The role of the C-terminal region in phosphoglycerate mutase

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Removal of the C-terminal seven residues from phosphoglycerate mutase from *Saccharomyces cerevisiae* by limited proteolysis is associated with loss of mutase activity, but no change in phosphatase activity. The presence of the cofactor 2,3-bisphosphoglycerate, or of the cofactor and substrate 3-phosphoglycerate together, confers protection against proteolysis. The substrate alone offers no protection. Replacement of either or both of the two lysines at the C-terminus by glycines has only limited effects on the kinetic properties of phosphoglycerate mutase, indicating that these residues are unlikely to be involved in crucial electrostatic interactions with the substrate, intermediate or product in the reaction. However, the double-mutant form of the enzyme is more sensitive to proteolysis and is no longer protected against proteolysis by the presence of cofactor. The proteolysed wild-type and two of the mutated forms of the enzyme show a reduced response to 2-phosphoglycollate, which enhances the instability of the phospho form of the native enzyme. The phosphoglycerate mutase from *Schizosaccharomyces pombe*, which lacks the analogous C-terminal tail, has an inherently lower mutase activity and is also less responsive to stimulation by 2-phosphoglycollate. It is proposed that the C-terminal region of phosphoglycerate mutase helps to maintain the enzyme in its active phosphorylated form and assists in the retention of the bisphosphoglycerate intermediate at the active site. However, its role seems not to be to contribute directly to ligand binding, but rather to exert indirect effects on the transfer of the phospho group between substrate, enzyme, intermediate and product.

INTRODUCTION

The enzyme phosphoglycerate mutase (EC 5.4.2.1) (PGAM) catalyses the interconversion of 2- and 3-phosphoglycerate in the glycolytic/gluconeogenic pathways. The cofactor-dependent PGAMs require 2,3-bisphosphoglycerate (BPG) to 'prime' the reaction in an initial phosphorylation step. The enzyme from *Saccharomyces cerevisiae* has been particularly well studied, with both the amino acid sequence and crystal structures determined [1–3]. A catalytic mechanism based on the known properties of the enzyme has been proposed (reviewed by Fothergill-Gilmore and Watson [4]). Briefly, a round of catalysis is initiated when a monophosphoglycerate molecule binds at the active site of the phosphorylated enzyme. The phospho group on His-8 at the active site is then transferred to the substrate to form BPG as an intermediate. It is suggested that BPG then changes its orientation in the active site before transferring its other phospho group to His-8 to regenerate the active form of the enzyme. The product is then released to allow another round of catalysis.

The phosphorylated enzyme is slightly unstable, leading to a low level of phosphatase activity (approx. 0.002 % that of the mutase activity). The phosphatase activity is conveniently measured by providing the enzyme with BPG and determining the release of inorganic phosphate. This phosphatase activity is stimulated some 20-fold in the presence of 2-phosphoglycollate or 30-fold by 2-phosphohydroxypyruvate [5]. The half-life of the phosphoenzyme is 35 min in the absence of ligands, but decreases to less than 20 s in the presence of 2-phosphoglycollate [6].

Sasaki et al. [7] showed that the effect of endogenous proteolysis on *S. cerevisiae* PGAM was to decrease the mutase activity by some 30-fold. However, the phosphatase activity was not affected by the proteolysis. In contrast, the proteolysed enzyme was almost unable to respond to the stimulatory effects of 2-phosphoglycollate [5]. Amino acid analysis indicated that the amino acids removed by proteolysis corresponded to those now known to constitute residues 235–246 at the C-terminus of the enzyme [7].

In the X-ray structure of *S. cerevisiae* PGAM (Figure 1) [3], there is no definite electron density beyond residue 234, 235 or 236, depending upon subunit. This indicates that the C-terminal segment of 10–12 amino acids (-AGAAAVANQGKK) adopts no well-ordered structure in the crystal.

It has been proposed [4,8] that the C-terminal region might be involved in 'enclosing' the active site of PGAM during the catalytic cycle, thereby preventing access of water which would lead to non-productive hydrolysis of the phosphorylated enzyme intermediate (i.e. the phosphatase reaction). All the sequences of PGAMs available up to 1989 (see Figure 2) showed that the C-terminal region contains several alanines and, in the distal portion, a glycine and at least two lysines. It was suggested that these lysines might be involved in charge interactions with phospho groups on the substrates or intermediate [4].

The more recent determination of the amino acid sequence of PGAM from *Zymomonas mobilis* [9] and of the small monomeric PGAM from the fission yeast *Schizosaccharomyces pombe* [10] revealed that not all PGAMs possess a comparable C-terminal region. The *S. pombe* enzyme has a deletion corresponding to the C-terminal 16 residues of the *S. cerevisiae* enzyme, and lacks a C-terminal basic residue (Figure 2). The fact that PGAM from *S. pombe* can function without the contribution of

Abbreviations used: PGAM, phosphoglycerate mutase; BPGAM, bisphosphoglycerate mutase; BPG, 2,3-bisphosphoglycerate; F2,6BPase, fructose 2,6-bisphosphatase.

