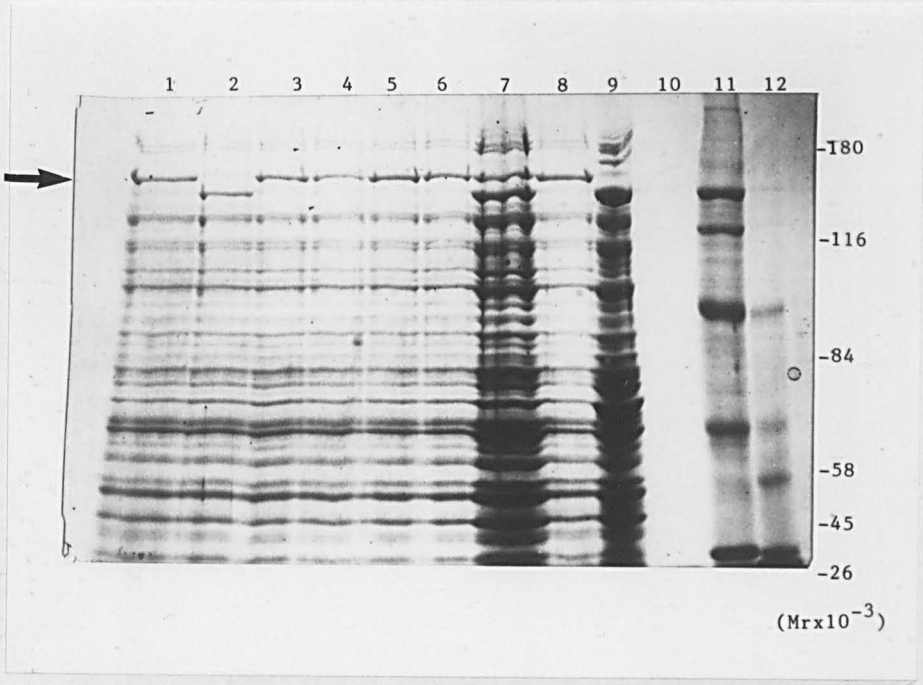


Figure 25: Coomassie stained 7% SDS-PAGE of fusion proteins from putative *S. pombe* phosphoglycerate mutase lambda clones. The arrow indicates the relative mobility of non-fused β-galactosidase (Mr 120kDa).

Lane	Sample
1-8	Crude extracts isolated from immunoreactive clones 1-8, showing fusion proteins at >120kDa
9	Cell lysate from non-recombinant clone, with non-fused β-galactosidase which has a relative mobility of 120kDa
10	Purified *S. pombe* phosphoglycerate mutase (Mr 23,000Da). This small protein has the same relative mobility as the dye front on 7% SDS-PAGE. Thus the protein is difficult to observe on the gel.
11	Prestained molecular weight markers
12	Low molecular weight markers

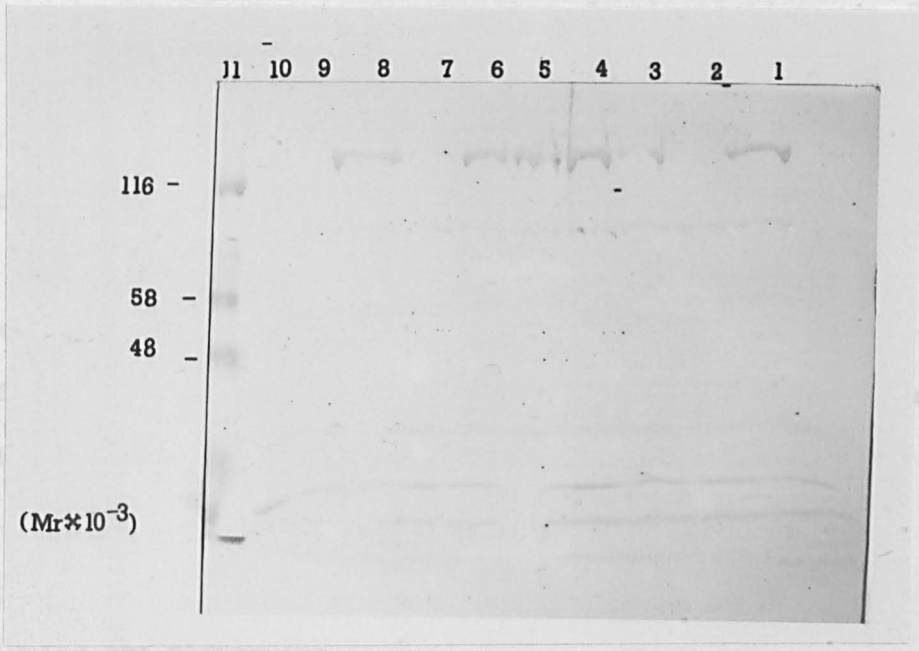


Figure 26: Western blot of fusion proteins following 7% SDS-PAGE (figure 25). Putative fusion proteins have a relative mobility of > 120kDa and *S. pombe* phosphoglycerate mutase was included as a positive control for the immunoscreen.

Lane	Sample
1-8	Crude extracts isolated from immunoreactive clones 1-8, with clones 1,3,4,5,6 and 8 showing immunoreactive fusion proteins at >120kDa
9	Cell lysate from non-recombinant clone, with non-fused β-galactosidase which has a relative mobility of 120kDa
10	Purified *S. pombe* phosphoglycerate mutase (Mr 23,000Da)
11	Prestained molecular weight markers

proteins, which run at a slower rate than β-galactosidase on SDS-PAGE. Figure 26 shows that the fusion proteins from all of the putative PGAM clones, with the exception of P2 and P7, cross-reacted with the *S. pombe* PGAM antibody.

Thus, it would appear that recombinant phage P1, P3, P4, P5, P6 and P8 encode a fusion protein which cross-reacts with the polyclonal antibody raised against *S. pombe* PGAM.

4.5.5 Subcloning and sequencing

The only definitive method available to validate the immunoreactive clones was to sequence them. As clones P1, P3, P4, P5, P6 and P8 contained a fragment of identical size and cross-reactivity with the polyclonal antibody, it was assumed that they all contained the same insert and so only one of these clones (P4) was selected for sequencing. To facilitate the sequencing of P4, the cDNA insert was subcloned into the phagemid pIBI30. pIBI30 is a phagemid measuring 2.9Kb which offers ampicillin resistance as a selectable marker and contains the f1 origin of replication allowing for the production and isolation of ssDNA when superinfected with M13K07 helper phage. Therefore, by subcloning into pIBI30 it was possible to achieve:

1. insert stability
2. a method of selecting transformants
3. DNA sequencing

As mentioned previously, the EcoRI site was not accessible in P4 and so the EcoRI site was not a candidate for subcloning the cDNA insert into pIBI30. The KpnI-MluI digest of P4 yielded a 1.6Kb fragment which was difficult to separate from the 1.7Kb fragment of λgt11 DNA which was generated in the same restriction reaction and so alternative subcloning sites were investigated. KpnI-SacI digestion of the P4 clone yielded a 2.3Kb fragment, containing the cDNA insert, which was possible to isolate by agarose gel electrophoresis. This fragment was excised from LMP agarose, ligated to dephosphorylated pIBI30 and then the entire ligation mix was used to successfully transform competent cells. Over 75% of the transformants contained recombinant DNA, as indicated by the presence of white colonies.

Restriction analysis of phagemid DNA from blue colonies and white colonies confirmed that the white colonies contain pIBI30 ligated to a KpnI-SacI fragment which measures 2.3Kb (0.2Kb cDNA insert + 2.1Kb λgt11). Thus, it would appear that the cDNA insert carried on a 2.1Kb fragment of λgt11 was successfully subcloned into the KpnI-SacI sites of pIBI30 (P4pIBI30), see figure 27.

With subcloning apparently achieved, sequencing commenced with the production of ssDNA.

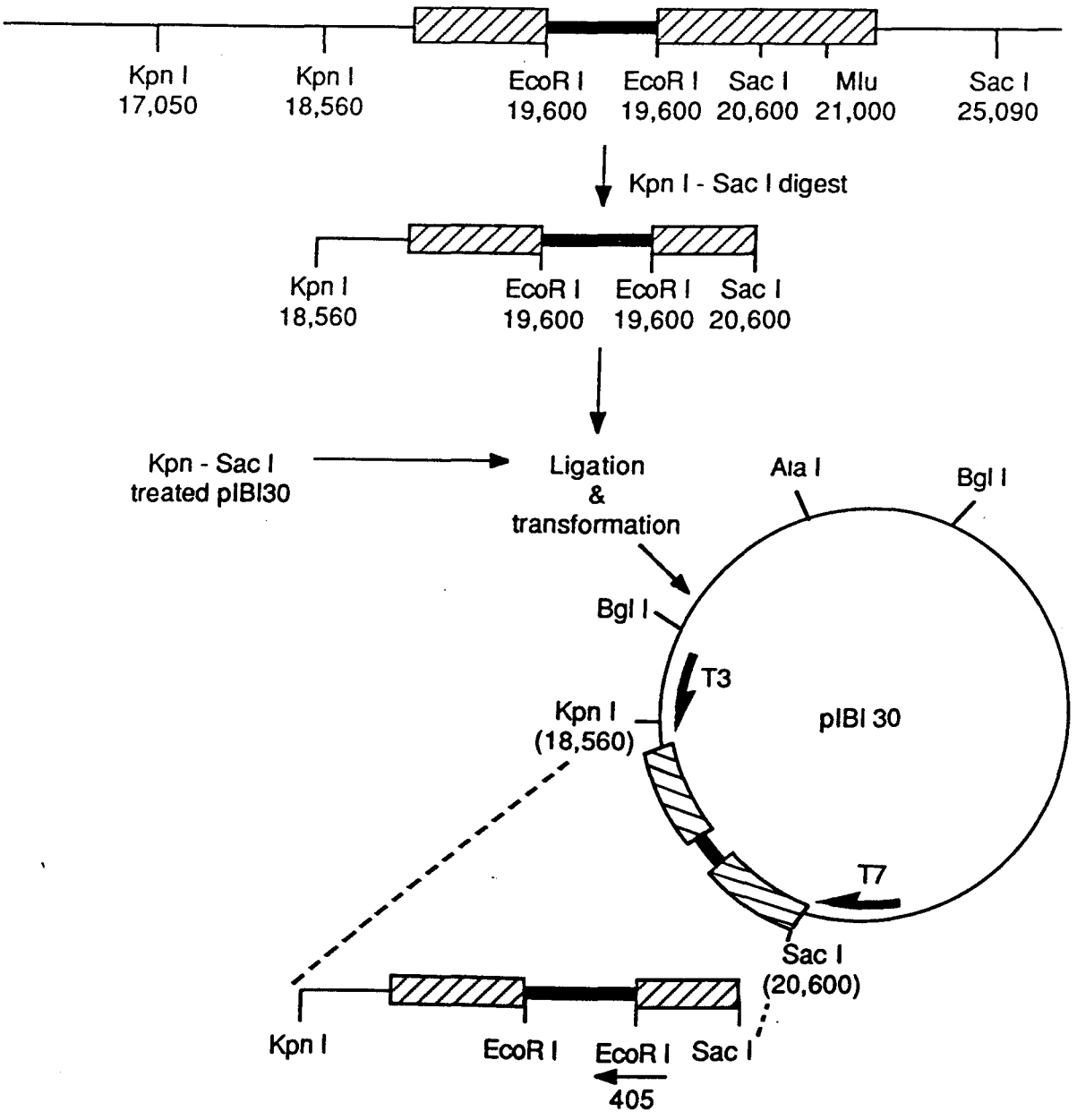


Figure 27: Subcloning and sequencing strategy. The boxed region represents the lacZ region of λgt11 and the thick black line indicates the coding region of the cDNA insert. Oligonucleotide 405, used as a sequencing primer, is also shown. Arrows indicate direction of sequence.

4.6 Sequence Analysis

The amino acid sequence of the insert of P4pIBI30, deduced from the DNA sequence, was compared with those of *S.cerevisiae* PGAM, human M-type PGAM, human B-type PGAM, human BPGAM, mouse BPGAM, rabbit BPGAM and the partial peptide sequence of *S.pombe* PGAM. Initial attempts to align the P4pIBI30 sequence with the mutase multiple alignment proved difficult with no obvious homologous or similar regions. A search of the Darsbury protein sequence database using the Hits;1 programme produced a list of 100 sequences which were, to some extent, similar to P4pIBI30: the best match to P4pIBI30 was human α-chain fodrin, with 29% identity. A number of other seemingly non-PGAM related sequences were listed. However, 6-PF-2-K/F-2,6-BPase from mouse, human and rat were also listed, with 24% identity over the 68 amino acids compared. As mentioned in section 1.8, the bisphosphatase domain of 6-PF-2-K/F-2,6-BPase was found to be homologous to the active site region of *S.cerevisiae* PGAM and so I have aligned the translated sequence of P4pIBI30 with the multiple alignment of mutase and phosphatase enzymes, see figure 28. A higher degree of identity (36%) was observed over residues 18-54 (P4pIBI30 numbering). This alignment does not require gaps to be inserted and it would appear that the residues that are highly conserved in the mutases and phosphatases (see figure 28) are shared by the primary structure of P4pIBI30. Most of the residues around those of identity show conservative changes.

