The role of the C-terminal lysine in the hinge bending mechanism of yeast phosphoglycerate kinase

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Abstract Treatment of yeast phosphoglycerate kinase (PGK) with trypsin results in a fourfold increase in the V_{max} of this enzyme, without affecting the K_m. This activation is shown to be due to the removal of the C-terminal lysine residue. The C-terminal sequence folds back over the N-terminal domain and contacts the extreme N-terminal sequence which folds onto the C-terminal domain, thus making many of the inter-domain contacts in this two domain protein. Previous studies have shown that this C-terminal region is important in mediating the conformational changes required during catalysis by yeast PGK. Observation of the three-dimensional structure of this enzyme suggests that removal of the C-terminal lysine residue will strengthen the interaction between K5 and E413. This indicates that this salt bridge stabilises the enzyme in the higher activity form, while the presence of K415 reduces the strength of that interaction.

Key words: Phosphoglycerate kinase; Enzyme activation; Enzyme catalysis; Hinge bending; Lysine

1. Introduction

Phosphoglycerate kinase (PGK) is a monomeric enzyme which catalyses the phosphorylation of 3-phosphoglycerate. The three-dimensional structures of this enzyme from horse muscle [1], yeast [2], pig muscle [3], and *Bacillus stearothermophilus* [4] have been determined. In all these species the fold of the protein chain is very similar, and they all consist of two continuous structural domains of similar size. In the native fold of the enzyme inter-domain contacts are made through helix V, which links the two domains, and the C-terminal helices XIII and XIV, which contact the N-terminal domain.

The two domains of PGK have been shown to be capable of folding independently into stable structural units [5-8]. Further folding studies have shown that the C-terminal, domain spanning helix XIV, although clearly involved in the inter-domain contacts, is not required for the structural integrity of either domain [9] but is important in stabilising their interactions across the waist region [10].

Such studies have provided dynamic support for the early proposal that PGK, along with other kinases, undergoes a conformational change during catalysis [1]. Upon binding substrates the two domains of the protein are thought to change their relative positions, both bringing the two substrates together, and excluding water from the active site. NMR [11], small angle X-ray scattering [12,13] and analytical ultracentrifugation [14] studies have been interpreted in terms of large conformational changes associated with hinge bending. In such a mechanism the two domains rotate about their interdomain contacts. Results from fluorescence anisotropy decay [15] suggest that this rotation is relatively small.

A mutant protein lacking the 15 C-terminal residues (helix XIV and the preceding loop) has been expressed [16]. This protein, although able to bind substrates, exhibits only 0.1% of the catalytic activity of the native protein. This supports a hypothesis in which the C-terminal helix, although not involved in the folding or substrate binding of phosphoglycerate kinase, has a key role in mediating the conformational change required for catalysis.

Here we report that removal of the C-terminal lysine residue, Lys-415, results in a large increase in the V_{max} of yeast phosphoglycerate kinase, while little or no change in the K_m is observed. This gives further support to the permissive model of hinge bending previously proposed in which the energy barrier between the two conformers is relatively low, allowing the binding of substrate to swing the hinge to the active state [15,17].

2. Materials and methods

Yeast phosphoglycerate kinase (EC 2.7.2.3) was obtained from Sigma Chemical Co., and was shown to run as a single band on electrophoresis in the presence of SDS, and have similar kinetic properties to those reported for this enzyme [18]. Chicken muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), DPCC treated bovine type XI trypsin, ATP and 3-phosphoglycerate were also obtained from Sigma Chemical Co. The OD_{300} Aquapore ODS column was purchased from Brownlee, acetonitrile from Rathburn Chemicals Ltd and sodium 1-octanesulfonate from Sigma Chemicals. Other chemicals were of analytical grade.

2.1. Tryptic digestion

Prior to treatment with trypsin, phosphoglycerate kinase was dissolved in a minimal volume of 100 mM Tris, 10 mM $CaCl_2$ (pH 8.0) and desalted into the same buffer using a PD10 column (Pharmacia) and diluted to a concentration of 4 mg/ml. The sample was then equilibrated at 37°C, and digestion initiated by the addition of trypsin to a concentration of 10 μg/ml. 40 μl samples were taken at various times after the addition of trypsin, and the reaction was stopped by the addition of 1 μl 10 mM PMSF.

