The amino acid sequence of the small monomeric phosphoglycerate mutase from the fission yeast *Schizosaccharomyces pombe*

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The amino acid sequence of the monomeric 2,3-bisphosphoglycerate (BPG)-dependent phosphoglycerate mutase (PGAM) from the fission yeast *Schizosaccharomyces pombe* has been determined. Amino acid sequencing of proteolytic fragments of the enzyme showed the *S. pombe* mutase to be similar in sequence to the tetrameric enzyme of baker's yeast (*Saccharomyces cerevisiae*). An *S. pombe* cDNA library was screened using a PCR fragment generated from two oligonucleotides complementary to sequences encoding the regions at the two active-site histidine residues. The 0.63 kb cDNA encoded an open reading frame of 210 amino acids. This sequence agreed completely with sequences of peptides derived from the purified protein. The amino acid sequence of *S. pombe* PGAM is 43% identical with that of *S. cerevisiae* PGAM and shows an equally high degree of identity with BPG-dependent PGAMs from other sources. However, the sequence of the *S. pombe* enzyme differs from other BPG-dependent enzymes in three important ways: (i) it does not contain the alanine- and lysine-rich sequence of amino acids at the C-terminus which have been proposed to constitute a flexible tail involved in catalysis; (ii) the sequence spanning residues 122–146 (*S. cerevisiae* PGAM numbering) is not present in the *S. pombe* PGAM sequence; in the *S. cerevisiae* PGAM crystal structure this stretch of sequence has been shown to occur as an extended loop, part of which is involved in inter-subunit interactions; (iii) the amino acid sequence in the region of a second *S. cerevisiae* inter-subunit contact (residues 74–78) shows radical mutations in the *S. pombe* enzyme.

INTRODUCTION

Phosphoglycerate mutase (PGAM) (EC 5.4.2.1) catalyses the interconversion of 2-phosphoglycerate and 3-phosphoglycerate (Meyerhof and Kiessling, 1935). There are broadly two classes of phosphoglycerate mutase: those which are active in the absence of 2,3-bisphosphoglycerate (BPG) and those which depend on BPG for activity. The mechanism of the latter group involves formation and breakdown of an intermediate in which a histidine side chain is phosphorylated. BPG-independent enzymes have been found in plant tissues, filamentous fungi, certain algae, invertebrates and strains of *Bacillus* (Carreras et al., 1982; Price et al., 1983). The BPG-dependent enzymes have been found in vertebrates, certain invertebrates, bacteria and fungi (Carreras et al., 1982). A comparative study by Price and Stevens (1983) showed that BPG-dependent enzymes were retained on Cibacron Blue–Sepharose and could be eluted by a pulse of BPG, whereas BPG-independent enzymes failed to bind to Cibacron Blue–Sepharose. During this study, *S. pombe* PGAM was found to be BPG-dependent, and was later purified from a crude cell extract using a one-step procedure involving the specific elution of the enzyme from Cibacron Blue–Sepharose with a pulse of BPG (Price et al., 1985). *S. pombe* PGAM was shown to be a monomer of M_r approx. 23000. Being monomeric and having a low subunit M_r, *S. pombe* PGAM differs from nearly all other BPG-dependent enzymes, which have been shown to be dimeric or tetrameric with a subunit M_r between 26000 and 30000 depending on the source (Table 1).

The most extensively studied PGAM is the tetrameric enzyme from *S. cerevisiae*: the amino acid sequence has been deduced by sequencing the protein (Fothergill and Harkins, 1982), the DNA encoding the enzyme has been isolated and sequenced (White and Fothergill-Gilmore, 1988) and the X-ray structure (Winn et al., 1981; Watson, 1982) has been determined. Combining this structural information with kinetic studies, a reaction mechanism has been proposed for *S. cerevisiae* PGAM (Fothergill-Gilmore and Watson, 1989). Currently this proposed mechanism is being tested by site-directed mutagenesis in combination with biophysical techniques, including high-resolution n.m.r. spectroscopy. However, owing to the size of the *S. cerevisiae* enzyme (M_r 108000), many aspects of the structure and activity remain inaccessible to high-resolution n.m.r. spectroscopy. The small PGAM from *S. pombe* (M_r 23000) is within the size range in which n.m.r. can be used. Thus the *S. pombe* enzyme presents an ideal system for probing the structure and mechanism of PGAM.