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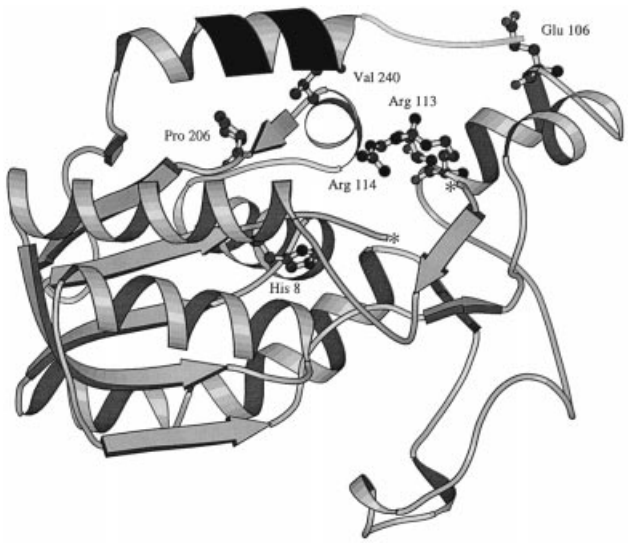

Figure 1 Diagram of the PGAM subunit

The predicted helix in the C-terminal region terminating at residue 242 is shown at the top of the diagram with a broader ribbon in black, and the polypeptide chain corresponding to the distal four residues is shown in extended conformation reaching Glu-106. The side-chains of certain residues referred to in the text are indicated. Residues between the * symbols have been omitted for clarity. (Drawn with MOLSCRIPT [26].)

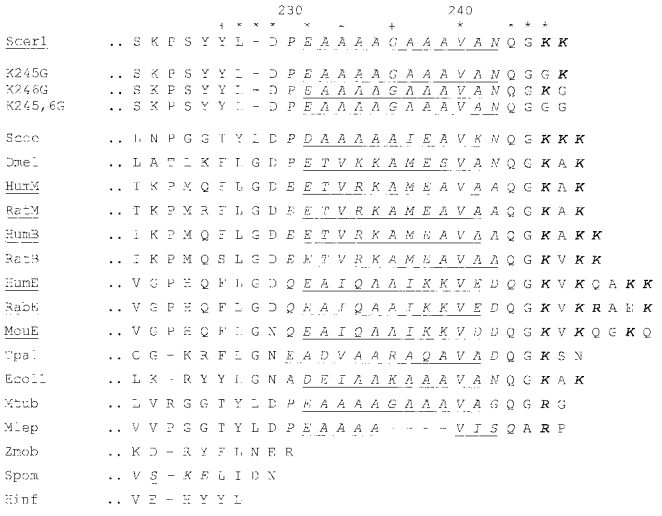

Figure 2 Alignment of the C-terminal sequences of PGAMs, including the tail mutants of *S. cerevisiae* PGAM

The regions which are predicted by the PHD program [27–29] to adopt helical conformations are shown in italic type, with the residues awarded maximum score underlined. The basic residues at the C-termini are indicated in bold type. The symbol * indicates that ten or more species are identical at that position, and the symbol + is used where ten or more species are similar. The sequences have been aligned using the CLUSTAL program [30], with subsequent modification of the extreme C-terminal region to facilitate direct comparisons. The names of the sequences available by 1989 are underlined. In the alignment, the abbreviations and sequence publications are as follows: Scer1, *S. cerevisiae* [1]; K245G, K246G and K245,6G are the tail mutants of *S. cerevisiae* PGAM; Scoe, *Streptomyces coelicolor* [31]; Dmel, *Drosophila melanogaster* [32]; HumM, human M-type [33]; RatM, rat M-type [34]; HumB, human B-type [35,36]; RatB, rat B-type [37]; HumE, human E-type [38]; RabE, rabbit E-type [39]; MouE, mouse E-type [40]; Tpal, *Treponema pallidum* [41]; Ecol1, *Escherichia coli* [42]; Mtub, *Mycobacterium tuberculosis* [43]; Mlep, *Mycobacterium leprae* [44]; Zmob, *Zymomonas mobilis* [9]; Spom, *Schizosaccharomyces pombe* [10]; Hinf, *Haemophilus influenzae* [45].

these residues prompted a further exploration of the role of the C-terminal region.

In addition to differences in the C-terminal region, the *S. pombe* enzyme has the deletion of a large loop in the middle of the chain as well as significant changes in another loop comprising residues 74–78 (*S. cerevisiae* numbering). These two alterations would be expected to weaken subunit interactions, and help to account for the monomeric nature of the *S. pombe* enzyme. However, molecular modelling indicates that the remainder of the sequence has the same fold as that of *S. cerevisiae* PGAM [11].

In this paper, we report on the effects of limited digestion of the *S. cerevisiae* and *S. pombe* enzymes with thermolysin. The protection afforded by substrate and cofactor is also examined. In addition, we investigate the importance of the two lysine residues at the C-terminus of *S. cerevisiae* PGAM by replacing either or both of them by glycine.