Figure 28: Alignment of the open reading frame of P4pIBI30 with the primary sequences of phosphoglycerate mutases and the phosphatase domain of Sco=*S. coelicolor* PGAM; Sce=*S. cerevisiae* PGAM; Hre2=human BPGAM; Mre=mouse BPGAM; Rre=rabbit BPGAM; Hmu=human M-type BPGAM; Rmu=rabbit M-type PGAM; Pgms=*S. pombe* PGAM; consensus=PGAM consensus as outlined in figure 3; pf2k consensus=consensus of phosphatase domain of 6PF2K/Fru-2,6-BPases; P4pIBI30=open reading frame of PGAM immunoreactive clone, see figure 18. The sequences have been aligned using BEST FIT, Devereux *et al.*,1984.

```
                1                                                  50
        Sco  ADAPYKLILL RHGESEWNEK NLFTGWVDVN LTPKGEKEAT RGGELLKDAG
        Sce  ..MP.KLVLV RHGQSEWNEK NLFTGWVDVK LSAKGQQEAA RAGELLKEKK
       Hre2  .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
        Mre  .MSKHKLIIL RHGEGQWNKE NRFCSWVDQK LNNDGLEEAR NCGRQLKALN
        Rre  .MSKYKLIML RHGEGAWNKE NRFCSWVDQK LNSEGMEEAR NCGKQLKALN
        Hmu  .MATHRLVMV RHGETTWNQE NRFCGWFDAE LSEKGTEEAK RGAKAIKDAK
        Rmu  .MATHRLVMV RHGESSWNQE NRFCGWFDAE LSEKGAEEAK RGATAIKDAK
       Pgms  .AAPNLLVLT RHGESEWNKL NLFTGWKDPA LSETGIKEAK LGGERLKSRG
  consensus  AMAPHKLVML RHGESEWNKE NWFCGWVDQK LSEKGMEEAK RGGKQLKDMN
   pf2kcon   VTPRSIYLC RHGESELNLW GRIGG..DSG VSARGKQYAY ALANFIQSQG

                51                                                 100
        Sco  LLPDVVHTSV QKRAIRTAQL ALEAADRHWI PVHRHWRLNE RHYGALQGKD
        Sce  VYPDVLYTSK LSRAIQTANI ALEKADRLWI PVNRSWRLNE RHYGDLQGKD
       Hre2  FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
        Mre  FEFDLVFTSI LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
        Rre  FEFDLVFTSV LNRSIHTAWL ILEELGQEWV PVESSWRLNE RHYGALIGLN
        Hmu  MEFDICYTSV LKRAIRTLWA ILDGTDQMWL PVVRTWRFNE RHYGGLTGFN
        Rmu  IEFDICYTSV LKRAIRTLWT ILDVTDQMWV PVVRTWRLNE RAYGGLTGLN
       Pgms  YKFDIAFTSA LNRANRT... .......... .........RQ RYYGDLQGLN
  consensus  FEFDIVYTSV LNRAIRTARL ILEELDQEWV PVEWSWRLNE RHYGALIGLN
   pf2kcon   ISSLKVRTSH MKRTIQTAEA LG..     .V PYEQWKALNE IDAGV     .

                101                                                150
        Sco  KAQTLAEFGE EQFMLWRRSY DTPPPALDRD AEYSQF..SD PRYAM.LPP.
        Sce  KAETLKKFGE EKFNTYRRSF DVPPPPIDAS SPFSQK..GD ERYKY.VDP.
       Hre2  REQMALNHGE EQVRLWRRSY NVTPPPIEES HPYYQEIYND RRYKVCDVPL
        Mre  REKMALNHGE EQVRLWRRSY NVTPPPIEES HPYFHEIYSD RRYKVCDVPL
        Rre  REKMALNHGE EQVRIWRRSY NVTPPPIEES HPYYHEIYSD RRYRVCDVPL
        Hmu  KAETAAKHGE EQVRSWRRSF DIPPPPMDEK HPYYNSISKE RRYA.GLKPG
        Rmu  KAETAAKHGE EQVKIWRRSF DTPPPPMDEK HNYYASISKD RRYA.GLKPE
       Pgms  KDDARKKWGA EQVQIWRRSY DIAPPNGESL KDTAERV... .......LPY
  consensus  KAETAMKHGE EQVRIWRRSY DVPPPPIEES HPYYQEICSD RRYKVCLVPL
   pf2kcon   .......... .......... .....TYEEI QEHYPEEFAL RDQDKYWY..
   P4pIBI30                                              F.REETKDFI..

                151                                                200
        Sco  ELRPQTECLK DVVGRMLPYW FDAIVPDLLT GRTVLVAAHG NSLRALVKHL
        Sce  NVLPETESLA LVIDRLLPYW QDVIAKDLLS GKTVMIAAHG NSLRGLVKHL
       Hre2  DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTILISAHG NSSRALLKHL
        Mre  DQLPRSESLK DVLERLLPYW KERIAPEILK GKSILISAHG NSSRALLKHL
        Rre  DQLPRSESLK DVLERLLPYW NERIAPEVLR GKTVLISAHG NSSRALLKHL
        Hmu  E.LPTCESLK DTIARALPFW NEEIVPQIKA GKRVLIAAHG NSLRGIVKHL
        Rmu  E.LPTCESLK DTIARALPFW NEEIAPKIKA GKRVLIAAHG NSLRGIVKHL
       Pgms  D.........P NLETERLEXL NSTIVAAILK GVKVLIAAHG NSLRALIMDL
  consensus  DQLPRSESLK DVIERMLPYW NERIAPEILK GKTVLIAAHG NSLRALVKHL
   pf2kcon   .RYPKGESYE DLVQRLEPVI MELERQE       NVLVICHQ AVMRCLLATF
   P4pIBI30..N.RSLESNQ DGFERLHPLA ERLERMS       QLSDLRKE SIHRIF

                201                                                250
        Sco  DGISDADIAG LNIPTGIPLS YELNAEFKPL NPGGTYLDPD AAAAAIEAVK
        Sce  EGISDADIAK LNIPTGIPLV FELDENLKPS KPSYYL DPE AAAAGAAAVA
       Hre2  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
        Mre  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGNQE AIQAAIKKVD
        Rre  EGISDEDIIN ITLPTGVPIL LELDENLRAV GPHQFLGDQE AIQAAIKKVE
        Hmu  EGMSDQAIME LNLPTGIPIV YELNKELKPT KPMQFLGDEE TVRKAMEAVA
        Rmu  EGMSDQAIME LNLPTGIPIV YELNQELKPT KPMRFLGDEE TVRKAMEAVA
       Pgms  EGLTGDQIVK RELATGVPIV YHLDKDGKYV SK.ELIDN.. ..........
  consensus  EGISDEDIIN LNLPTGVPIV FELDENLKPV GPHQFFGDQE AIQAAIEAVA
   pf2kcon   LDKSSDELPY LKCPL HTVL KLTPVAYGCK VESIYL NVE AVNTHRDKPE

        Sco  NQGKKK....
        Sce  NQGKK.....
       Hre2  DQGKVQ....
        Mre  DQGKVKQGKQ
        Rre  DQGKVKRAEK
        Hmu  AQGKAK....
        Rmu  AQGKAK....
```

Secondary structure prediction analyses of residues 18-54 of P4pIBI30 imply the presence of an α-helix in the same position predicted for the mutases and phosphatases shown in figure 29. Thus it would appear that part of the sequence of P4pIBI30 is similar to that of PGAM, BPGAM and the phosphatase domain of 6-PF-2-K/F-2,6-BPase. However, alignment of this sequence with the *S.pombe* PGAM partial peptide sequence implies that it is not *S.pombe* PGAM. This in turn implies that either the antibody used to isolate P4pIBI30 was not specific for *S.pombe* PGAM or that the screening conditions used were not specific enough. Therefore the entire screening procedure was repeated using a 1:2000 dilution of the anti-PGAM polyclonal antibody. Three immunoreactive λgt11 clones were isolated, subcloned into pIBI30 and sequenced. All three clones were identical to P4pIBI30.

Thus, using the anti-PGAM polyclonal antibody, I have successfully cloned a isolated and sequenced a partial gene which appears to be homologous to PGAM, BPGAM and the phosphatase domain of 6-PF-2-K/F-2,6-BPase. The cross-reactivity of the fusion protein produced by P4pIBI30 may reflect either:
(a) the ability of the *S.pombe* PGAM, injected into the rabbit for antibody production, to refold and so the antibody may simply be raised against certain secondary and tertiary structural elements, and/or,
(b) the antibody recognises general primary structural arrangements of charge and shape.
Nevertheless, the method used has not been successful in isolating the gene encoding *S.pombe* PGAM. The fact that λgt11 clones carrying small inserts (measuring around 0.3Kb) were isolated on each screen suggests that during several rounds of amplification, the proportion of full-sized clones has

```
                150                       170
P4pIBI30    DTSLESNQDG FERLHPLAER LERMSQLS
     Sce    LPETESLALV IDRLLPYWQD VIAKDLLS
     Hmu    LPTCESLKDT IARALPFWNE EIVPQIKA
     Hre    LPSCESLKDT IARALPFWNE EIVPQIKE
     Rmu    LPRSESLKDV LERLLPYWNE RIAPEVLR
     Mre    LPRSESLKDV LERLLPYWKE RIAPEVLK
     Rre    LPRSESLKDV LERLLPYWNE RIAPEVLR
pf2kcons    YPKGESYEDL VQRLEPVIME LERQENVL
```

Figure 29: Alignment of the predicted secondary structural elements of the amino acid sequence encoded by P4pIBI30 with the predicted secondary structures for PGAM and F-2,6-BPase sequences (over the region where similarity appears possible). Predicted α-helical structure is indicated by underlining. Numbering and abbreviations as in figure 28.

been reduced in the λgt11 library. Lack of size fractionation of mRNA during the synthesis of the library may have added to this problem.

4.7 Attempted Isolation of the Gene encoding PGAM from *S.pombe*, by PCR

Preliminary PCR reactions were carried out as a final attempt to isolate the gene encoding *S.pombe* PGAM. An *S.pombe* cDNA library, either in λgt11 or 2μM URA3 plasmid, was used as a template and degenerate oligonucleotides as primers. It was hoped that primer 1 would anneal to the coding region of *S.pombe* PGAM and primer 2 would anneal to the poly-A tail of cDNA inserts. Following 30 cycles of PCR, each reaction should, in theory, contain multiple copies of DNA encoding the C-terminal region of *S.pombe* PGAM.

Analysis of the PCR products by agarose gel electrophoresis revealed the production of one major DNA species in each reaction, see figure 30. The products were of similar size (≈1Kb), which would be expected if the primers annealed to the regions they were planned to prime. However, lanes 4 and 5 reveal that with only one primer present in the reaction, a 1Kb fragment was formed. Therefore, the conditions will have to be optimised to produce specific products.

The only definitive way to characterise the PCR products would be to subclone and sequence them. However, with limited time, this was not possible.

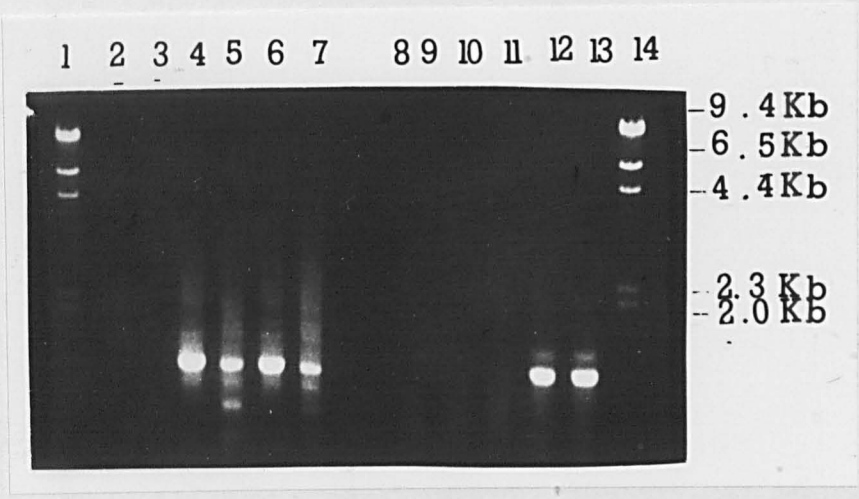


Figure 30: Gel electrophoresis (0.8% agarose) of PCR products, conditions as outlined in section 3.18.3.