In this paper we present the amino acid sequence of *S. pombe* PGAM deduced from a cDNA encoding this enzyme and from sequencing peptides isolated from *S. pombe* PGAM. The sequence of this monomeric enzyme is compared with the sequences of other oligomeric BPG-dependent enzymes, especially the well-characterized tetrameric PGAM from *S. cerevisiae*. The polypeptide chain of the *S. pombe* enzyme is 36 residues shorter than the *S. cerevisiae* enzyme, with significant deletions in the middle of the chain and at the C-terminus. The sequence of the *S. pombe* enzyme offers a structural explanation of the monomeric nature of the enzyme.

Abbreviations used: BPG, 2,3-bisphosphoglycerate; PGAM, phosphoglycerate mutase; DIG, digoxygenin; CR, contact region; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate.

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The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X75385.

Table 1 Quaternary structures of BPG-dependent PGAMs

References: [1]Price et al. (1985); [2]Hill and Attwood (1976); [3]Pawluk et al. (1988); [4]LeBoulch et al. (1988); [5]Joulin et al. (1986); [6]Yanagawa et al. (1986); [7]Shankse et al. (1987); [8]Castella-Escola et al. (1989); [9]White et al. (1992); [10]White and Fothergill-Gilmore (1988).

Source	Subunit M_r	No. of subunits
S. pombe[1]	23000‡	1
Pseudomonas AM1[2]	32000†	(1)†
Zymomonas mobilis[3]	26000‡	2
Mouse E-type[4]	30000*	2
Human E-type[5]	30000*	2
Rabbit E-type[6]	30000*	2
Human M-type[7]	29000*	2
Rat M-type[8]	29000*	2
Strep. coelicolor[9]	29000*	4
S. cerevisiae[10]	27000*	4

*Subunit M_r calculated from the amino acid sequence.

†The total M_r of the enzyme was determined by gel filtration of an enzyme preparation which was 40% pure; the subunit M_r was not determined.

‡Subunit M_r was determined by SDS/PAGE.

EXPERIMENTAL

Materials

All growth media were from Difco. The *Escherichia coli* strains NM522 and XL1-Blue were purchased from Stratagene. *S. pombe* (CMI 39917) was supplied by the Commonwealth Mycological Institute, Kew, Surrey, U.K. Novozym 234 was supplied by Novo Enzymes Ltd.. The TA cloning kit was Invitrogen, and the *S. pombe* cDNA library in λZAP II was kindly given by Dr Dallan Young, University of Calgary, Calgary, Alberta, Canada. Helper phages (MK407 and R408), and the sequencing primers (T3 and T7) were supplied by Stratagene. Custom-made oligonucleotides were from Oswell DNA Service, University of Edinburgh. [α-^{35}S]dATP and Hybond-N were supplied by Amersham International. Digoxygenin (DIG)-11-cyclic UTP (AMPPD), and associated detection reagents were purchased from Boehringer Mannheim. Sequenase and sequenase sequencing kits were from United States Biochemical Corporation, via Cambridge Bioscience. Clostripain was purchased from Worthington, and all other enzymes were from Boehringer.

Growth of *S. 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, 20 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 25 g of KH_2PO_4 and 20 g of glucose, in an orbital incubator (150 rev./min) at 30 °C. Initially, a 10 ml culture was grown overnight; this was then used to inoculate 5 × 200 ml batches, which were grown to early stationary phase (48–60 h).

Purification of *S. pombe* PGAM

PGAM from *S. pombe* was extracted and purified as described previously (Price et al., 1985; Johnson and Price, 1987).