EXPERIMENTAL

Purification and mutagenesis of *S. cerevisiae* PGAM

The *S. cerevisiae* PGAM gene carried by a pVT-type shuttle phagemid (pVT-GPM) was subjected to site-directed mutagenesis as described previously [12]. Expression was in a yeast strain in which the wild-type PGAM gene had been deleted. In each case approx. 25% of the protein in a crude extract was the mutated PGAM, as indicated by SDS/PAGE. The phagemids with the mutated genes were rescued from transformed yeast cells and the genes were sequenced to verify that only the desired mutations were present. Purification of the mutant enzymes was by ammonium sulphate and QA-cellulose fractionation, essentially as reported previously [12], with the addition of a final FPLC Superose-12 gel-filtration step. The Superose column was eluted with 20 mM Tris/HCl, pH 8.0. All enzymes showed only a single band on SDS/PAGE [13] with subunit M_r 27000. The concentrations of enzyme were determined spectrophotometrically, assuming a value of 1.45 for the A_{280} of a 1 mg/ml solution [14].

Purification of recombinant *S. pombe* PGAM

Recombinant wild-type PGAM from *S. pombe* was isolated as described by Nairn et al. [10], with the addition of a final FPLC step as for the *S. cerevisiae* enzyme. The concentrations of the *S. pombe* enzyme were determined by a Coomassie Blue binding method [15] using BSA as a standard. This method gave values within 5% of those determined spectrophotometrically, using a value for the A_{280} calculated from the aromatic amino acid content of the enzyme [16]. [The value calculated (1.40 for a 1 mg/ml solution) refers to the enzyme in 6 M guanidine hydrochloride, but for most proteins the value in buffer is generally within 10% of this value].

Enzyme assays

The assays of mutase, phosphatase and synthase activities were done as described by White and Fothergill-Gilmore [12].

Limited proteolysis with thermolysin

The treatment of PGAM with thermolysin (Boehringer) was carried out essentially as described previously [17], i.e. 10% (w/w) thermolysin (500 μg/ml PGAM, 50 μg/ml thermolysin) in 50 mM Tris/HCl buffer, pH 8.0, at 20 °C. Samples removed from the incubation mixture were treated with EDTA (final concentration 5 mM) in order to inactivate the thermolysin [18]. The experiments were done in triplicate, and the results of

individual experiments differed by less than 10% from each other.

Protein sequencing

Sequencing studies on *S. cerevisiae* PGAM (native and thermolysin-treated forms) were done on an Applied Biosystems 477A microsequencer operated as described previously [19]. Samples containing approx. 1 nmol of PGAM were taken at zero time and after a 10 min treatment with thermolysin and were spotted directly on to glass fibre discs for sequencing.

MS

Analysis by MS was done on a VG Platform quadropole mass spectrometer (200–3000 atomic mass units range) fitted with a pneumatically assisted electrospray source and controlled via the VG Mass-Lynx software (VG Biotech Ltd., Altrincham, Cheshire, U.K.), as described previously [6,11].

CD spectroscopy

CD measurements were performed on a JASCO J-600 spectropolarimeter. The molar ellipticities were calculated assuming a value of 112 for the mean residue mass of each enzyme [1,10].

RESULTS AND DISCUSSION

Structure of the C-terminal region of PGAM

An alignment of the C-terminal sequences of currently available cofactor-dependent PGAMs and bisphosphoglycerate mutases (BPGAMs) is given in Figure 2. This portion of PGAM had been implicated as playing an important role in the activity of the enzyme because its removal from *S. cerevisiae* PGAM by an endogenous protease caused a devastating 97% loss of activity [7]. The fact that these residues did not contribute to the electron-density map of the crystalline enzyme was intriguing, and served to stimulate ideas about their possible function (see for example, Fothergill-Gilmore and Watson [4]). However, the subsequent discovery that not all PGAMs possess this C-terminal region (see Figure 2) seemed at odds with the proteolysis results, and demanded an explanation. The limited proteolysis and site-directed mutagenesis experiments reported here were done in an attempt to clarify the function of the C-terminal region.

An examination of the recently determined high-resolution structure of *S. cerevisiae* PGAM [3] gives some clues about the structure of the C-terminal region. The final residues that are observed in the electron-density map (231–234) are arranged into a single turn of helix. This can be seen in Figure 1 as a narrow ribbon at the beginning of the C-terminal helix shown at the top of the diagram.

Secondary structure predictions were thus made to ascertain whether more residues might be involved in this helix. It was found that indeed residues 231–242 were awarded a maximum score for adopting a helical conformation. Similar secondary structure predictions were made for all the PGAMs, and all but the three short PGAMs possess a maximally predicted helix in the same region, despite considerable sequence variation. Every third or fouth residue of the helix (residues 233, 236 and 240) is hydrophobic and shows only conservative replacements. Moreover, the helix is preceded and followed by short stretches of highly conserved sequences, especially the distal Gln-Gly-Lys at positions 243–245. All sequences have at least one basic residue in the distal portion, and this is always in the 245 position. The conservation of these sequences would suggest that one of the main functions of the helix is to hold and present the distal C-terminal residues in the correct orientation.