Lane	Sample
1	Molecular weight markers
2	2μM URA library, no primers
3	No 2μM URA library, primers only
4	2μM URA library + oligo 82-90 only
5	2μM URA library + oligo 107-114 only
6	2μM URA library + oligo 82-90 + oligo dT
7	2μM URA library + oligo 107-114 + oligo dT
8	λgt11 library, no primers
9	No λgt11 library, primers only
10	λgt11 library + oligo 82-90 only
11	λgt11 library + oligo 107-114 only
12	λgt11 library + oligo 82-90 + oligo dT
13	λgt11 library + oligo 107-114 + oligo dT
14	Molecular weight markers

CHAPTER 5

CONCLUSIONS

Genes encoding enzymes of central metabolic pathways, such as glycolysis, are highly conserved due to the crucial role played by these pathways (Fothergill-Gilmore, 1986). *S.pombe* and *S.cerevisiae* PGAM are thought to have diverged 800-1200 million years ago (Sharp and Wright, unpublished). *S.pombe* and *S.cerevisiae* PGAM have evolved to retain similar activity, active sites and secondary structure, see section 2.2. However, a number of structural differences exist between the PGAM from *S.pombe* and *S.cerevisiae* as reflected by DNA hybridisation and immunological studies. The primary structures of PGAM from the two yeasts are sufficiently different that the *S.cerevisiae* PGAM gene failed to detect gene sequence homologies in *S.pombe* and the polyclonal antibody raised against *S.pombe* PGAM failed to cross-react with *S.cerevisiae* PGAM. Weak cross-reactivity of this antibody with rabbit muscle PGAM reflects the fact that *S.pombe* PGAM shares more antigenic determinants with the dimeric rabbit muscle enzyme than with the tetrameric *S.cerevisiae* enzyme, implying that *S.pombe* PGAM is more homologous to the mammalian enzyme than the *S.cerevisiae* enzyme. This is not unusual as other mammalian proteins have been found to be more similar to *S.pombe* than is the *S.cerevisiae* homologue e.g. calmodulin and β-tubulin (Russell, 1989).

Immunological screening of the *S.pombe* expression library for the *S.pombe* PGAM gene resulted in the isolation of a number of identical clones which contained cDNA inserts encoding 69 residues. Part of this sequence was similar to residues 362-385 of fructose-2,6-bisphosphatase and residues 143-167 of PGAM, see alignment in section 4.6. This alignment reveals that this stretch of the primary structure of the immunoreactive clones is more similar to F-2,6-BPase than to the mutases. Assuming the antibody against

S. pombe PGAM is specific, it would appear that part of a F-2,6-BPase-like gene has been isolated from the *S. pombe* expression library due to either this part of the F-2,6-BPase-like protein sharing determinants with *S. pombe* PGAM (which are more antigenic in the F-2,6-BPase-like protein than *S. pombe* PGAM) or under-representation of the PGAM gene in the expression library, or a combination of both of these factors.

The inability to isolate immunoreactive clones containing cDNA inserts measuring >500bp implied that the *S. pombe* expression library had not been size-fractionated during its construction. As a result, amplification of such a library leads to enhancement of the proportion of small cDNA-containing recombinants (Kaiser and Murray, 1986). Thus, the chance of isolating a full-length clone was reduced by virtue of screening a cDNA library which had not been size-fractionated.

CHAPTER 6

PLANS FOR FUTURE WORK

The main priority for future work on this project would be to isolate the gene encoding PGAM from the fission yeast *S. pombe*. A number of approaches are now available to acheive this:

(i) synthesis of a new size-fractionated cDNA library in λgt11. This library would then be subjected to immunoscreening with the polyclonal antibody raised against *S. pombe* PGAM. A size-fractionated library would increase the chances of isolating a full-length clone encoding *S. pombe* PGAM.

(ii) probing Southern blots of digested *S. pombe* genomic DNA with oligonucleotides designed against alternative regions of the *S. pombe* PGAM peptide sequences e.g. oligonucleotides 82-90 and 107-114, see figure 13. Determination of the hybridisation conditions would facilitate screening of a new cDNA library or a genomic sublibrary using oligonucleotides as molecular probes. Screening of a genomic sublibrary was successful in isolating the gene encoding PGAM from *S. coelicolor* (White *et al.*, 1992).

(iii) complementation of the *S. cerevisiae* strain (DBY gpm$^-$) devoid of any wild type PGAM with the *S. pombe* cDNA library in 2μm URA3 plasmid. DBY gpm$^-$ was made by M.F. White, University of Edinburgh and the cDNA library in 2μm URA3 was synthesised by J.D. Fikes *et al.*, 1990. A number of *S. pombe* genes have been successfully isolated by complementation with *S. cerevisiae* mutants e.g. alcohol dehydrogenase (Russell and Hall, 1983), triose phosphate isomerase (Russell, 1985) and acid phosphatase (Elliott *et al.*, 1986).

(iv) from preliminary PCR work, using *S.pombe* cDNA libraries as templates and degenerate oligonucleotides as primers, it would appear that specific 1Kb products were obtained (section 4.7). Sequencing of these products should be undertaken. If the products appeared to represent multiple copies of DNA encoding the C-terminal region of *S.pombe* PGAM, they could be used as molecular probes to isolate the entire coding region of the *S.pombe* PGAM gene.

If putative *S.pombe* PGAM genes are isolated using one of the techniques outlined above, or by some other method, the next step would be to sequence these genes to confirm that they encode PGAM. Following this, the next obvious step would be to develop a suitable overexpression system which would produce large quantities of *S.pombe* PGAM. Such a system would also allow site-directed mutagenesis studies. The availability of large quantities of native and mutant forms of mutase would facilitate biophysical techniques e.g. X-ray, circular dichroism and NMR, thus leading to a better understanding of the structure and catalytic mechanism of phosphoglycerate mutase:

1. The overall structure of *S.pombe* PGAM could be studied by a range of biophysical techniques. Far U.V. circular dichroism spectra of the enzyme could be used to derive the secondary structure. Near U.V. circular dichroism and fluorescence studies would reveal features of the tertiary structure of *S.pombe* PGAM. Trials should be undertaken to crystallise *S.pombe* PGAM and following this, X-ray diffraction studies may yield the high resolution crystal structure of the enzyme. In conjunction with this,

the primary structure of *S.pombe* PGAM could be modelled into the coordinates of the three dimensional structure of PGAM from *S.cerevisiae*.

2. The stability of *S.pombe* PGAM could be assessed by detailed studies of guanidine hydrochloride denaturation titration curves. Preliminary results (Johnson and Price, 1987) implied that the presence of BPG conferred stability to the enzyme, so experiments could be carried out in the absence and presence of BPG and its phosphonate analogues.

3. The role of the C-terminal tail could be assessed by kinetic, binding and stability properties of *S.pombe* PGAM, with and without this C-terminal portion. Measurements of the rates of exchange of substrate and cofactor would help elucidate the effect that the C-terminal portion has on controlling the accessibility of the active site.

4. The catalytic properties of the active site of *S.pombe* PGAM could be studied by a combination of kinetics, site-directed mutagenesis and NMR spectroscopy. Measurements of the rates of the phosphatase, synthetase and mutase reactions (see figure 2) of *S.pombe* PGAM would establish the relationship between this enzyme and other mutases.

X-ray and chemical modification studies of *S.cerevisiae* PGAM have proposed the role of a number of residues to be important in its activity e.g. His-8, His-181, Arg-7, Ser-11, Thr-20 and Arg-59. Site-directed mutagenesis of each of these residues in *S.pombe* PGAM would produce a range of mutant enzymes. Kinetic and structural (NMR and CD) measurements of each of these mutants may reveal the role played by each of these amino acids.

5. The consequences of the monomeric nature of *S. pombe* PGAM may be studied using site-directed mutagenesis, kinetic and structural studies. Once the complete amino acid sequence of *S. pombe* PGAM is available, alignment with other mutases should help decide whether the monomeric nature of the enzyme is the result of localised changes in amino acids known to be involved in intersubunit contacts (see figure 4) or whether an entire portion of polypeptide chain involved in such contacts is deleted. SDM, kinetic and structural studies would help explore the consequences of these possibilities.

Attempts to isolate the gene encoding *S. pombe* PGAM by immunoscreening the expression library supplied by V. Simanis has led to the isolation of a DNA sequence with an open reading frame of 207 bases. It may be of interest to use this DNA sequence as a molecular probe to isolate a complementary full-length clone. Isolation and sequencing of the full-length clone may lead to a more complete characterisation by sequence alignments and eventual isolation of the protein encoded by this sequence.

CHAPTER 7

INTRODUCTION TO PURIFICATION AND CHARACTERISATION OF PHOSPHOGLYCERATE MUTASE FROM *STREPTOMYCES COELICOLOR*

7.1 Aim of project

The aim of this part of the project was to purify the enzyme phosphoglycerate mutase from *Streptomyces coelicolor* and then conduct some preliminary kinetic and stuctural studies. Purification of the enzyme was intended to lead to obtaining the N-terminal amino acid sequence of PGAM from *S.coelicolor*.

This work was part of an intensive study (based at the University of Glasgow) of the regulation of the biosynthetic pathways of *S.coelicolor*.

7.2 Background to project

The shikimate pathway is the biosynthetic route by which micro-organisms and plants synthesize aromatic amino acids (Haslam E., 1974). This pathway is being studied extensively in the filamentous bacterium *Streptomyces coelicolor* by J.R. Coggins, I.S. Hunter and H.G. Nimmo at the University of Glasgow. This group have undertaken the purification and characterisation of the enzymes in the shikimate pathway in this organism. In addition, a number of the genes encoding these enzymes have been isolated. In an attempt to isolate the gene encoding shikimate dehydrogenase from *S.coelicolor*, the enzyme was purified as outlined in appendix II. The final purification step yielded a single band on SDS-PAGE (Mr 29,000), which was subjected to N-terminal amino acid sequence analysis. This analysis gave a sequence of 14 amino acid residues in low yield, suggesting that the major protein species in the sample (shikimate dehydrogenase) was not being

sequenced. In fact, the N-terminal sequence information was very similar to the N-terminal sequences of a number of eukaryotic phosphoglycerate mutases (E.C.2.7.5.3.). An oligonucleotide (24-mer) was designed from this N-terminal sequence information. The oligonucleotide was used to screen digests of *S.coelicolor* J13456 genomic DNA. A number of hybridisation signals were obtained, including a 3.1Kb SalI band which was further characterized. This fragment was subcloned, sequenced and the amino acid sequence was deduced. The amino acid sequence corroborated the N-terminal sequence information obtained and alignment with known protein sequences of phosphoglycerate mutases revealed a high degree of similarity, see figure 3. The isolation and sequencing of the gene was carried out by Peter White at the University of Glasgow.

Thus, by chance contamination of shikimate dehydrogenase preparations, the N-terminal sequence of PGAM from *S.coelicolor* was obtained which in turn led to the isolation and sequencing of the gene encoding *S.coelicolor* PGAM. A number of prokaryotic PGAMs have been studied, see table 1, but sequence information is not available for any of these enzymes. *S.coelicolor* PGAM was the first prokaryotic PGAM gene to be sequenced and so it was decided to take advantage of this finding to further the understanding of the mechanism and evolution of PGAM. Therefore, the opportunity arose to develop a purification scheme for *S.coelicolor* PGAM and to characterise this enzyme.

7.3 Information available at the outset of the project

As outlined in section 7.2, *S.coelicolor* PGAM was present as a trace contaminant in preparations of shikimate dehydrogenase. Therefore, the PGAM must have been co-purified using the method outlined in appendix II. From this it can be deduced that PGAM precipitates at 40-60% ammonium sulphate and binds the triazine dye Procion Red in the presence of 50mM Tris-HCl, pH 8.0, 0.4mM DTT and can be eluted from this dye with increasing concentrations of NaCl in 50mM Tris-HCl, pH 8.0, 0.4mM DTT, 0.1mM NADPH. These initial purification steps are similar to those adopted for the purification of PGAM from other sources such as *S.pombe* and *S.cerevisiae*. Thus, the ability of the *S.coelicolor* PGAM to bind triazine dyes could be exploited for its purification.

Analysis of the deduced amino acid sequence of the *S.coelicolor* PGAM reveals the presence of the two histidine residues which are known to be present in the active site of *S.cerevisiae* PGAM and chemical modification studies have implicated His-8 to be phosphorylated during the course of the reaction in PGAMs from numerous sources. As outlined in chapter 1, there are two classes of phosphoglycerate mutase: BPG-dependent and BPG-independent. Inhibition of PGAMs by vanadate has been used as a diagnostic tool for BPG-dependence. Carreras *et al.*, 1980, reported that cofactor dependent PGAMs were inhibited by μM concentrations of vanadate, but that cofactor-independent enzymes remained fully active in the presence of vanadate. Vanadate is a potent inhibitor of phospho-transfer reactions as it adopts a trigonal bipyramidal structure which resembles the presumed transition state of the phospho-group during transfer. However, in the case

of PGAM, inhibition by vanadate cannot be assigned to this competitive inhibition by a similar structure. ^{51}V-NMR studies (Stankiewicz *et al.*, 1987) have shown PGAM to bind divanadate, rather than monovanadate, which promotes the dephosphorylation of the phospho-form of the enzyme (Carreras *et al.*, 1982). Sensitivity of *S.coelicolor* PGAM to vanadate could be used to determine BPG-dependence.

Far U.V. circular dichroism studies have been used to study the structural properties of PGAM from *S.cerevisiae*, rabbit muscle and *S.pombe*, and have shown that these enzymes share similar structures. In addition this technique has revealed the importance of the presence of BPG to the overall stability of these enzymes.