Ultracentrifugation

The M_r of *S. pombe* PGAM was determined by sedimentation-equilibrium measurements using a Beckman Spinco model E analytical ultracentrifuge as described by Price and Jaenicke

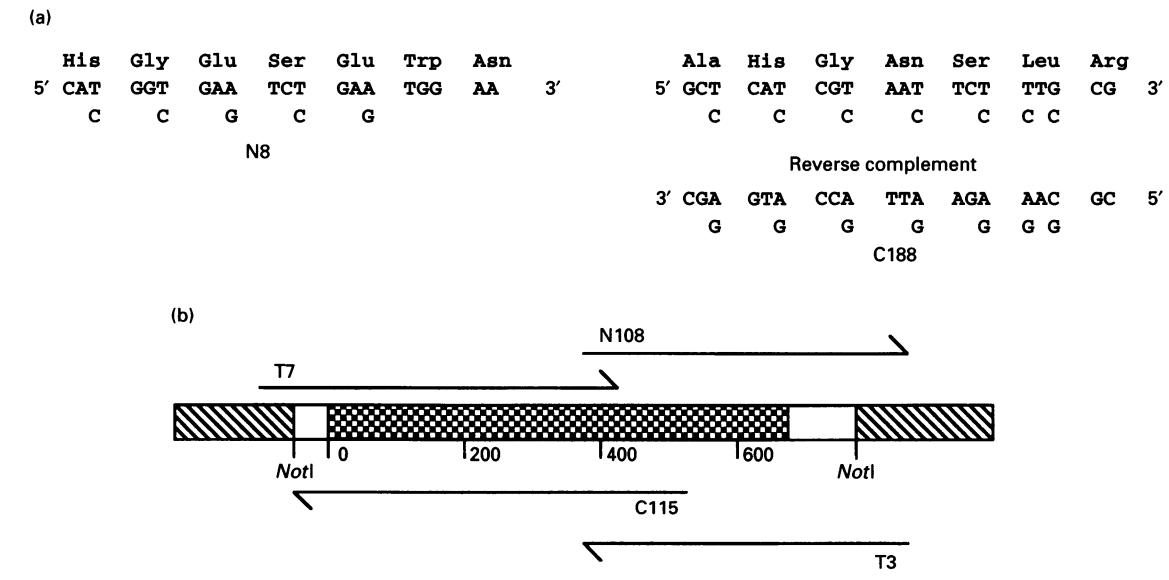

Figure 1 Sequencing strategy for *S. pombe* PGAM

(a) Design of PCR primers. Oligonucleotides were designed using the amino acid sequence around the two active-site histidine residues. The amino acid sequences of these two regions were determined by sequencing the *S. pombe* PGAM peptides. Primer N8 is 32-fold degenerate and C188 (the reverse complement of the coding strand) is 128-fold degenerate. Restriction sites (*Sal*I, N8; *Bam*HI, C188) were added to the 5′ end of each of the primers to facilitate subcloning. (b) Sequencing of the cDNA. The sequence of *S. pombe* PGAM was determined by sequencing both strands using double-stranded DNA generated from pBluescript containing the cDNA of interest. The primers used (T3, T7, N108 and C115) and the corresponding sequenced regions are shown. The pBluescript sequence is indicated by hatched lines. Non-coding DNA is represented by the empty boxes and the open-reading frame of *S. pombe* PGAM is represented by the chequered region. *Not*I sites were used in the construction of the cDNA library.

(1982). A value of 0.734 ml/g for the partial specific volume (calculated from the amino acid composition of the enzyme) was used in the calculation of M_r.

Digestion of protein and h.p.l.c. separation of peptides

The purified mutase was digested separately with clostripain, Glu-C endopeptidase and Asp-N endopeptidase at a 1:50 ratio of protease to substrate. Peptides were separated by reverse-phase h.p.l.c. using a Waters C-18 μBondapak column (3.9 mm × 300 mm) equilibrated in 0.1% trifluoroacetic acid using a linear gradient of acetonitrile or methanol/acetonitrile/propan-2-ol (1:1:1, by vol.) as described previously (Carter et al., 1983).