Molecular modelling of the helix, with a continuation in the same direction as residues 231–234, shows that it follows along one edge of the active-site cleft (but not across it), and would serve to bury the hydrophobic residues Pro-206 and Val-240 (Figure 1). It is noteworthy that Pro-206 is conserved in all the 'long' sequences, but is replaced by an alanine in the *S. pombe* 'short' sequence. In this position the helix could constrain residues Arg-113 and Arg-114 that provide positive charges to the active site where they can interact with negatively charged mono and bisphosphoglycerates. The distal portion of the C-terminal region could easily approach Glu-106 in such a way that one of the basic residues might form an electrostatic interaction. Glu-106 is also highly conserved in all 'long' PGAMs, but is replaced by Ala in the *S. pombe* enzyme. Glu-106 is in the middle of the only region in PGAM (residues 104–110) that has a high B-factor in the crystal [3] and is thus implicated as being relatively mobile. This in turn may have implications for conformational changes during the catalytic cycle.

Limited proteolysis with thermolysin

S. cerevisiae PGAM

The kinetic constants of untreated wild-type PGAM in the mutase and phosphatase assays and the degree of stimulation of the phosphatase activity by 2-phosphoglycollate (Table 1) were found to be within 10% of the values reported previously [12]. On treatment with thermolysin, there was a pronounced loss of mutase activity (reduced to 55% after 10 min and to 30% after 30 min), whereas the phosphatase activity was unaffected (Table 1; see Figure 5, left). The loss of mutase activity was not as marked as that observed by Sasaki et al. [7], in which the enzyme was degraded by an endogenous protease. The degree of stimulation of the phosphatase activity by 2-phosphoglycollate was reduced from 18-fold in the native enzyme to 6.2-fold after 10 min and to 3.2-fold after 30 min (Table 1). There was no significant change in the far-UV CD spectrum over a 2 h period (results not shown), suggesting that no major changes in secondary structure have occurred on treatment with thermolysin.

N-terminal sequence analysis (six cycles) of PGAM from the zero time point of the thermolysin digest revealed a high-yield sequence corresponding to the N-terminal six residues of PGAM (Pro-Lys-Leu-Val-Leu-Val, initial yield approx. 400 pmol) plus a minor sequence from the N-terminus of thermolysin [Ile-Thr-Gly-Thr-Ser(?)-Thr, initial yield approx. 80 pmol]. Sequence analysis of the 10 min sample from the thermolysin digest showed, in addition, a strong sequence of Val-Ala-Asn-Gln-Gly-Lys(?) (initial yield approx. 500 pmol). This sequence corresponds to residues 240–245 near the C-terminus of PGAM. The C-terminal peptide showed a dramatic decrease in yield as the sequence progressed, which is consistent with washing from the sequencer sample disk, judging by the small size of the peptide and the nature of the sequence. The relatively high initial yield of the peptide compared with that of the parent protein can be attributed to the typical poor cyclization of the proline residue at the N-terminus of PGAM during Edman degradation.

Electrospray mass spectrometric analysis showed that the parent PGAM molecule (M_r 27474) was almost completely converted into a smaller protein with M_r 26748 after 5 min incubation with thermolysin (Figure 3). The mass difference corresponds to the C-terminal seven amino acid residues (Val-Ala-Asn-Gln-Gly-Lys-Lys). The small peak at M_r 26675 corresponds to the removal of an additional Ala residue.

Table 1 Kinetic constants of PGAMs

Kinetic values were determined in at least three separate experiments; the values shown are the means of these experiments with S.E.M. of less than 5%. The stimulated phosphatase activity was measured in the presence of 1 mM 2-phosphoglycollate. *These values were determined by Sasaki et al. [5,7] and Chiba et al. [46] using a different assay system from that used in the present work; the values of k_{cat} and of K_m for BPG shown for the proteolysed enzyme are calculated by reference to the values for native PGAM in this study. †This value was calculated by interpolation from a 2 h value of 1.8-fold stimulation. 3PGA, 3-phosphoglycerate; 2-PG, 2-phosphoglycollate; nd, not determined.