7.4 Strategy of project

Initial attempts to purify *S.coelicolor* PGAM would make use of affinity chromatography on the triazine dyes. If necessary, further purification steps would be used. Once *S.coelicolor* PGAM had been purified to a single band on SDS-PAGE, the overall molecular weight of the enzyme would be determined by gel filtration on Sephacryl S-300.

Purified enzyme would be subjected to extensive dialysis to remove any traces of BPG and hence any PGAM present as a putative phospho-form. The dialysed enzyme would then be assayed in the absence of BPG to test for cofactor dependence. Vanadate inhibition studies would also be carried out to test for cofactor dependence.

Preliminary circular dichroism spectra would be measured to determine the relatedness of the structure to those from other sources.

Finally, the N-terminal amino acid sequence of the purified *S.coelicolor* PGAM would be determined to confirm that the DNA sequence, isolated by the Glasgow group, actually encodes *S.coelicolor* PGAM.

CHAPTER 8

METHODS AND MATERIALS

8.1 Purification of phosphoglycerate mutase from *Streptomyces coelicolor*

8.1.1 Growth of *Streptomyces coelicolor*

Growth of the organism was conducted in the Institute of Genetics, University of Glasgow. *S. coelicolor* strain, JI3456 was grown to mid-log phase for 48 hours at 30°C on a rotary shaker (180rpm). Cultures were grown in 2 litre flasks containing 400ml YEME. Per litre, YEME contains 16g malt agar, 10g bacto-yeast agar and 5g NaCl.

Prior to inoculation of YEME, spores were prepared from frozen spore suspensions. Frozen spore suspensions were spread on agar plates (2% mannitol, 2% soya bean meal/flour, 1.6% agar) which were incubated at 30°C for 10 days. Following this, 5ml SDW was added to the plates and spores were removed from the plate by careful scraping. The spores were washed twice in SDW and then used to innoculate the YEME.

8.1.2 Cell lysis and ammonium sulphate fractionation

All the following procedures were conducted at 4°C. A 20g batch (wet weight) of *S. coelicolor* cells was suspended in 25ml of 100mM potassium phosphate buffer, pH 7.0, containing 5mM EDTA, 1.2mM PMSF, and 0.4mM DTT. The cells were lysed by passage through a French pressure cell (98MPa, internal pressure). This step was repeated with the lysate to ensure a high

degree of lysis. Cell debris was removed from the cell lysate by centrifugation at 100,000g for 1 hour.

The crude extract was subjected to ammonium sulphate fractionation. The fraction precipitating between 50% and 70% saturation was collected and resuspended in a small volume (around 5ml) 10mM Tris-HCl. pH 8.0 and then dialysed overnight against this buffer.

8.1.3 Affinity chromatography

The dialysed sample was then applied to a column (2.5cm x 5.0cm^2) of Cibacron-Blue Sepharose, equilibrated with 10mM Tris-HCl, pH 8.0. The PGAM activity failed to bind Cibacron-Blue when the column was washed with 10mM Tris-HCl, pH 8.0. Fractions containing PGAM activity were pooled and applied to a column (12cm x 0.8cm^2) of Procion Red Agarose (Reactive Red Agarose). The column was then washed with several column volumes of 10mM Tris-HCl, pH8.0, to remove unbound proteins. PGAM activity was eluted with one column volume of 4mM BPG, followed by two column volumes of buffer. PGAM activity emerged from the column as a single peak of activity in a volume of around 10ml.

To regenerate the affinity column, the Procion Red Agarose was removed and mixed with 50ml 5M NaCl for 30 minutes. The column was then repacked and washed with 10mM Tris-HCl, pH8.0, until the conductivity of the effluent matched that of this buffer.

8.2 Characterisation of Structure of *S.coelicolor* PGAM

8.2.1 Sephacryl-300 Gel Filtration

A gel filtration column measuring 40cm x 6.2cm^2 was equilibrated with 0.1M sodium phosphate buffer, pH 7.4. The following proteins served as Mr standards: lactate dehydrogenase, aspartate amino transferase, bovine serum albumin, ovalbumin, trypsinogen and ribonuclease.

8.2.2 SDS-PAGE

As oulined in section 3.10.

8.2.3 Circular Dichroism

Far U.V. circular dichroism spectra were recorded at 20°C in a Jasco J-600 Spectropolarimeter, using 0.1cm path length quartz cells. The ellipticity of PGAM was monitored between 190nm and 260nm. All measurements were made using 10mM Tris-HCl. pH 8.0.

8.2.4 Determination of N-terminal amino acid sequence

To obtain N-terminal sequence information from *S.coelicolor* PGAM, the protein was purified by SDS-PAGE and electroblotted onto PDVF (Immobilon P) membranes. Using the minigel apparatus (Biorad), 2μg of purified PGAM was loaded onto each lane of a 12% acrylamide gel which was run at 30mA. The

PDVF filter was prepared as follows: the PDVF filter was soaked in 100% methanol for a few seconds and then in SDW for a few seconds. The filter was then equilibrated in transfer buffer (10mM CAPS, 10% methanol, pH 11) for at least 15min. The gel was also equilibrated in transfer buffer for 5min prior to transfer. The gel and PDVF filter were sandwiched between Whatman 3MM paper and assembled into the blotting apparatus. Transfer was conducted at 500mA for 30min in transfer buffer. The filter was washed in deionised water after transfer for 5min. and then stained with Coomassie blue R-250 (0.1% in 50% methanol) for 5min. The filter was then destained with several changes of 50% methanol, 10% acetic acid for 10min at room temperature. The filter was finally rinsed in deionised water for 5-10min and air dried. The electroblotted *S.coelicolor* PGAM was sequenced by Bryan Dunbar (University of Aberdeen) on an applied Biosystems 407A gas phase sequencer, as described by Russell *et al.*, (1986).

8.3 Kinetic Properties of *S.coelicolor* PGAM

8.3.1 Km for 3-PGA

The Km value for 3-PGA was determined using the enolase coupled assay outlined in section 3.6. The assay was carried out in the presence of 0.3mM BPG with 3-PGA concentrations ranging from 0.1mM to 10mM.

8.3.2 BPG Dependence

S.coelicolor PGAM was subjected to extensive dialysis against 10mM Tris-HCl, pH 8.0, to attempt to remove any traces of BPG. The dialysed enzyme was then assayed using the enolase coupled assay. BPG was omitted from the assay mix and grade I 3-PGA was used in the assay system as it is virtually BPG-free (<0.005%). Rabbit muscle PGAM, a known BPG-dependent enzyme, was dialysed alongside the *S.coelicolor* enzyme to check that the system used was BPG-free.

8.3.3 Vanadate Inhibition

The inhibition of PGAM caused by vanadate was tested as described by Carreras *et al.*,1980. This method involved assaying PGAM by the enolase coupled method, as described in section 3.6, and then adding metavanadate to final concentrations of 10μM and 100μM in the 1ml assay mix.

CHAPTER 9

RESULTS AND DISCUSSION

9.1 Purification of *S.coelicolor* PGAM

Initially, attempts were made to purify PGAM from *S.coelicolor* using the one step affinity chromatography method outlined for *S.pombe* PGAM, see section 3.4.4. The cells were lysed using a French press and the lysate was centrifuged at 100,000g for 1 hour to remove all cell debris. PGAM activity was then precipitated with ammonium sulphate between 50% and 70% saturation. The precipitate was dissolved in 2ml 10mM Tris-HCl, pH 8.0, dialysed overnight and applied to a Cibacron-Blue Sepharose column. The PGAM activity failed to bind to the Cibacron-Blue column and so it would appear that PGAM from *S.coelicolor* differs from other cofactor dependent enzymes which bind Cibacron-Blue under the same conditions (Price and Stevens, 1983). An alternative purification method had to be found. On checking the purification scheme employed for shikimate dehydrogenase from *S.coelicolor*, which resulted in the co-purification of PGAM, it was noted that PGAM bound to the triazine dye Procion-Red in the presence of 50mM Tris-HCl, pH 8.0, 0.4mM DTT. In an attempt to repeat this, the effluent from the Cibacron-Blue column, which contained PGAM activity, was applied to Procion-Red Agarose. Non-specifically bound proteins were removed from the Procion-Red by washing with 2 column volumes of 10mM Tris-HCl, pH 8.0. PGAM activity remained bound to the column under these conditions and was specifically eluted by a pulse of 4mM BPG in 10mM Tris-HCl, pH 8.0.

The purification of PGAM from *S.coelicolor* is summarized in table 4.

Table 4: Purification scheme for PGAM from *S.coelicolor* (for 20g wet weight cells)

STEP	TOTAL PROTEIN(mg)	TOTAL ACTIVITY(units)	SPECIFIC ACTIVITY(U/mg)	YIELD (%)
Crude extract	91	1450	15	100
Ammonium sulphate	26	1040	40	72
Cibacron-Blue	14.2	1080	76	75
Procion-Red	0.17	490	2880	34

From SDS-PAGE, see figure 31, it would appear that following the Procion-Red step, the purified PGAM was at least 95% hmongeneous.

9.2 Structure of *S.coelicolor* PGAM

From the relative mobililities of marker proteins on SDS-PAGE, the subunit M_r of *S.coelicolor* PGAM is 28,800 ± 2,000. This is consistent with the deduced amino acid sequence, see figure 3. *S.coelicolor* PGAM subunits consist of 253 residues which would give a subunit Mr of 27,830. The Mr of the protein under non-denaturing conditions was determined by gel filtration on Sephacryl S-300 and monitoring the activity of the eluted fractions, see figure 32. From gel filtration, the native Mr was estimated at 120,000 ± 10,000. The enzyme activity was eluted from Sephacryl S-300 as a single peak and the recovery was >90%. These results indicate that *S.coelicolor* PGAM is a tetramer,similar to that from *S.cerevisiae*. The two

Figure 31: Coomassie stained SDS-PAGE of *S.coelicolor* phosphoglycerate mutase at various stages of purification.

Lane	Sample
1	Molecular weight markers
2	Cibacron blue eluant
3	2,3-BPG elution from Procion red

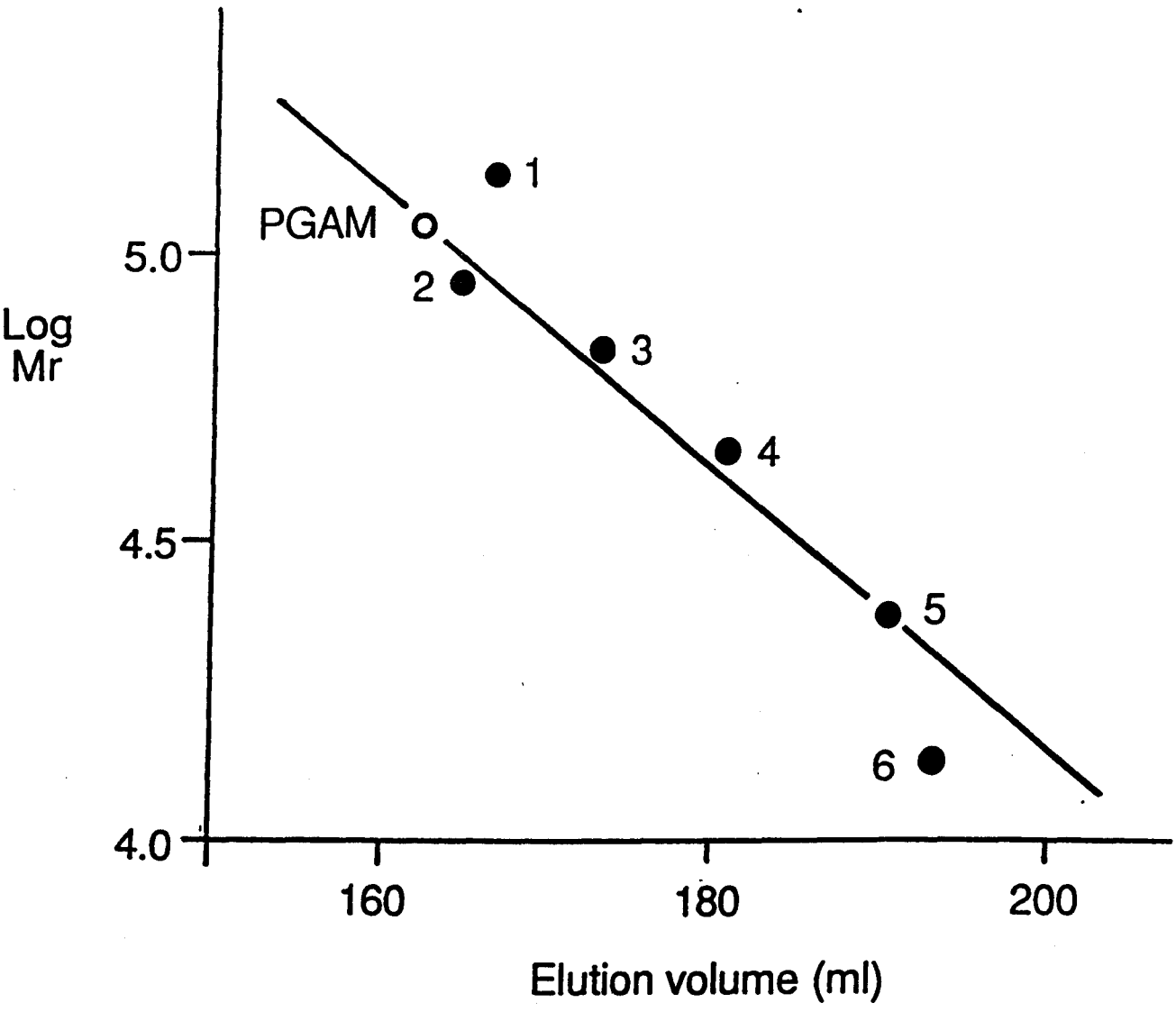


Figure 32: Sephacryl S-300 standard curve constructed to determine the native molecular weight of phosphoglycerate mutase from *S.coelicolor*, where:
1 = rabbit muscle lactate dehydrogenase (M_r 140,000)
2 = aspartate amino transferase (M_r 90,000)
3 = bovine serum albumin (M_r 66,000)
4 = ovalbumin (M_r 45,000)
5 = trypsinogen (M_r 24,000)
6 = RNase (M_r 13,600)
From this curve the M_r of native *S.coelicolor* PGAM is ≈120,000.

enzymes not only share similar quaternary structures but also similar overall secondary structure, as determined by far U.V. circular dichroism spectra, see figure 33.