Sequencing of peptides

Amino acid sequencing was done by sequential Edman degradation in an Applied Biosystems model 470A gas-phase sequencer equipped with on-line h.p.l.c. for characterization of the phenythiohydantoin derivatives as described previously (Lambert et al., 1989).

Isolation of *S. pombe* genomic DNA

High-M_r genomic DNA from *S. pombe* was prepared as outlined by Moreno et al. (1991), with the exception that Novozym 234 was used to generate protoplasts rather than Zymolase 20T.

Oligonucleotide design for PCR

The primers used for PCR were oligonucleotides designed using the *S. pombe* PGAM amino acid sequence around the two active-site histidine residues (see Figure 1a). Attention was paid to the *S. pombe* codon-usage patterns determined by Sharp et al. (1988). To facilitate analysis and subsequent manipulation of the PCR product, restriction sites were included in the oligonucleotides.

PCR conditions

The optimal conditions for 100 μl of reaction mixture were as follows: either 2 μg of *S. pombe* genomic DNA or 10^6 plaque-forming units of *S. pombe* cDNA λZAP II library, 1.75 mM $MgCl_2$, 200 μM NTPs, 120 pmol of each primer (N8 and C188) and 2.5–5 units of *Taq* polymerase. After 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), the products were separated by electrophoresis on a 1.5%-agarose gel and revealed by ethidium bromide staining.

Cloning and sequencing the 0.47 kb PCR product

The 0.47 kb PCR product was directly inserted into the pCR™II vector which is supplied as part of the TA cloning system. As the pCR™II phagemid contains the F1 origin of replication, single-stranded DNA could be rescued in the presence of helper phage (MK407) and sequenced using oligonucleotide C188 as the primer. DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al., 1977) using [α-^{35}S]dATP. The sequence of this 0.47 kb PCR product confirmed that the amplified DNA encoded PGAM, as the deduced amino acid sequence revealed a high degree of similarity to other PGAM sequences.

Labelling PCR product

DIG was incorporated into the 0.47 kb PCR product using *Taq* polymerase. This method involved repeating the PCR reaction with two changes to the original conditions: the template was the pCR™II vector containing the 0.47 kb PCR product which was sequenced and the TTP concentration was lower and substituted with DIG-11-dUTP. The conditions for a 50 μl reaction mixture were as follows: 200 pg of 0.47 kb PCR product in pCR™II, 1.75 mM $MgCl_2$, 200 μM ATP, CTP, GTP, 130 μM TTP, 70 μM DIG-11-dUTP, 60 pmol of each primer and 2.5–5 units of *Taq* polymerase. After 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), the labelled PCR product was analysed on a 1.5%-agarose gel and revealed by staining with ethidium bromide.

cDNA library screening

An *S. pombe* cDNA library constructed in λZAP II was screened using the DIG-labelled PCR fragment. For the primary screen, 300000 plaques were plated out as outlined in the Stratagene manual. The plaques were transferred on to Hybond-N. The filters were incubated in prehybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5% blocking agent, 0.1% Sarcosyl, 0.02% SDS and 50% (v/v) formamide] at 42 °C for 1 h. The filters were then transferred to 30 ml of fresh prehybridization solution containing 700 ng of denatured DIG-labelled PCR product and incubated overnight at 42 °C. The filters were washed: twice in 2 × SSC/0.1% SDS at room temperature for 15 min, twice in 0.1 × SSC/0.1% SDS at room temperature for 15 min, and twice in 0.1 × SSC/0.1% SDS at 68 °C for 15 min. Hybridized DIG-labelled probe was detected using the chemiluminescence method outlined in the Boehringer-Mannheim protocol. The plaques selected were purified by plating at a low density and rescreening with DIG-labelled PCR product.