Enzyme	Mutase			Phosphatase		Stimulation by 2-PG (fold)
	k_{cat} (s^{-1})	K_m 3PGA (μM)	K_m BPG (μM)	k_{cat} (s^{-1})	K_m BPG (μM)	
S. cerevisiae PGAM						
Wild-type	530	470	3.2	0.009	220*	18
Proteolysed	18*	nd	3.2*	0.009*	220*	3.5
Thermolysin-treated						
10 min	290	nd	nd	0.009	nd	6.2
30 min	160	nd	nd	0.009	nd	3.2†
Mutants						
Lys-245 → Gly	210	400	2.4	0.010	nd	4.1
Lys-246 → Gly	520	630	3.2	0.011	nd	16
Lys-245,6 → Gly	560	1430	7.9	0.010	nd	4.0
S. pombe PGAM						
Wild type	82	580	4.6	0.020	nd	2.3

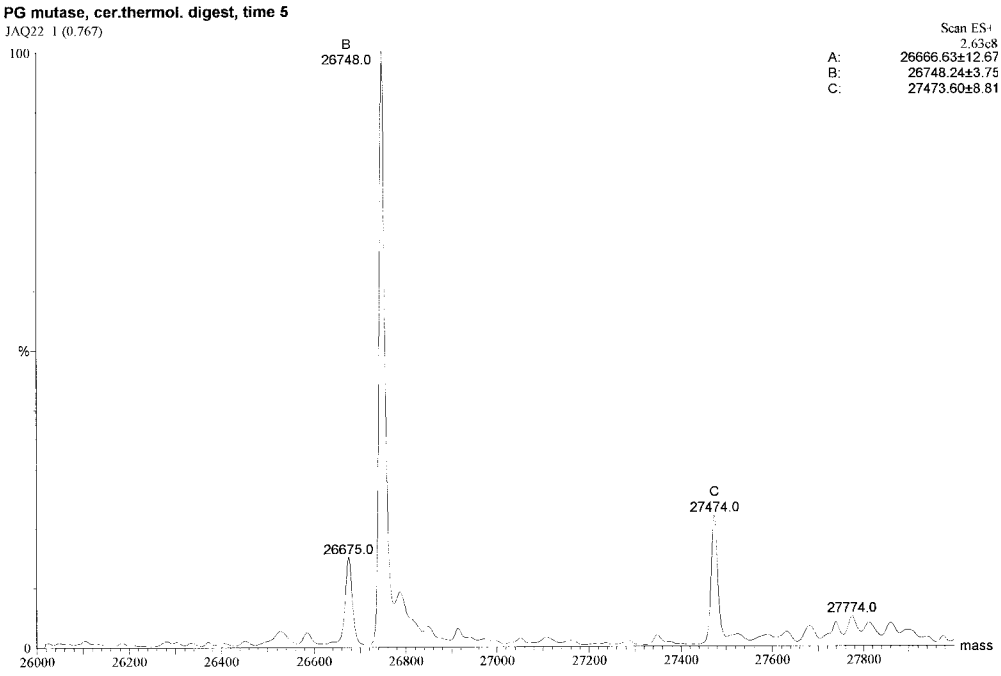

Figure 3 Electrospray mass spectrometric analysis of a sample of *S. cerevisiae* PGAM treated with thermolysin for 5 min

The M_r of each protein detected is given above the peak.

After periods of digestion longer than approx. 30 min, SDS/PAGE analysis revealed the formation of a distinct band just below the major band, corresponding to an apparent decrease in subunit M_r of approx. 1500 (i.e. M_r 25 500) compared with that of the undigested polypeptide chain (Figure 4). The intensity of this band increased at the expense of that corresponding to the intact chain over a 2 h period of digestion. When a sample taken after 2 h digestion was subjected to sequence analysis, a complex pattern of N-terminal sequences was obtained and the reverse-phase HPLC profile of the mixture indicated that a large number of peptides was present.

It is therefore probable that the initial site of thermolytic attack on PGAM is the bond between Ala-239 and Val-240, consistent with the specificity of thermolysin. This leads to a substantial loss of mutase activity, but does not generate a species that can be distinguished from undigested PGAM on SDS/PAGE under the conditions used in the present study. This initial attack does not affect the phosphatase activity but does

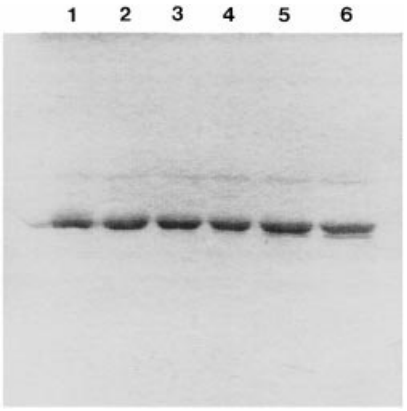

Figure 4 SDS/PAGE analysis of *S. cerevisiae* PGAM digested with thermolysin

Incubation time: lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 60 min; lane 6, 120 min.

reduce the stimulation by 2-phosphoglycollate (Table 1). Further digestion then occurs, possibly at the bond between Tyr-227 and Leu-228 (which could account for the band of apparent M_r 25500 detected on SDS/PAGE), but this is followed by other cleavages of the polypeptide chain to generate the multiple peptides detected at the later stages of the digestion.

These observations are consistent with the biphasic nature of the inactivation curves seen in Figure 5, left. The initial rapid loss of activity appears to correlate with the removal of the C-terminal seven residues of the enzyme. The slower and more gradual loss of activity probably corresponds to more extensive general proteolysis.