The initial work conducted by the Glasgow group, resulted in the determination of the N-terminal sequence of *S.coelicolor* PGAM by its co-purification with shikimate dehydrogenase (ADAPYKLILRHG). From the results described above i.e. specific activity and behaviour on SDS-PAGE, the *S.coelicolor* PGAM appeared to have been purified and so the N-terminal sequence was determined to confirm that the DNA sequence isolated by the Glasgow group actually encoded PGAM from *S.coelicolor* (ADAPYKLILLRHG). The sequence information obtained differed by one residue from the original data, from which the oligonucleotide was designed to isolate the gene encoding *S.coelicolor* PGAM.

Alignment of the deduced amino acid sequence of this enzyme with PGAMs from other sources, shows a high degree of identity, see figure 3. Points to note are (*S.cervisiae* numbering used):

1. The presence of Arg 7, His 8, Thr 20, Arg 59 and His 181. All of these residues are thought to be of catalytic importance (Winn *et al.*, 1981).
2. The presence of a cluster of residues with small side chains followed by three consecutive lysines at the C-terminus. This structure is like the flexible C-terminal tail known to occur in the *S.cerevisiae* enzyme, see section 1.6.4.
3. Residues 59-82, which encompasses α-helix 2 and β-sheet 1, are similar to other PGAMs implying contact region CR1/2 is similar, see section 1.6.6.
4. Residue 168, which lies in α-helix 6 is a lysine residue in the *S.cerevisiae* PGAM. α-helix 6 forms the weaker of the two types of subunit

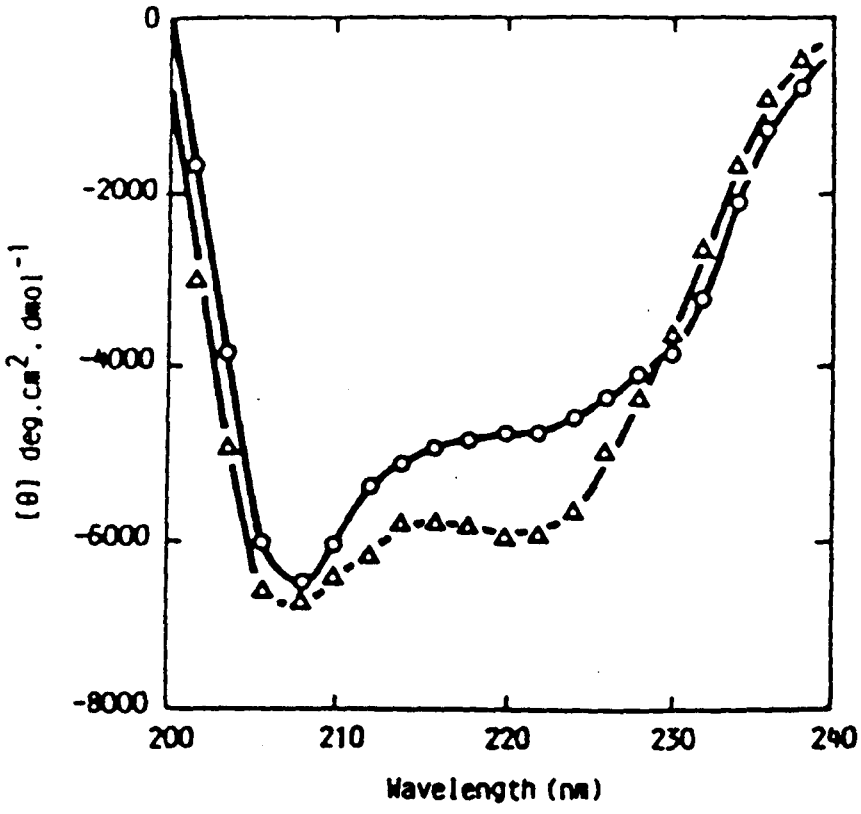


Figure 33: Circular dichroism spectrum of (Δ) *S.coelicolor* and (O) *S.cerevisiae* phosphoglycerate mutase (data from Herman *et al.*, 1983)

interface (CR5/6) in the *S.cerevisiae* enzyme and in all dimeric forms of the enzyme residue 168 is a proline residue. This has led to the theory that CR5/6 no longer contributes to subunit contacts in the dimeric forms of the enzyme. However, residue 168 is a proline residue in the *S.coelicolor* enzyme thus discounting the theory that the presence of a proline residue at this position necessarily results in the formation of a dimeric form of the enzyme. Recent site directed mutagenesis work on the *S.cerevisiae* enzyme has revealed that the K168P mutant exists as a tetramer. However, cross-linking studies have revealed a tendency for dimers to occur as the concentration of this mutant enzyme is lowered (Malcolm F. White, personal communication).

From the data outlined above, the structure of the *S.coelicolor* PGAM would appear to be very similar to the enzyme from *S.cerevisiae*. However, unlike the *S.cerevisiae* enzyme, and other BPG-dependent enzymes which have been tested, *S.coelicolor* PGAM fails to bind to Cibacron-Blue. This was not only indicative of some structural difference but also brought into question the BPG-dependence of *S.coelicolor* PGAM.

9.3 Kinetics of *S.coelicolor* PGAM

PGAM from *S.cerevisiae* is BPG-dependent (Ray and Peck, 1973). To determine the BPG-dependence of the *S.coelicolor* enzyme, it was subjected to extensive dialysis (5 days, 12 changes) against 10mM Tris-HCl, pH 8.0, at 4°C. The enzyme was assayed in the absence of BPG but it was found to retain 100% activity. This suggested one of three possibilities:

1. BPG-independence

2. the presence of a stable phosphoenzyme intermediate, formed in the presence of BPG, which was used in the final purification step to elute PGAM from the Procion-Red column

3. *S.coelicolor* PGAM binds BPG very tightly

Substitution of BPG by 3-PGA in the affinity chromatography purification step, also resulted in the specific elution of *S.coelicolor* PGAM but when the enzyme was dialysed and assayed in the absence of BPG, the activity of the enzyme was depleted to 20% (590units/mg). Activity was restored to 100% (3,000units/mg) by including 0.3mM BPG in the enolase coupled assay.

From these results, it would appear that *S.coelicolor* PGAM is at least partially BPG-dependent. However, the enzyme must bind BPG tightly or exist as a stable phosphoform, unlike other BPG-dependent PGAMs. Following extensive dialysis of rabbit muscle PGAM, 80% of the activity was lost after 6 hours and no activity was found in the absence of BPG after 10 hours dialysis. In the case of *S.coelicolor* PGAM (purified and assayed in the absence of BPG), 20% activity remained after 6 days of dialysis.

Carreras and Bartrons, 1980, reported the use of vanadate as a diagnostic reagent to differentiate BPG-dependent and BPG-independent PGAMs. Cofactor dependent enzymes were found to be inhibited by μM concentrations of vanadate. Later work (Carreras *et al.*, 1982) revealed the phosphoenzyme intermediate of BPG-dependent PGAMs is destablised in the presence of vanadate. BPG-independent PGAMs were unaffected by vanadate.

S.coelicolor PGAM was assayed in the presence of 10μM and 100μM metavanadate, which resulted in an 80% and 100% inhibition, respectively.

Inhibition did not occur immediately but became fully effective 2-3 minutes after the addition of vanadate. Thus, *S.coelicolor* PGAM was inhibited in a manner similar to the range of BPG-dependent mutases studied by Carreras and Bartrons, 1980.

Therefore, the vanadate inhibition studies imply that *S.coelicolor* PGAM is fully BPG-dependent. However, the difficulties encountered when trying to prove cofactor dependence by subjecting the enzyme to dialysis imply either BPG binds very tightly to the enzyme or that the phospho-form of *S.coelicolor* PGAM is stable towards hydrolysis. The putative phospho-form of *S.coelicolor* PGAM would have a $t_{½}$ of 3hours (80% of the activity was lost following ≈10hours dialysis in the absence of BPG) whereas the phospho-form of the *S.cerevisiae* enzyme has a $t_{½}$ of 2min, Winn *et al.*, 1981.

The Km value for 3-PGA was determined (at a BPG concentration of 0.3mM) over a range of 3-PGA concentrations from 0.1mM to 10mM. The Km value obtained was 1.3mM ± 0.1mM, a value similar to those reported for other BPG-dependent enzymes under similar assay conditions (Price *et al.*,1985).

CHAPTER 10

CONCLUSIONS

A number of prokaryotic PGAMs have been isolated and partially characterised, see table 1. However, *S.coelicolor* PGAM is the first prokaryotic PGAM for which the primary structure has been determined. Alignment of the *S.coelicolor* PGAM sequence with sequences of BPG-dependent PGAMs from eukaryotic sources, figure 3, revealed a high degree of identity, thus reinforcing the view (Fothergill-Gilmore, 1986) that the glycolytic genes are highly conserved.

S.coelicolor PGAM exists as a tetramer with subunit M_r 28,000. This quaternary structure is similar to the *S.cerevisiae* PGAM. Sequence alignment and CD studies reveal that *S.coelicolor* and *S.cerevisiae* PGAMs have similar primary structure and overall secondary structure, respectively. Both enzymes share common active site residues, a C-terminal region with an unusual run of alanine and lysine residues and residues 59-82 are similar implying contact region CR1/2 is conserved.

Although *S.coelicolor* PGAM shares high sequence identity with the eukaryotic BPG-dependent enzymes, it possesses some unusual properties. It failed to bind to Cibacron-Blue, a property previously correlated with BPG-dependence (Price and Stevens, 1983). Extensive dialysis failed to demonstrate complete BPG-dependence; purification in the absence of BPG followed by prolonged dialysis revealed considerable BPG-dependence (≈80%) but not total BPG-dependence.

Assuming *S.coelicolor* PGAM was inhibited by vanadate in a manner similar to other BPG-dependent mutases, it would appear that *S.coelicolor* PGAM either exists as a stable phospho-form or binds BPG very tightly.

CHAPTER 11

PLANS FOR FUTURE WORK

The availability of the gene encoding *S.coelicolor* PGAM and the purification scheme for *S.coelicolor* PGAM means that it will be possible to investigate the structure of the enzyme using both molecular and biochemical techniques. Of the particular interest is the nature of the phosphorylation site of this enzyme. Measurement of the rates of the synthetase and phosphatase reactions of *S.coelicolor* PGAM may resolve whether the enzyme binds BPG tightly or the phosphoform is particularly stable. Incorporation of phosphate into *S.coelicolor* could be monitored by incubation with ^{32}P-labelled BPG followed by TCA precipitation. Precipitation of ^{32}P with the PGAM would be indicative of a ^{32}P-labelled phosphoenzyme. A stable ^{32}P-labelled phospho-enzyme would permit measurements of stoichiometry. Isolation of the phospho-form of PGAM, followed by its enzymatic cleavage into peptide fragments would produce material for sequence analysis which may reveal the nature of the phosphorylation site. In view of the sequence homology between *S.cerevisiae* PGAM and *S.coelicolor* PGAM, it is very likely that His-8 is phosphorylated.

The presence of Pro-168 in *S.coelicolor* PGAM has raised some questions with respect to contact region CR5/6, see section 1.6.6. Residue 168 is a lysine residue located in helix 6 of the *S.cerevisiae* enzyme and it was thought that replacement of this lysine residue by a proline would result in the formation of a dimer. Until now, the occurrence of a proline at this position has been particular to dimeric forms of the mutase, see figure 3. However, *S.coelicolor* remains in a tetrameric form, despite the presence of of a proline at residue 168. It would be of interest to compare the strength of the subunit contacts of the *S.coelicolor* PGAM with those of

S. cerevisiae PGAM to determine whether this amino acid substitution has any effect. From denaturation titration curves, it would be possible to estimate the relative stabilities of these enzymes and hence assess any differences in intersubunit forces. Denaturation of both enzymes with guanidine hydrochloride would be studied using ultracentrifugation, cross-linking and light scattering to record any changes in quaternary structure. Denaturation of the enzymes could also be studied by monitoring changes in activity, protein fluorescence and circular dichroism. These techniques could be used to monitor refolding and reassociation of the enzymes, which in turn could reveal any differences in the rates of the steps involved in renaturation. The effect of proteinases on the enzymes during refolding may give an indication of the stability of the intermediates involved in the refolding process (Johnson and Price, 1987).