Analysis of selected λZAP II clones

Using the protocol outlined by Stratagene, pBluescript phagemids were prepared from four of the λZAP II clones selected from the screening procedure. cDNA inserts were ligated into the unique *Not*I site of the λZAP II vector during the synthesis of the library. Thus the phagemids were digested with *Not*I to release the cDNA inserts, which were then analysed on a 1.5%-agarose gel stained with ethidium bromide.

Sequencing-selected λZAP II clones

The four phagemids isolated following the screening procedure were sequenced directly by the dideoxy chain-termination method (Sanger et al., 1977) using [α-^{35}S]dATP. Alkaline-denatured double-stranded phagemid served as a template, and using the primers T3, T7, N108 and C115, the cDNA inserts of all four recombinants were sequenced in both directions (see Figure 1b).

The DNA sequences were analysed using the sequence analysis program of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

RESULTS

Amino acid sequencing

The intact mutase gave no amino acid sequence, indicating that the N-terminus was blocked. Sequences were obtained from many peptides (Figure 2), and they showed substantial similarity

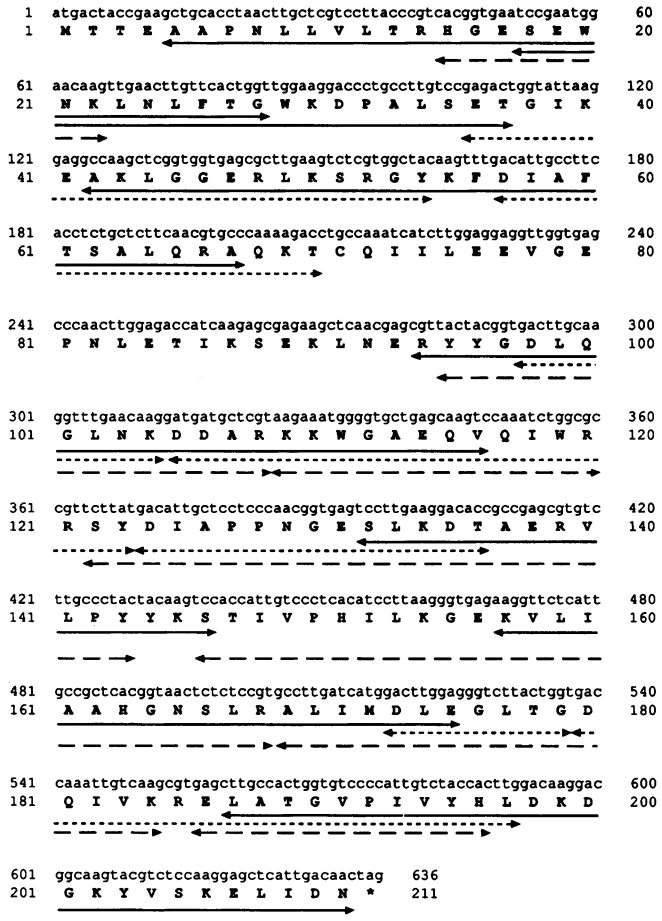

Figure 2 Amino acid sequence of *S. pombe* PGAM

The top line (lower-case letters) shows the nucleotide sequence of the sense strand of the cDNA. The bottom line shows the amino acid sequence in the one-letter code. The underlined regions show sequences which have been obtained by Edman degradation of peptides derived from digestion of the purified enzyme with Glu-C (←——→), clostripain (←– – –→), or Asp-N (←------→).