In the case of the experiments reported by Sasaki et al. [7], it appears that the site of cleavage by the endogenous protease was probably at the C-terminal side of residue 234, and thus involved the removal of the C-terminal 12 residues of the enzyme. This more extensive modification is likely to correlate with the greater loss of mutase activity observed (Table 1). It is relevant to note that neither the phosphatase activity nor the K_m for BPG in the phosphatase reaction was affected by endogenous proteolysis.

S. pombe PGAM

The effect of incubating *S. pombe* PGAM with thermolysin was found to be dramatically different from the pattern observed with the *S. cerevisiae* enzyme. As shown in Figure 5 (middle), the mutase activity and phosphatase activity, in both the absence and presence of 2-phosphoglycollate, all decline at the same rate, which is relatively slow compared with the initial rate of loss of mutase activity in the *S. cerevisiae* enzyme (Figure 5, left). The rate of disappearance of the band on SDS/PAGE that corresponds to the intact polypeptide chain (M_r 23000) matches the rate of loss of the various activities in Figure 5 (middle). There was no evidence for the formation of products of $M_r > 14000$ from *S. pombe* PGAM during the thermolysin treatment, indicating that proteolysis yielded only relatively small fragments. In earlier studies it had been shown that *S. pombe* PGAM was relatively resistant to short periods of treatment with thermolysin and other proteases [20], in agreement with the present results.

Ligand protection

The addition of the cofactor BPG to *S. cerevisiae* PGAM afforded substantial protection against inactivation by limited thermolysin digestion (Figure 5, left). In contrast, the addition of the substrate 3-phosphoglycerate alone gave very little protection. The two ligands in combination were indistinguishable from the cofactor alone.

Properties of C-terminal mutants of *S. cerevisiae* PGAM

The two single mutants (Lys-245 → Gly and Lys-246 → Gly) and the double mutant (Lys-245,6 → Gly) had very similar far UV CD spectra to wild-type PGAM, with a distinct minimum at 208 nm and a shoulder in the 220–230 nm region in each case (results not shown). In addition, the near UV CD spectra of the wild-type and mutant enzymes were very similar, with a sharp maximum at 291 nm and a broader maximum at 282 nm in each case (results not shown). Taken together, these results indicate that the mutations had little if any effect on the secondary or tertiary structure of the enzyme. The mutants showed mutase activity ranging from 40 to 110 % of the wild-type enzyme (Table 1). The K_m values for the substrate and cofactor of the single mutants were similar to those of the wild-type enzyme, although the double mutant showed 3–4-fold elevated values. Unfortu-

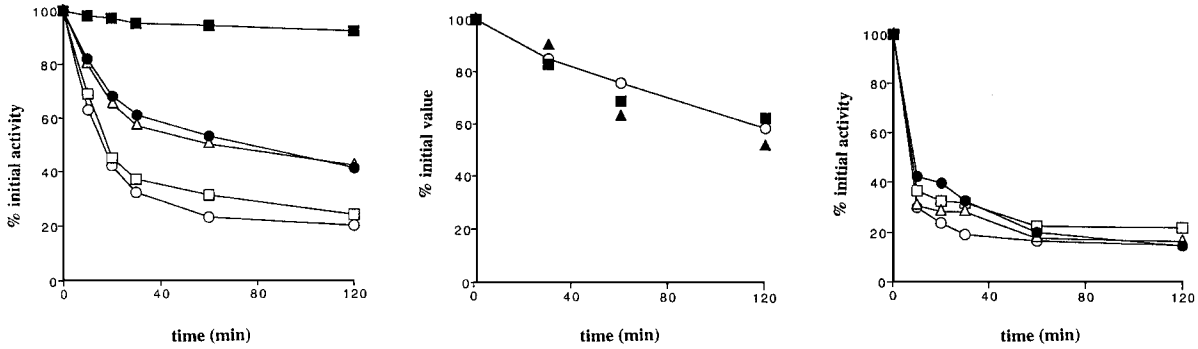

Figure 5 Loss of enzyme activity after limited digestion by thermolysin

Left: PGAM from *S. cerevisiae* was incubated with thermolysin, and samples were taken for analysis at the time intervals indicated. Mutase activity was measured with: (○) no ligands; (□) 10 mM 3-phosphoglycerate; (△) 0.5 mM BPG; (●) 10 mM 3-phosphoglycerate + 0.5 mM BPG; and phosphatase activity (■) with no ligands and in the absence of 2-phosphoglycollate. Middle: PGAM from *S. pombe* was incubated with thermolysin as above. (○) and (■) represent the mutase and phosphatase activities respectively; (▲) represents the amount of intact (M_r 23000) *S. pombe* protein as determined by SDS/PAGE. The changes in the relative levels of phosphatase activity in the presence of 2-phosphoglycollate (1 mM) ran in parallel with the changes in the phosphatase activity measured in the absence of 2-phosphoglycollate. Right: the double mutant of *S. cerevisiae* PGAM, Lys-245,6 → Gly, was incubated with thermolysin in the absence and presence of ligands. Mutase activity was measured with: (○) no ligands; (□) 10 mM 3-phosphoglycerate; (△) 0.2 mM BPG; (●) 10 mM 3-phosphoglycerate + 0.5 mM BPG. In all cases the results shown are the average of three independent experiments.

nately the multi-step Ping Pong mechanism of the mutase reaction and the very rapid phospho transfer steps [21] mean that it is inappropriate to interpret differences in K_m values simply in terms of ligand-binding affinity.