Glycolytic genes and the enzymes they encode have been isolated from numerous sources. The glycolytic enzymes form a large percentage of soluble cell protein. The glycolytic genes are among the most highly expressed genes and due to this, the promoters of many glycolytic genes have served as the basis of high level expression vectors e.g. PGK, GAP.DH, ADH (Schena *et al.*, 1991). Isolation and characterisation of the *S. coelicolor* PGAM may be useful in the expression of heterologous genes. Many glycolytic genes e.g. *S. cerevisiae* PGK (Tuite *et al.*, 1982) share the property of being inducible by glucose and so it will be important to determine if the *S. coelicolor* PGAM promoter is induced in a similar fashion.

Development of an overexpression system for the *S. coelicolor* PGAM gene would result in the production of large quantities of *S. coelicolor* PGAM.

Large quantities of protein would facilitate X-ray studies to determine the structure of *S.coelicolor* PGAM. The overexpression system would also allow site-directed mutagenesis studies. Native and mutant forms of *S.coelicolor* PGAM could be studied using biophysical techniques to address the problems similar to those outlined in points 1-5 of chapter 6.

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APPENDIX 1

SDS-PAGE

Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970). Slab gels were prepared and run using a Bethesda Research Laboratories Inc. vertical slab gel elecrophoresis apparatus. The height of the separating gel was 10cm and the stacking gel 4cm. Gels were prepared to a uniform thickness of 0.8mm. Separating and stacking gels were prepared from a stock solution of 30% (w/v) acrylamide containing acrylamide and N,N'-bisacrylamide in the ratio 30:0.8, respectively.

Separating gels comprised: 0.375M Tris.HCl, pH 8.8, 0.1% (w/v) SDS and the appropriate amount of acrylamide solution to give the required acrylamide concentration. The gels were polymerised by the addition of 0.075% N,N,N',N'-Tetramethylenediamine (TEMED) and 0.35% (v/v) 10% ammonium persulphate.

Stacking gels comprised: 0.125M Tris-HCl, pH 6.8, 0.1% w/v SDS and 10% (v/v) 30% acrylamide stock, and were polymerised as for the separating gel.

Samples for SDS-PAGE were prepared by mixing with an equal volume of 'boiling mix' (10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and

0.005% w/v bromophenol blue). Samples were then boiled for 2 min prior to loading onto a gel.

The electrode buffer contained 0.025M Tris-HCl, pH 8.3, 0.192M glycine and 0.1% w/v SDS. Electrophoresis was carried out with a current of 50mA, until the bromophenol blue reached the bottom of the gel.

The proteins were stained and fixed in the gels with 0.1% Coomassie blue ρ250 (CI 42660) in acetic acid:methanol:distilled water (2:2:5) for 1 hour. The gels were destained in 10% acetic acid.

APPENDIX II

Purification of shikimate dehydrogenase from *S.coelicolor*

This method was developed by Peter J. White (personal communication) and resulted in the copurification of *S.coelicolor* phoshoglycerate mutase.

METHODS AND MATERIALS

Step 1 **Growth of *S.coelicolor* as outlined in 8.1.1.**

Step 2 **Cell lysis and ammonium sulphate fractionation**

All the following procedures were carried out at 4°C. A 20g (wet weight) batch of *S.coelicolor* cells was resuspended in 100mM KP_i buffer, pH 7.0, containing 2mM EDTA, 1.2mM PMSF and 0.4mM DTT. The cells were lysed by passage through a French pressure cell (98MPa, internal pressure). Cell debris was removed from the lysate by centrifugation at 100,00g for 1 hour.

The crude extract was subjected to ammonium sulphate fractionation. The fraction precipitating between 40% and 60% saturation was collected and resuspended in extraction buffer before dialysing against 2 litres of 50mM Tris-HCl, pH 7.5, 0.4mM DTT (buffer A).

Step 3 Chromatography on Mono-Q

The dialysed material from step 2 was sujected to anion exchange chromatography on a Mono Q column using a standard FPLC apparatus. Samples were loaded onto the column in the presence of buffer A. The column was then washed with buffer A, containing an increasing concentration of NaCl (0M to 0.5M NaCl over a period of 30min). The flow rate of the column was 4ml/min, collecting 2ml fractions. Shikimate dehydrogenase activity elutes at ≈190mM NaCl. Peak fractions were pooled and dialysed against 50mM Tris-HCl, pH 8.0, 0.4mM DTT (buffer B).

Step 4 Chromatography on Procion-Red

Dialysed sample from step 3 was loaded onto a column of Procion-Red (10ml bed volume) which had been equilibrated with buffer B. The column was then washed with several volumes of buffer B, containing 0.1M NaCl and 0.1mM NADPH, to remove non-specifically bound proteins. Shikimate dehydrogenase activity was eluted with a column volume of buffer B, containing 0.8M NaCl and 0.1mM NADPH. Active fractions were pooled and dialysed against buffer A, containing 1M ammonium sulphate.

Step 5 Chromatography on Phenyl-Sepharose

Enzyme from the affinity chromatography step was subjected to hydrophobic interaction chromatography using the FPLC apparatus. Samples were loaded onto the column in the presence of buffer A, containing 1M ammonium sulphate. The column was then washed with buffer A, containing a decreasing

concentration of ammonium sulphate (1M to 0M ammonium sulphate over a period of 25min). The flow rate of the column was 0.5ml/min, collecting 0.5ml fractions. Fractions containing shikimate dehydrogenase activity were pooled and dialysed against 50mM Tris-HCl, pH 7.5.

Step 6 Chromatography on Mono-Q

Chromatography on Mono-Q was used as a final purification step. Sample from step 5 was applied to the column in the presence of 50m Tris-HCl, pH 7.5. The column was washed with 50mM Tris-HCl, pH 7.5, containing increasing concentrations of NaCl (0M to 0.5M in 50min). The flow rate was 1ml/min with 0.5ml fractions collected. Shikimate dehydrogenase activity was pooled and dialysed exhaustively against 0.5% ammonium bicarbonate in preparation for sequencing.

RESULTS AND DISCUSSION

SDS-PAGE of the material eluted from the final purification step gave a single band when stained with silver nitrate. SDS-PAGE implied that the enzyme preparation was at least 95% homogeneous and the subunit M_r of the purified protein was 29 kDa.

The enzyme was purified >2,000-fold to yield 50μg protein (from 20g wet weight cells).

APPENDIX III

The data presented below is a summary of the codon usage patterns in *S. pombe* and *S. cerevisiae* from Sharp *et al.*, 1988. The 'Relative Synonymous Codon Usage values' are presented for highly expressed genes and lowly expressed genes from both species.

		S. cerevisiae		*S. pombe*	
		high	low	high	low
Phe	UUU	0.19	1.38	0.44	1.28
	UUC	1.81	0.62	1.56	0.72
Leu	UUA	0.49	1.49	0.28	1.79
	UUG	5.34	1.48	2.16	0.80
Leu	CUU	0.02	0.73	2.44	1.55
	CUC	0.00	0.51	1.13	0.31
	CUA	0.15	0.95	0.00	0.87
	CUG	0.02	0.84	0.00	0.68
Ile	AUU	1.26	1.29	1.53	1.77
	AUC	1.74	0.66	1.47	0.59
	AUA	0.00	1.05	0.00	0.64
Met	AUG	1.00	1.00	1.00	1.00
Val	GUU	2.07	1.13	1.61	2.04
	GUC	1.91	0.76	2.39	0.65
	GUA	0.00	1.18	0.00	1.06
	GUG	0.02	0.93	0.00	0.24
Ser	UCU	3.26	1.56	3.14	1.33
	UCC	2.42	0.81	2.57	0.52
	UCA	0.08	1.30	0.00	1.56
	UCG	0.02	0.66	0.00	0.67
Pro	CCU	0.21	1.17	2.00	1.21
	CCC	0.02	0.75	2.00	0.83
	CCA	3.77	1.38	0.00	1.51
	CCG	0.00	0.70	0.00	0.45
Thr	ACU	1.83	1.23	1.89	1.52
	ACC	2.15	0.78	2.11	1.04
	ACA	0.00	1.38	0.00	1.04
	ACG	0.01	0.60	0.00	0.40
Ala	GCU	3.09	1.07	2.30	1.79
	GCC	0.89	0.76	1.49	0.50
	GCA	0.03	1.49	0.21	1.14
	GCG	0.00	0.68	0.00	0.57
Tyr	UAU	0.06	1.13	0.48	1.24
	UAC	1.94	0.87	1.52	0.76
ter	UAA	--	--	--	--
	UAG	--	--	--	--

		S. cerevisiae		*S. pombe*	
		high	low	high	low
His	CAU	0.32	1.16	0.56	1.44
	CAC	1.68	0.84	1.44	0.56
Gln	CAA	1.98	1.10	1.85	1.67
	CAG	0.02	0.90	0.15	0.33
Asn	AAU	0.06	1.28	0.30	1.41
	AAC	1.94	0.72	1.70	0.59
Lys	AAA	0.16	1.24	0.10	1.27
	AAG	1.84	0.76	1.90	0.73
Asp	GAU	0.70	1.38	0.78	1.56
	GAC	1.30	0.62	1.22	0.44
Glu	GAA	1.98	1.29	0.69	1.20
	GAG	0.02	0.71	1.31	0.80
Cys	UGU	1.80	1.10	0.14	1.56
	UGC	0.20	0.90	1.86	0.44
ter	UGA	--	--	--	--
Trp	UGG	1.00	1.00	1.00	1.00
Arg	CGU	0.63	0.64	5.17	1.89
	CGC	0.00	0.39	0.83	0.26
	CGA	0.00	0.65	0.00	0.86
	CGA	0.00	0.34	0.00	0.43
Ser	AGU	0.06	0.97	0.14	1.48
	AGC	0.16	0.70	0.14	0.44
Arg	AGA	5.37	2.51	0.00	1.71
	AGG	0.00	1.47	0.00	0.85
Gly	GGU	3.92	1.32	3.36	1.87
	GGC	0.06	0.92	0.59	0.27
	GGA	0.00	1.22	0.05	1.60
	GGG	0.02	0.55	0.00	0.27

APPENDIX IV

Published Paper

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Phosphoglycerate Mutase from *Streptomyces coelicolor* A3(2): Purification and Characterization of the Enzyme and Cloning and Sequence Analysis of the Gene

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The enzyme 3-phosphoglycerate mutase was purified 192-fold from *Streptomyces coelicolor*, and its N-terminal sequence was determined. The enzyme is tetrameric with a subunit M_r of 29,000. It is 2,3-bisphosphoglycerate dependent and inhibited by vanadate. The gene encoding the enzyme was cloned by using a synthetic oligonucleotide probe designed from the N-terminal peptide sequence, and the complete coding sequence was determined. The deduced amino acid sequence is 64% identical to that of the phosphoglycerate mutase of *Saccharomyces cerevisiae* and has substantial identity to those of other phosphoglycerate mutases.

The enzymes of central metabolism in streptomycete species have been studied very little, despite the considerable commercial importance of these organisms as sources of antibiotics. Many fermentations are glucose based, and it is usually assumed that glucose is metabolized principally by the Embden-Meyerhoff pathway. However, to date, for *Streptomyces* spp., no enzyme of the pathway has been characterized fully and none of the genes of the pathway have been cloned and analyzed. As part of a program to study the genes and enzymes of central metabolism in *Streptomyces coelicolor*, we purified to apparent homogeneity the aromatic biosynthetic enzyme shikimate dehydrogenase. Our purified preparation of shikimate dehydrogenase showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an M_r of 29,000, but when the amino-terminal sequence analysis of this preparation was attempted, a 14-residue sequence was obtained which was in low yield (3%) compared with the estimated number of picomoles of protein presented to the sequencer. The sequence corresponded closely to the N-terminal sequences of a number of eukaryotic 3-phosphoglycerate mutases (EC 2.7.5.3). Apparently the shikimate dehydrogenase in the preparation could not be sequenced, but it contained phosphoglycerate mutase as a minor contaminant. This chance observation prompted us to characterize this important glycolytic enzyme for which there is no primary structure information from any bacterial source. In this paper we report the purification of 3-phosphoglycerate mutase from *S. coelicolor* A3(2), biochemical characterization of the enzyme and comparison with some other 3-phosphoglycerate mutases, cloning and sequencing of the gene, analysis of the coding region, and comparison of the deduced amino acid sequence with those of the enzymes from other species.

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MATERIALS AND METHODS

Reagents. Reactive Blue 2-Sepharose CL-6B and Reactive Red 120-Agarose (type 3000-CL) were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom; rabbit muscle phosphoglycerate mutase and 3-phosphoglycerate (grade 1) were obtained from Boehringer Corp., Lewes, East Sussex, United Kingdom. Restriction endonucleases, bacteriophage-T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of *Escherichia coli* DNA polymerase were purchased from Gibco-BRL, Paisley, Scotland, United Kingdom; Taq DNA polymerase and Taquence sequencing kits were from U.S. Biochemical Corp. via Cambridge Bioscience, Cambridge, United Kingdom.