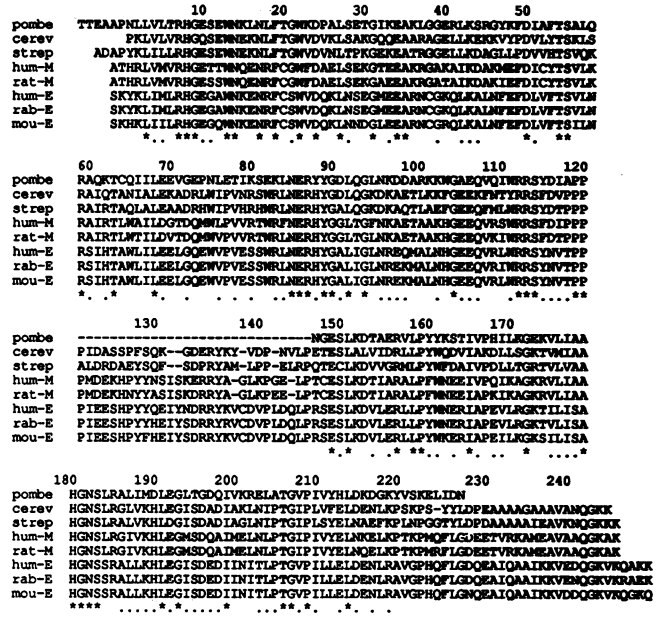

Figure 3 Alignment of the sequences of PGAM

In this alignment, abbreviations are as follows: pombe, *S. pombe* PGAM; cerev, *S. cerevisiae* PGAM; strep, *Streptomyces coelicolor* PGAM; hum-M, human M-type PGAM; rat-M, rat M-type PGAM; hum-E, human E-type PGAM; rab-E, rabbit E-type PGAM; mou-E, mouse E-type PGAM. The sequences have been aligned using the CLUSTAL program (Devereux et al., 1984), with subsequent modification of the N- and C-terminal regions to allow direct comparisons of these regions. Asterisks (*) indicate residues which are conserved in all of the sequences aligned, and bold dots (.) are indicative of positions in which conservative changes have occurred. The numbering is according to that of the *S. cerevisiae* enzyme.

to those of other BPG-dependent mutases and clearly showed conservation of the two regions containing the active-site histidine residues.

Amplification of DNA encoding *S. pombe* PGAM

Degenerate oligonucleotides were designed around the sequences of the active-site-histidine-containing peptides (Figure 1a), taking into account the codon bias for highly expressed *S. pombe* genes (Sharp et al., 1988) in order to minimize degeneracy. These oligonucleotides served as primers for amplification of *S. pombe* PGAM. The resultant 0.47 kb PCR fragment was characterized by sequencing, and this confirmed that *S. pombe* PGAM had been amplified. During initial PCR studies it was noted that the size of the amplified product remained constant regardless of whether the cDNA library or genomic DNA was used as the template, suggesting that the *S. pombe* PGAM gene contains no introns. The 0.47 kb PCR product was used to screen the *S. pombe* cDNA library cloned in λZAP II.

Isolation and sequencing of *S. pombe* PGAM cDNA

Approx. 300000 plaques from the *S. pombe* cDNA library were screened using the DIG-labelled PCR product. Four positive clones were purified, and phagemid DNA was rescued from each as described in the Experimental section. All four clones were found to contain cDNA inserts of 0.8 kb, which were sequenced as outlined in Figure 1(b). The complete nucleotide sequence and deduced amino acid sequence are shown in Figure 2.

Amino acid sequence of *S. pombe* PGAM

An open reading frame of 630 bases (excluding the start signal) encoded a protein of 210 amino acids. The codon usage for this protein shares the same bias pattern as displayed by other highly expressed *S. pombe* genes (Sharp et al., 1988). The complete sequence of *S. pombe* PGAM as deduced from the cDNA sequence is shown in Figure 2, together with the sequences of peptides derived from the protein by cleavage with clostripain, Glu-C endopeptidase and Asp-N endopeptidase. Figure 3 shows the alignment of *S. pombe* PGAM with the available sequences of other PGAMs. (To facilitate comparison with the X-ray structure, the numbering used is that of the *S. cerevisiae* PGAM.) Overall, the sequences share 52 identical residues, around 21% of the total amino acids, and a further 72 (29% of the total) represent conservative substitutions. The *S. pombe* and *S. cerevisiae* enzymes show 45% identity, with a further 17% conservative substitutions. The polypeptide chain of the *S. pombe* enzyme is 210 amino acids (plus the initiating methionine) in length, some 36 amino acids shorter than the chain of the *S. cerevisiae* enzyme. The two principal regions of deletion are a stretch of 25 amino acids in the middle of the chain (residues 122–146 inclusive, using the *S. cerevisiae* numbering) and a stretch of 17 amino acids at the C-terminus. The nucleotide sequence (Figure 2) suggests that the *S. pombe* enzyme has an

extension of six amino acids at the N-terminus. However, direct amino acid sequencing of the whole protein gave no sequence, suggesting that the protein had undergone post-translational modification at the N-terminus.