The phosphatase activites were within 20 % of the wild-type enzyme. In the cases of the Lys-245 → Gly and the Lys-245,6 → Gly mutants, the level of stimulation by 2-phosphoglycollate was markedly reduced (from 18-fold to 4-fold). In contrast, the stimulation of the Lys-246 → Gly mutant (16-fold) was similar to that of wild-type enzyme.

It was noted that the addition of the cofactor BPG to wild-type PGAM afforded substantial protection against thermolysin treatment (Figure 5, left). In marked contrast, the double mutant Lys-245,6 → Gly was unable to respond to the protective effects of BPG (Figure 5, right). The addition of BPG or of the substrate 3-phosphoglycerate afforded no protection, either singly or in combination. Moreover, the double mutant showed a more rapid loss of activity than the wild-type enzyme. These results suggest that Lys-245 may play a role at the active site. Further encouragement for this proposal comes from mutagenesis of the corresponding residue in BPGAM (see below).

Properties of *S. pombe* PGAM

As shown in Table 1, the normal mutase activity of native *S. pombe* PGAM is only about 15 % that of the *S. cerevisiae* enzyme. The K_m values for BPG and 3-phosphoglycerate, however, are only slightly elevated. The phosphatase activity of the *S. pombe* enzyme is similar to that of the *S. cerevisiae* enzyme, but the stimulation of the phosphatase activity by 2-phosphoglycollate is only 2.3-fold. The phospho form of the enzyme is markedly less stable than that of the *S. cerevisiae* enzyme, with a half-life of less than 1 min [6]. Thus, in terms of the mutase and phosphatase activities and the effect of 2-phosphoglycollate, the properties of *S. pombe* PGAM are comparable with those of the proteolysed *S. cerevisiae* enzyme described by Sasaki et al. [5,7] and with the enzyme treated with thermolysin for 30 min.

The role of the C-terminal region

It is clear that the C-terminal region is not essential for activity, but that its removal leads to enzymes with markedly less mutase activity. Excision of 12 residues from the *S. cerevisiae* enzyme has a more drastic effect than the removal of seven residues. The phosphatase activity of the enzyme is also affected, but in an indirect manner, shown by the lack of responsiveness to stimulation by 2-phosphoglycollate. A consideration of the likely steps of the catalytic pathway of PGAM may help to reveal those aspects that may be favoured by the participation of the C-terminal region.

PGAM must be phosphorylated in order to be active. The phospho form of the enzyme is apparently in a different conformation from the unphosphorylated form, as seen in the crystal structure. Justification for this statement comes from the observation that crystals crack when soaked with BPG [8], and from NMR spectroscopic changes that occur on the binding of a transition-state analogue of BPG, 2-vanadio-3-phosphoglycerate [22].

The essential preliminary step is thus the binding of BPG to the unphosphorylated enzyme. The active-site cleft is a relatively roomy cavity that is rich in basic residues, including Arg-113, Arg-114 and Lys-97 [3]. The cleft is sufficiently large and the basic side-chains are sufficiently flexible to allow the BPG to bind in two different modes, with either the 2-phospho or the 3-phospho group near to His-8 ready for transfer. There is no need to invoke the C-terminal region in the initial binding of BPG. This statement is supported by the fact that the phosphatase activity (which involves an initial binding of BPG) is unaffected in the various proteolysed or mutated forms of the enzyme. Moreover, the K_m for BPG determined for the phosphatase reaction catalysed by the endogenously proteolysed enzyme was also unchanged.

Examination of the active site shows that Glu-86 and a water molecule are suitably placed to provide the catalytic network to enable the phospho transfer to His-8 to take place. Both side-chains would be uncharged before the transfer and charged afterwards. It seems likely that the phospho transfer provokes the structural changes mentioned above and concomitantly brings the C-terminal region into play. This in turn might help to stabilize the phospho form of the enzyme. The enzyme would remain in this different catalytically competent conformation as long as it retains the phospho group.

The monophosphoglycerate product of this initial phosphorylation reaction would then be released. The structural explanation for the release is not readily apparent from the crystal structure of the unligated enzyme, but presumably is a consequence of the new phosphoenzyme conformation. One possibility is that the C-terminal region constrains the positions of Arg-113 and Arg-114 such that they can no longer have favourable interactions with the monophosphoglycerate. In addition, the monophosphoglycerate would have only three negative charges compared with the five negative charges of BPG.