Bacterial strains, vectors, and growth of cells for enzyme isolation. *S. coelicolor* JI3456 (SCP1^{NF}, SCP2$^-$) was provided by D. A. Hopwood, John Innes Institute. *E. coli* DS941 (23) and plasmid pUC18 were used in the primary genomic cloning. *E. coli* TG1 and phages M13 mp18 and M13 mp19 (18) were used for DNA sequencing. *S. coelicolor* JI3456 was grown in YEME medium (9).

Assay of 3-phosphoglycerate mutase activity. The enolase coupled assay was used with an assay volume of 1 ml (7). One unit is defined as a change in A_{240} of 0.1 per min. Under these conditions 1 μmol of 3-phosphoglycerate consumed per min is reported to be equivalent to a change in A_{240} of 0.87 (7). Protein was determined by the method of Sedmak and Grossberg (20) with bovine serum albumin as a standard.

Purification of 3-phosphoglycerate mutase. All steps in the purification of 3-phosphoglycerate mutase were performed at 4°C unless otherwise stated.

(i) Step 1: preparation of crude extract. A 20-g (wet weight) batch of *S. coelicolor* grown to the midlogarithmic phase for 48 h was harvested and resuspended in 25 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 1.2 mM phenylmethylsulfonyl fluoride, and 0.4 mM dithiothreitol. The cells were broken by passage through a French pressure cell (98-MPa internal pressure). The cell lysate was centrifuged at 100,000 × *g* for 1 h.

(ii) Step 2: fractionation with $(NH_4)_2SO_4$. The supernatant of the crude extract (20 ml) was subjected to fractionation

with ammonium sulfate. The fraction that precipitated between 50% and 70% saturation contained the enzyme activity. It was dissolved in 2 ml of 10 mM Tris-HCl (pH 8.0) (buffer A) and dialyzed overnight against buffer A.

(iii) Step 3: chromatography with Cibacron-Blue Sepharose. The solution from step 2 was applied to a column (2.5 cm by 5.0 cm^2) of Cibacron-Blue Sepharose, previously equilibrated with buffer A. The phosphoglycerate mutase activity was not retained when the column was eluted with buffer A. Fractions (3 ml) containing enzyme activity were pooled.

(iv) Step 4: chromatography with Procion-Red Agarose. The enzyme solution from step 3 was applied to a column (12 cm by 0.8 cm^2) of Procion-Red Agarose (Reactive Red 120-agarose) previously equilibrated with buffer A. After the column was washed with 2 column volumes of buffer A, phosphoglycerate mutase activity was eluted by including 4 mM 2,3-bisphosphoglycerate (BPG) in buffer A. Fractions (3 ml) containing enzyme activity were pooled for long-term storage at 4°C. Elution from the Procion-Red Agarose column could also be achieved by using 5 mM 3-phosphoglycerate instead of BPG in buffer A.

Circular dichroism spectra. Circular dichroism spectra were recorded at 20°C on a Jasco J-600 spectrophotometer. The enzyme (0.1 mg/ml) was in 10 mM Tris-HCl (pH 8.0); the cell path length was 0.1 cm.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (12) with 12% (wt/vol) polyacrylamide gels. Protein was detected by staining with Coomassie blue. A calibration curve was constructed by using the following M_r markers: bovine serum albumin (66,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200).

Native M_r of phosphoglycerate mutase. The M_r of the enzyme was determined by gel filtration with a column (40 cm by 6.2 cm^2) of Sephacryl S-300 eluted with 0.1 M sodium phosphate buffer (pH 7.4). The following proteins were used as native M_r standards: lactate dehydrogenase (144,000), aspartate aminotransferase (92,000), bovine serum albumin (66,000), ovalbumin (43,000), trypsinogen (24,000), and RNase (14,000).

Determination of amino-terminal amino acid sequence. A sample of the purified 3-phosphoglycerate mutase was sequenced by B. Dunbar (University of Aberdeen) on an Applied Biosystems model 470A gas-phase sequencer as described by Russell et al. (17). The analysis gave a 25-residue sequence.

The protein sample, which contained shikimate dehydrogenase purified to apparent homogeneity by gel electrophoresis criteria, was sequenced by J. Young (ICI Pharmaceuticals) as described by White et al. (25). Whereas 200 pmol was presented to the analyzer, the initial yield (3%) indicated that the major protein species in the sample (shikimate dehydrogenase) was not being sequenced. Fourteen amino acids of a minor species were detected with a step yield of 94%.

Oligonucleotides. Oligonucleotides for screening of genomic DNA and genomic libraries and for primers in DNA sequencing were synthesized by V. Math, Department of Biochemistry, University of Glasgow, with an Applied Biosystems model 380A DNA synthesizer or an Applied Biosystems PCR-Mate.

Molecular biological methods. Total DNA of *S. coelicolor* J13456 was prepared essentially as described by Hopwood et al. (9). Other molecular biological procedures were carried out as described by Sambrook et al. (18). Genomic digests were transferred to Hybond-N (Amersham) as described by Southern (22). The filter was incubated with radiolabeled oligonucleotide as described by Binnie (2). DNA sequencing was performed by the dideoxy-chain termination method (19) with [α-^{32}P]dATP. To overcome problems of primer extension associated with secondary structure of template DNA, sequencing reactions were carried out at 70°C with Taq DNA polymerase, usually with 7-deaza-dGTP as a replacement for dGTP in the reactions. Electrophoresis was in 8 M urea–6% (wt/vol) polyacrylamide linear gels. Sequences were compiled and analyzed by using the sequence analysis programs of the University of Wisconsin Genetics Computer Group (5).

Nucleotide sequence accession number. The data shown in Fig. 5 have been deposited with the EMBL data base under accession number X123456.

TABLE 1. Purification scheme for 3-phosphoglycerate mutase of *S. coelicolor*[a]

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude extract	91	1,450	15	100
Ammonium sulfate	26	1,040	40	72
Cibacron-Blue chromatography	14.2	1,080	76	75
Procion-Red chromatography eluted with BPG	0.17	490	2,880	34

[a] These data are from cell samples of 20 g (wet weight).

RESULTS

Purification of enzyme. The purification of the 3-phosphoglycerate mutase of *S. coelicolor* is summarized in Table 1. The enzyme preparation was at least 95% homogeneous, as judged by SDS-PAGE (Fig. 1). The specific activity (2,880 U/mg) corresponded to a 192-fold purification.

Quaternary structure of enzyme. The subunit of 3-phosphoglycerate mutase migrated on SDS-PAGE gels with a

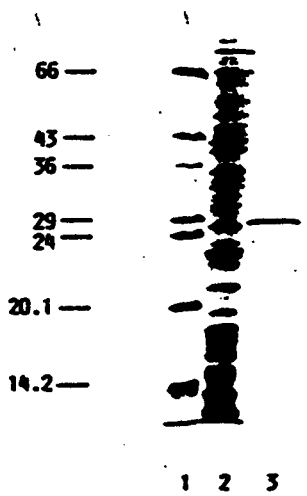

FIG. 1. SDS-PAGE of phosphoglycerate mutase at various stages of purification. Lanes: 1, molecular weight markers; 2, after elution through the Cibacron-Blue column; 3, after elution through the Procion-Red column. The numbered bars show the M_r values (10^3) of marker proteins.

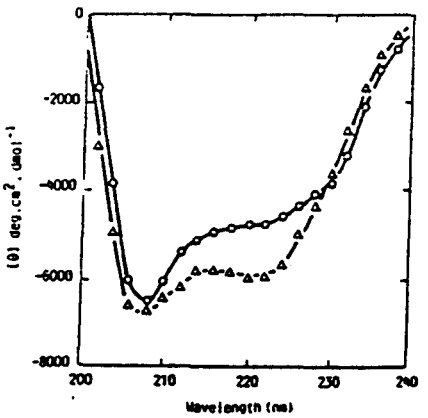

FIG. 2. Circular dichroism spectrum Δ. *S. coelicolor* enzyme; O. *S. cerevisiae* enzyme (data from Hermann et al. [8]). For further details, see the text.

mobility consistent with an M_r of 28,800 ± 2,000. The native M_r, determined by gel filtration, was estimated at 120,000 ± 10,000. Taken together, these results indicate that the *S. coelicolor* enzyme is a tetramer, similar to that from *Saccharomyces cerevisiae*.

Circular dichroism spectrum. In a preliminary experiment, it was shown that the far-UV circular dichroism spectrum of the enzyme (Fig. 2) was similar in shape and magnitude to that determined previously for the *S. cerevisiae* enzyme (8). It would appear that the two enzymes have similar overall secondary structures, although this conclusion would have to be confirmed when larger quantities of the *S. coelicolor* enzyme are available.

Kinetic properties. (i) Dependence of enzyme activity on BPG. The enzyme from *S. cerevisiae* is dependent on BPG as a cofactor for full activity (10). After prolonged dialysis (5 days, 12 changes) against 10 mM Tris-HCl (pH 8.0), the enzyme from *S. coelicolor* was active in the absence of added BPG. This could have been due to the stability of the putative phosphoenzyme intermediate formed in the presence of BPG, which was used to elute the enzyme from the Procion-Red Agarose column at step 4 of the purification. When the enzyme was prepared by elution of the column with 3-phosphoglycerate and assayed in the absence of BPG, the specific activity was low (590 U/mg). The activity was restored (to 3,000 U/mg) by including 0.3 mM BPG in the assay (i.e., under normal assay conditions).

(ii) Inhibition by vanadate. Inhibition of phosphoglycerate mutases by vanadate has been proposed as a diagnostic tool for BPG-dependent enzymes (3). The addition of sodium metavanadate to the assay mixture resulted in marked inhibition of the *S. coelicolor* enzyme; 10 and 100 μM metavanadate led to 80 and 100% inhibition, respectively. These values are very similar to those observed by Carreras et al. (3) for a variety of BPG-dependent enzymes. The enzyme from *S. coelicolor* behaved similarly to others in that inhibition became fully effective only 2 to 3 min after the addition of metavanadate.

(iii) K_m for 3-phosphoglycerate. In the presence of 0.3 mM BPG, the K_m for 3-phosphoglycerate was 1.3 ± 0.1 mM, a value similar to those reported for other BPG-dependent enzymes under these assay conditions (16).

Cloning of the phosphoglycerate mutase gene. The minor protein species in the (apparently pure) shikimate dehydrogenase preparation gave the amino-terminal sequence ADAPYKLILRHG. By using the TFASTA program within the GCG DNA manipulation package, this peptide sequence was compared with all sequences in the GenBank data base (release 60). The streptomycete protein had high sequence identity with phosphoglycerate mutases. A fresh batch of cells was prepared, and phosphoglycerate mutase was purified as described above (Table 1). The N-terminal sequence of the preparation of pure enzyme confirmed the original data and extended it to 25 amino acids. Streptomycete genes have an unusual codon bias due to the high G+C composition (73%) of total DNA (1), which simplifies the design of oligonucleotide probes to clone genes based on peptide sequence data. A 24-nucleotide probe (24-mer) was designed against amino acids 1 through 9 of the protein (Fig. 3). The probe had two redundancies.

Digests of total DNA of *S. coelicolor* J13456 were transferred (22) to nylon membranes. Conditions for hybridization and washing were varied until a unique signal of labeled probe was obtained. The optimum conditions were as follows: radiolabeled oligonucleotide (10 ng/ml; $>10^8$ dpm/μg) incubated for 1.5 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (18)–0.5% (wt/vol) sodium pyrophosphate–0.5% (wt/vol) SDS–200 μg of heparin per ml and then washed twice at 65°C in 5× SSC–0.5% (wt/vol) SDS. Of the various signals obtained with restriction digestions of genomic DNA with different enzymes, a 3.1-kb *SalI* band was judged to be optimal for subcloning. A genomic sublibrary containing *SalI* fragments from 2.8 to 3.5 kb in size was subcloned into the vector pUC18. Recombinants containing the hybridizing sequence were identified by colony hybridization; pGLW105 was taken as a representative recombinant.

DNA sequence. The genomic insert of pGLW105 was subcloned into M13 mp18 (to give mGLW106) and into M13 mp19 (to give mGLW107). DNA sequence was obtained (Fig. 4 gives the overall strategy) with a universal primer and with oligonucleotide primers constructed sequentially. The complete nucleotide sequence and deduced amino acid sequence are shown in Fig. 5.

DISCUSSION

The biochemical properties of phosphoglycerate mutase from *S. coelicolor* (subunit molecular weight, quaternary

amino acid residue		1	2	3	4		5	6	7	8	9
		A	D	A	P		Y	K	L	I	L
oligo	5'	C	GAC	GCC	CC(CG)		TAC	AAG	CT(GC)	ATC	CT	3'
		*	***	**	**	*	***	***	**	*	***	**

FIG. 3. Amino-terminal sequence of phosphoglycerate mutase and design of the oligonucleotide probe. Asterisks indicate bases of the probe that are identical to those of the genomic DNA sequence.