The M_r of the protein from the sequence is 23600, a value in close agreement with that (23000) obtained from SDS/PAGE and gel filtration (Price et al., 1985; Johnson and Price, 1987) and from equilibrium ultracentrifugation (22500).

DISCUSSION

Several features of interest emerge from a comparison of the sequence of *S. pombe* mutase with those from other sources (Figure 3).

Active site

Residues identified as being at or near the active site in the *S. cerevisiae* enzyme (Watson, 1982; Fothergill-Gilmore and Watson, 1989) are conserved in the *S. pombe* enzyme. These are Arg-7, His-8, Gly-9, Ser-11, Thr-20, Gly-21, Trp-22, Glu-86, Ala-179, Ala-180, His-181 and Gly-182 (*S. cerevisiae* PGAM numbering used). Gln-10 in the *S. cerevisiae* enzyme is replaced by Glu, a substitution made in all the other mutases for which sequence data are available (Figure 3). It is thus likely that the active site of the *S. pombe* enzyme is very similar to that of the *S. cerevisiae* enzyme, since the residues identified as playing a role in the catalytic process, the binding of substrate and the maintenance of the integrity of the active site of the latter enzyme (Fothergill-Gilmore and Watson, 1989) are conserved in the *S. pombe* enzyme.

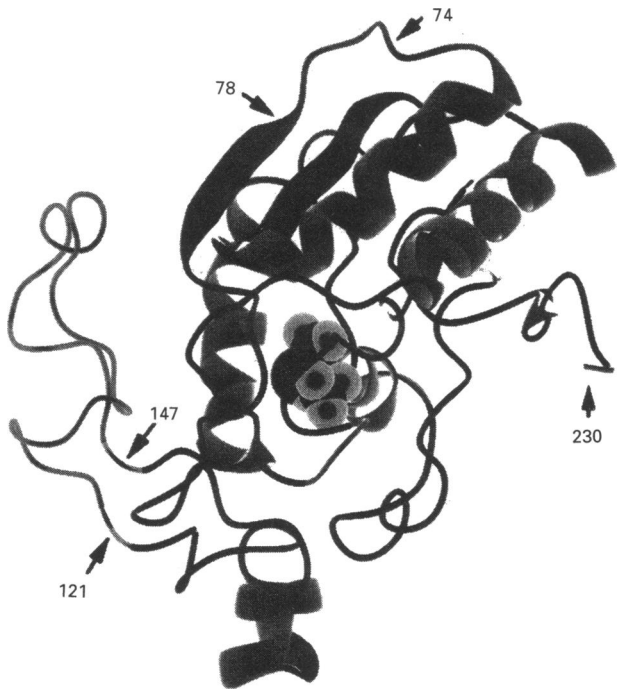

Figure 4 Structure of one subunit of *S. cerevisiae* PGAM

This cartoon representation of the structure is based on the X-ray structure determined by Watson (1982). The polypeptide chain is represented as a ribbon for helix and sheet, and as a thread for irregular structure; the substrate is shown as a space-filling model. Arrows identify the regions corresponding to significant differences between the *S. pombe* and *S. cerevisiae* sequences. Region 74–78 is responsible for a major subunit contact in the *S. cerevisiae* enzyme, but is drastically modified in the *S. pombe* sequence. Residues corresponding to positions 121 and 147 are covalently linked in the *S. pombe* enzyme by deletion of the 122 to 146 loop, thereby removing another of the subunit contacts. Residue 230 of the *S. cerevisiae* enzyme is the last residue seen in the X-ray structure, and corresponds to a position two residues beyond the end of the *S. pombe* sequence.