It is thus envisaged that monophosphoglycerates are relatively free to dissociate from the phosphoenzyme. However, the presence of a monophosphoglycerate at the active site of the phosphoenzyme could also encourage the reverse phospho transfer reaction to take place, such that a molecule of BPG is formed as an intermediate, leaving an uncharged, unphosphorylated His-8. It is tempting to speculate that the Arg-113 and Arg-114 side-chains would now be in a particularly suitable position to interact with the BPG and thus to prevent its dissociation and thereby facilitate its reorientation within the active-site cavity. If reorientation occurs, followed by another phospho transfer to His-8, then the product monophosphoglycerate has been formed, and this can in turn dissociate. Thus the C-terminal region is primarily invoked as a means to constrain enzyme flexibility so that the phosphoenzyme form is stabilized, and so that Arg-113 and Arg-114 would interact favourably with BPG, but relatively weakly with monophosphoglycerates.

The effect of 2-phosphoglycollate can be explained by envisaging that it would bind to the phosphoenzyme in a manner sufficiently similar to that of a monophosphoglycerate so as to provoke phospho transfer. However, in this case the hydroxyl group of 2-phosphoglycollate is not in a suitable position to receive the phospho group, which would thus be lost to an abortive hydrolysis reaction. This stimulation of the phosphatase reaction would not be observed in the proteolysed or 'short' forms of the enzyme because the phosphoenzyme is so much less stable and would thus relatively seldom be in the active, phosphoenzyme conformation.

In a similar way, the low mutase activity of the proteolysed and 'short' enzymes can be explained by the same instability of the phosphoenzyme conformation.

Comparison with BPGAM

BPGAM and PGAM share approx. 50 % sequence identity, and catalyse the same three reactions, albeit at substantially different relative rates (reviewed by Fothergill-Gilmore and Watson [4]).

The main reaction catalysed by BPGAM is the synthesis of 2,3-bisphosphoglycerate from 1,3-bisphosphoglycerate, designated the synthase reaction. However, the k_{cat} for this reaction is only 8 s^{-1}, and that for the mutase reaction is 4 s^{-1} (a factor of 130 less than the PGAM mutase reaction). The phosphatase reaction has a k_{cat} comparable with PGAM, but shows a much greater responsiveness to 2-phosphoglycollate, as the reaction is stimulated 300-fold.

A major difference between BPGAM and PGAM is that the synthase reaction requires that the bisphospho compound BPG be released as a product, but the mutase reaction demands that the BPG be retained as an intermediate. Moreover, the synthase reaction does not involve an initial priming phosphorylation step, and there is thus no requirement for the enzyme to be maintained in a phosphoenzyme conformation.

What can be proposed with regard to the role of the C-terminal region of BPGAM? The absence of a crystal structure of the enzyme means that any proposals must be tentative and place reliance upon a consideration of sequence comparisons and site-directed mutagenesis. It can be seen in Figure 2 that the C-terminal region of BPGAM contains the conserved helix and lysine at position 245, is longer than the C-terminal regions of monoPGAMs and has more basic residues. Mutagenesis studies showed that deletion of the C-terminal seven residues (corresponding to Lys-245–end, PGAM numbering) caused loss of all three activities, although the assays were done on unpurified enzymes in cell extracts [23]. The deletion of two or four residues gave partial loss of synthase activity. In addition, mutagenesis of Lys-245 implied its involvement at the active site [24]. These results would suggest that the role of the C-terminal region is to hold and to present Lys-245 in such a way as to contribute to ligand binding at the active site. Further speculation is probably not justified in the absence of a crystal structure of BPGAM, and the possible contribution of the C-terminal region to the binding and release of BPG remains enigmatic. However, it seems likely that this region plays different roles in PGAM and BPGAM.

Comparison with fructose 2,6-bisphosphatase (F2,6BPase)

PGAMs belong to a wider family of enzymes that all involve the formation of a phospho-histidine intermediate. Among these enzymes are some types of phosphatases, such as the F2,6BPase domain of the bifunctional enzyme responsible for the synthesis and breakdown of fructose 2,6-bisphosphate. F2,6BPase shares only very low sequence identity with PGAM, but a number of active-site residues are conserved and the enzymes have similar topologies [3]. A site-directed mutant form of F2,6Bpase, in which the C-terminal 30 residues were deleted, showed a 5-fold enhancement of phosphatase activity [25]. This observation would be consistent with the C-terminal region of F2,6BPase interacting with the active site, such that the phosphoenzyme intermediate would be destabilized in its absence. In this respect, the C-terminal region of F2,6BPase might provide a similar function to the analogous region in PGAM.

We wish to thank the Wellcome Trust and the Biotechnology and Biological Sciences Research Council (BBSRC) for financial support. We are grateful to Dr. M. F. White for preparing the mutant *S. cerevisiae* PGAM genes. The protein sequencing was done in the Welmet Protein Characterisation Facility that was established by grants from the Wellcome Trust, the University of Edinburgh, Salvesen's Trust and Heriot–Watt University, and we are grateful to Mr. Douglas Lamont and Dr. Andrew Cronshaw for their assistance. We thank Dr Tino Krell for the mass spectrometric analyses.

Received 10 August 1998/24 September 1998; accepted 21 October 1998

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