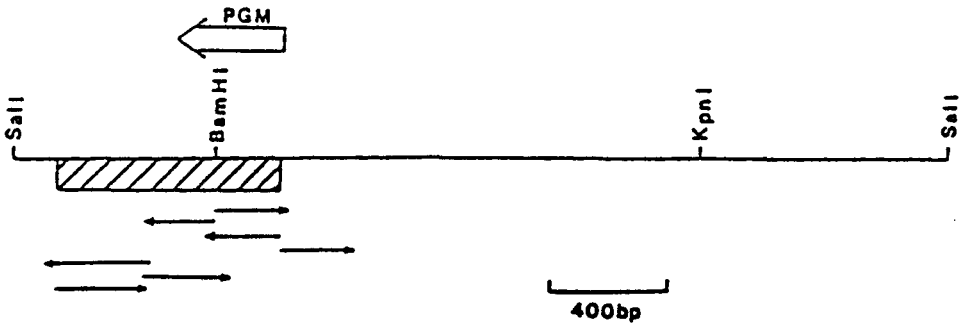

FIG. 4. Sequencing strategy for the phosphoglycerate mutase (PGM) gene.

structure, circular dichroism spectrum, cofactor or inhibitor dependence) implied that the enzyme was similar to that described previously from *S. cerevisiae* (10). At the level of deduced primary amino acid sequence, the *S. coelicolor* enzyme shows high similarity to the other phosphoglycerate mutases for which primary structures are available (Fig. 6). As far as we are aware, this is the first prokaryotic sequence to be reported. The sequence identity with phosphoglycerate

```
   1  CCGTCCAACCGTCCGCCCACCGGGGCGCACGCGCGGGGGATCAGGCCTTGGATTACGCTC   60
      RBS  M  A  D  A  P  Y  K  L  I  L  L  R  H  G  E  S  E  W
  61  GGAAGCATGGCCGACGCACCGTACAAGCTGATCCTCCTCCGCCACGGCGAGAGCGAGTGG  120
 121  AACGAGAAGAACCTGTTCACCGGCTGGGTGGACGTCAACCTCACCCCGAAGGGCGAGAAG  180
      N  E  K  N  L  F  T  G  W  V  D  V  N  L  T  P  K  G  E  K
 181  GAGGCGACGCGCGGCGGCGAGCTGCTCAAGGACGCCGGCCTGCTGCCCGACGTGGTCCAC  240
      E  A  T  R  G  G  E  L  L  K  D  A  G  L  L  P  D  V  V  H
 241  ACGTCCGTCCAGAAGCGCGCGATCCGCACGGCCCAGCTCGCGCTGGAGGCCGCCGACCGC  300
      T  S  V  Q  K  R  A  I  R  T  A  Q  L  A  L  E  A  A  D  R
 301  CACTGGATCCCGGTCCACCGCCACTGGCGCCTGAACGAGCGCCACTACGGCGCCCTCCAG  360
      H  W  I  P  V  H  R  H  W  R  L  N  E  R  H  Y  G  A  L  Q
 361  GGCAAGGACAAGGCCCAGACCCTCGCCGAGTTCGGCGAGGAGCAGTTCATGCTGTGGCGC  420
      G  K  D  K  A  Q  T  L  A  E  F  G  E  E  Q  F  M  L  W  R
 421  CGCTCCTACGACACCCCGCCGCCCGCGCTGGACCGCGACGCCGAGTACTCCCAGTTCTCC  480
      R  S  Y  D  T  P  P  P  A  L  D  R  D  A  E  Y  S  Q  F  S
 481  GACCCGCGTTACGCGATGCTCCCGCCGGAGCTGCGCCCGCAGACGGAGTGCCTGAAGGAC  540
      D  P  R  Y  A  M  L  P  P  E  L  R  P  Q  T  E  C  L  K  D
 541  GTCGTCGGCCGGATGCTCCCGTACTGGTTCGACGCGATCGTCCCCGACCTCCTCACCGGC  600
      V  V  G  R  M  L  P  Y  W  F  D  A  I  V  P  D  L  L  T  G
 601  CGCACGGTCCTGGTGGCGGCGCACGGCAACTCCCTCCGCGCCCTCGTCAAGCACCTCGAC  660
      R  T  V  L  V  A  A  H  G  N  S  L  R  A  L  V  K  H  L  D
 661  GGCATCTCCGACGCCGACATCGCGGGCCTGAACATCCCGACGGGCATCCCGCTCTCGTAC  720
      G  I  S  D  A  D  I  A  G  L  N  I  P  T  G  I  P  L  S  Y
 721  GAACTCAACGCCGAGTTCAAGCCCCTGAACCCGGGCGGCACGTACCTCGACCCGGACGCG  780
      E  L  N  A  E  F  K  P  L  N  P  G  G  T  Y  L  D  P  D  A
 781  GCCGCGGCGGCGATCGAGGCCGTGAAGAACCAGGGCAAGAAGAAGTAAGCGCGCACGAAC  840
      A  A  A  A  I  E  A  V  K  N  Q  G  K  K  K  *
 841  AGGCCCCCTACCTGCGGTTTCTCCGCGAGTAGGGGGCTTTGTGTTGTCGTGGGCCGTCTC  900
 901  TGGGCCGTTTCTTGCTCGGCG  921
```

FIG. 5. DNA sequence and deduced protein sequence.

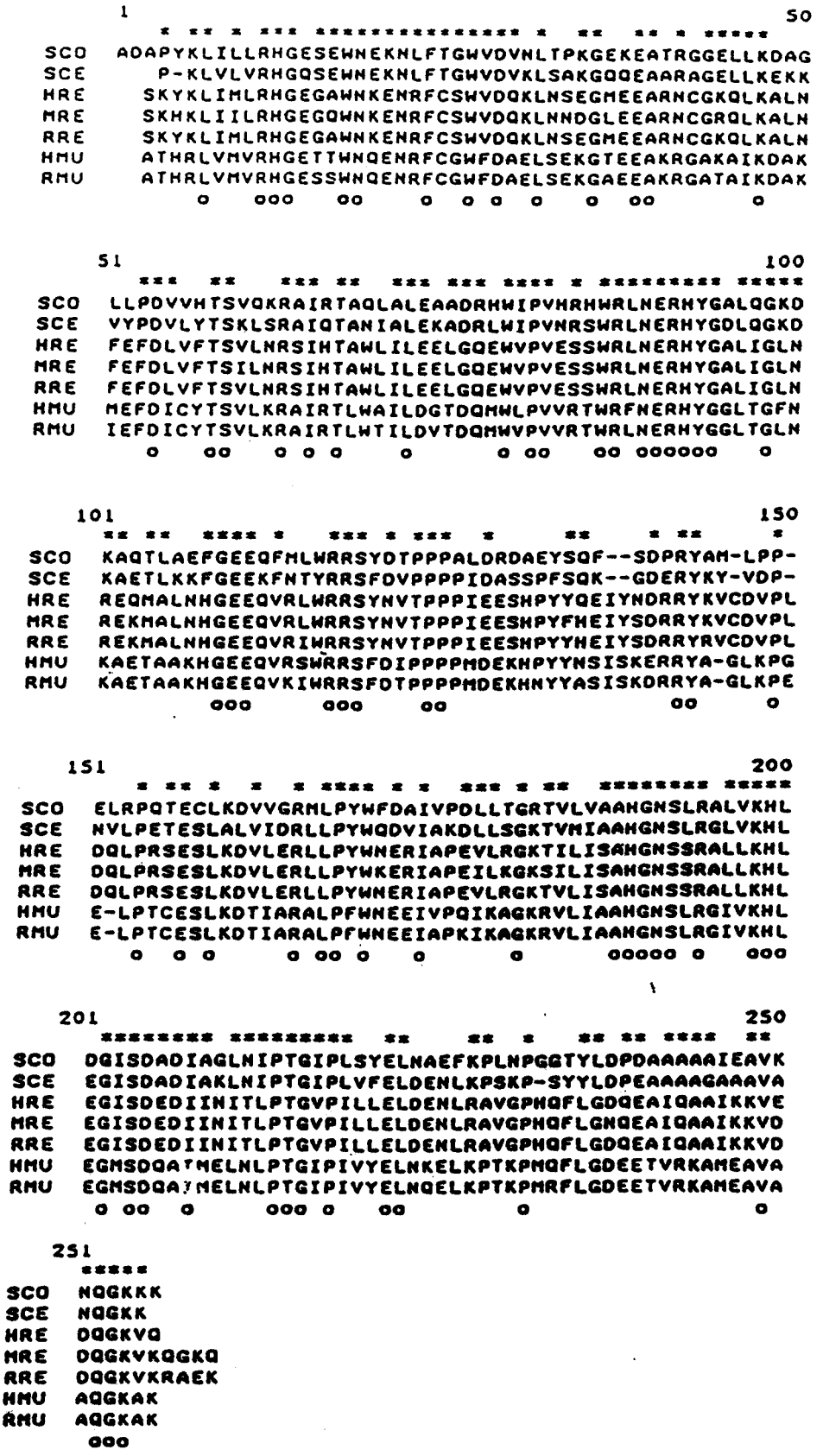

FIG. 6. Comparison of protein sequences of phosphoglycerate mutase from *S. coelicolor* (SCO) (this work), *S. cerevisiae* (SCE) (24), human reticulocyte (HRE) (11), mouse reticulocyte (MRE) (14), rabbit reticulocyte (RRE) (27), human muscle (HMU) (21), and rat muscle (RMU) (4). Sequences of the enzymes of *S. coelicolor* and *S. cerevisiae* that are identical (•) and by residues that are identical in all seven proteins (O) are indicated.

mutase from *S. cerevisiae* is particularly striking, but the high sequence identity with the mammalian proteins also reinforces the view (6) that glycolytic genes have been highly conserved during evolution. Of particular note, the *S. coelicolor* enzyme has at its carboxy terminus an unusual run of alanine residues and several lysines. The codons for lysine (AAA, AAG) are A+T rich. Because of the high G+C content of streptomycete DNA, lysine codons are relatively rare. Where there is a requirement for a positively charged residue, arginine (which has six codons, some of which contain only G+C) is often substituted for lysine (24a). The flexible tail of lysine residues, which is highly conserved between *S. coelicolor* and *S. cerevisiae*, has been proposed to be involved in limiting the access of substrate to the active site. The crystal structure of the *S. cerevisiae* enzyme has been determined (26), so it will be possible to use it to model the likely structure of the streptomycete protein.

Despite the high sequence identity with the other mutases, the enzyme from *S. coelicolor* has some unusual properties. It did not bind to Cibacron-Blue, a property previously correlated with dependence on BPG (16). Even after prolonged dialysis, it was difficult to demonstrate BPG dependence when the enzyme was eluted with BPG during the last step in purification (Table 1). It was only when the enzyme was eluted with 3-phosphoglycerate that BPG dependence could be demonstrated effectively. The difficulty in establishing this dependence implies either that the *S. coelicolor* binds BPG very tightly compared with the enzyme from other sources or that the putative phosphorylated form of the *S. coelicolor* enzyme is very stable toward hydrolysis. Inhibition was observed when vanadate was added, which is consistent with the proposed BPG dependence of the enzyme (3).

The DNA sequence revealed that the 24-mer oligonucleotide probe designed to clone the gene (Fig. 3) had only one mismatch. The predicted amino acid sequence was identical to the first 25 residues determined by sequencing the purified protein. In common with many bacterial proteins, the nascent polypeptide is processed to remove the *f*-methionyl residue to give the native form of the protein. A reasonable ribosome binding site (CGGA) was situated just upstream of the ATG start codon. The coding region of the gene displayed the G+C bias that is usual for streptomycete genes (1): 69% G+C in the first position, 42% G+C in the second position, and 99% G+C in the third position. By using the program Codonpreference (5) with threshold of 0.10, only two rare codons were identified within the coding region. Thus, the *S. coelicolor* gene shows the paucity of rare codons observed with glycolytic genes of other species, and this is likely to be a feature of other glycolytic genes from streptomycetes. Most of the streptomycete genes sequenced to date have been involved in differentiation or antibiotic biosynthesis and resistance. Expression of at least 1 tRNA species ($tRNA^{Leu}_{TTA}$ [13]) is regulated during the streptomycete life cycle, which results in temporal regulation of translation of genes containing TTA codons. The phosphoglycerate mutase gene of *S. coelicolor* has no TTA codons. It has also been suggested that antibiotic-related genes could have a codon usage that is different from those of central metabolism. The codon usage of the (highly expressed) phosphoglycerate mutase gene is not significantly different from those of other streptomycete genes sequenced so far, so this is unlikely to be the case. Although the peptide sequence translated from the DNA sequence of this gene corresponds at the amino terminus to the purified protein and has 64% identity with the corresponding protein of *S. cerevisiae*, it remains to be proven by overexpression or gene disruption that the cloned gene actually encodes the enzyme.

Under the growth conditions employed in this study, phosphoglycerate mutase was some 0.5% of the total protein of *S. coelicolor*. In other species many glycolytic genes are highly expressed and have formed the basis of high-level expression vectors. It will be important to identify and characterize the promoter of this gene, which could be useful in the expression of heterologous genes, and to study its activity during growth on glycolytic and gluconeogenic substrates.

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