C-terminal region

The *S. pombe* enzyme is unique among all mutases sequenced to date in that it lacks a C-terminal 'tail' rich in alanine and lysine residues. In the case of the *S. cerevisiae* enzyme, the C-terminal region (231–246) is not observed in the X-ray structure of the enzyme, presumably indicating that this region is highly flexible (Figure 4). The results of Sasaki et al. (1966), who showed that this region is readily removed by proteolysis, leading to loss of the mutase activity of the enzyme, are consistent with this interpretation. It has been suggested (Fothergill-Gilmore and Watson, 1989) that the tail could (a) adopt a conformation which allows it to stabilize the transition state of the reaction by ionic interactions between one or more of the lysine side chains in the tail and the negative charges on the substrate, and/or (b) modulate access of water to the active site.

It is thus of some interest that the *S. pombe* enzyme lacks this tail. Although the catalytic-centre activity (expressed per active site) of the *S. pombe* enzyme (95 s^{-1}) (Johnson and Price, 1987) is somewhat lower than that of the *S. cerevisiae* enzyme (490 s^{-1}) (White and Fothergill-Gilmore, 1992) or the dimeric rabbit muscle enzyme (400 s^{-1}) (Grisolia and Carreras, 1975), it is clear that presence of this tail is not an absolute prerequisite for the mutase activity of the *S. pombe* enzyme.

Monomeric nature of the *S. pombe* enzyme

Inspection of the sequence of the *S. pombe* enzyme offers several clues to the monomeric nature of the enzyme. In the tetrameric *S. cerevisiae* enzyme, there are two sets of intersubunit contacts (Fothergill-Gilmore and Watson, 1989). The more extensive set [termed the CR (contact region) 1/2] involves residues in strand 1 of the β-sheet, helix 2 and the connecting loop, i.e. from Leu-57 to Ser-81. Within this stretch of 25 residues, only eight identities occur between the *S. cerevisiae* and *S. pombe* enzymes (i.e. 32% identity compared with the overall value of 45%). The side chains of the five residues in the connecting loop (Leu-74 to Val-78) (Figure 4) are thought to stabilize the interactions across this interface of the *S. cerevisiae* enzyme by filling a hydrophobic pocket in the adjacent subunit. The amino acids in this loop are essentially conserved in all the tetrameric and dimeric PGAMs (Figure 3). However, in the *S. pombe* enzyme there are a number of radical substitutions, including Asn for Trp at position 75 and Glu for Pro at position 77 (*S. cerevisiae* numbering).

The effect of these substitutions would presumably be to weaken the interactions across this interface. The less-extensive set of intersubunit contacts (termed the 'region CR5') in the *S. cerevisiae* enzyme involves interactions between the side chains of residues in the loop preceding helix 5 (Tyr-139 to Val-144) which fit between the side chains in helix 6 and the loop region between strands 5 and 6 of the β-sheet of the symmetry-related subunit. In the *S. pombe* enzyme there is no equivalent to the 139–144 loop region; this is within the stretch of 25 amino acids which is deleted (Figure 3). Hence the weakening of subunit interactions across this interface can also be understood.

Deletion of residues 122–146

The deletion of 25 residues which occurs in the middle of the *S. pombe* sequence could be accommodated without substantial distortion to the overall structure of the *S. cerevisiae* enzyme. The α-carbon atoms of the equivalent residues at the ends of this deleted region in the *S. cerevisiae* enzyme (Pro-121 and Glu-147) are some 0.83 nm apart, with the intervening residues forming part of an extended loop structure (Figure 4).

In conclusion, the amino acid sequence of the *S. pombe* PGAM offers a number of insights into the structure and properties of this unusually small enzyme and its relationship to the *S. cerevisiae* enzyme. We shall explore these aspects in detail using a combination of site-directed mutagenesis and biophysical techniques.

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