Samples from the tryptic digests were analysed by electrophoresis in the presence of SDS on 12% polyacrylamide according to [19] and 16.5% polyacrylamide according to [20].

2.2. Enzyme kinetics

The specific activity of PGK was measured, in the absence of sulphate, by a modification of a method of Scopes [21]. The assay mixture contained 10 mM Tris/HCl, pH 7.2, 5 mM magnesium chloride, 15 mM 3-phosphoglycerate, 5 mM ATP, and 50 μg/ml rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. Activity was measured by following the change in absorbance at 340 nm. All measurements were made at 25°C.

K_m values for wild-type and activated PGK were determined for both substrates in the presence of 50 mM sodium sulphate as described [18]. The assay solution contained 10 mM Tris/HCl, pH 7.2, 50 mM sodium sulphate, 0.2 mM NADH, 150 μg/ml glyceraldehyde-

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3-phosphate dehydrogenase and either 5 mM ATP or 5 mM 3-phosphoglycerate. The concentration of the second substrate was then varied over a range from 0.03 to 5 mM, and the concentration of free magnesium kept to 1 mM. Activity at 25°C was measured by following the change in absorbance at 340 nm.

2.3. N-terminal sequencing

200 pmol of PGK sample to be sequenced was spotted onto PVDF paper. The samples were then subject to automated Edman sequencing using a Beckman LF 3000.

2.4. Lysine determination

Samples were analysed for free lysine by a modification of the method of Scalia et al. [22]. An ABI Model 172 Series HPLC system was used to analyse samples on a 4.6 mm internal diameter 100 mm OD_{300} aquapore C18 column with 300 Å pore size and 7 μm spherical packing. Lysine standards were prepared by dissolving lysine in 100 mM Tris, 10 mM $CaCl_2$ (pH 8.0) and mixing them 1:1 with HPLC buffer (20 mM sodium phosphate buffer (pH 6.5):acetonitrile (85:15, v/v) containing 0.01 M sodium 1-octanesulphonate), and filtering through a 0.22 μm filter. Samples for analysis were diluted 1:1 in HPLC buffer and filtered through a 0.22 μm filter. 40 μl were loaded on to the column, and eluted isocratically with HPLC buffer (filtered through a 0.45 μm filter) at 0.2 ml/min at ambient temperature. The eluent was monitored for absorbance at 210 nm.

3. Results

3.1. Limited treatment with trypsin results in an activation of PGK

When yeast phosphoglycerate kinase at 4 mg/ml in 100 mM Tris, 10 mM $CaCl_2$ pH 8.0 is treated with trypsin at 0.01 mg/ml, at 37°C, there is a marked increase in the PGK activity in the forward direction, under saturating substrate conditions. This increase in the specific activity occurs over 20 min and goes to four times the initial activity (Fig. 1). At later times there is a fall in the activity as the protein is further digested.

3.2. There is no accompanying change in the apparent molecular mass

When samples from the trypsin digest are subjected to electrophoresis in 12% acrylamide gels in the presence of SDS, there is no apparent change in the migration of the protein. This suggests that the activation of the protein is due to the removal of either a small peptide or a single amino acid from either the N- or C-terminus of the protein. Analysis of the same samples on 16.5% acrylamide gels, which normally visualise peptides as small as 2.5 kDa [20], showed no evidence for any peptide of this size or above being liberated.

Both native and activated PGK were subjected to N-terminal sequence analysis. When we attempted to sequence 200 pmol of native PGK, no sequence was obtained. This is consistent with previous reports that the N-terminal serine of this protein is acetylated [23]. When a sample of PGK, mixed first with PMSF and then trypsin (zero time of digestion), was subjected to sequencing, the only sequence obtained was that of trypsin, and that just at the limit of detection (1 pmol). When a sample treated with trypsin for 20 min was sequenced, in addition to the trypsin sequence, the following amino acids were detected at less than 5 pmol: position 1 – A, Y, and T; position 2 – H, R, and L; position 3 – T and E; position 4 – D, P, and N; position 5 – A, and K; position 6 – E; and position 7 – G, and P.

3.3. Lysine is released concurrently with the activation

Samples from the tryptic digest of yeast phosphoglycerate kinase were analysed by HPLC for the presence of free lysine [22]. This method proved to be highly sensitive, and able to quantify as little as 20 ng of free lysine. There is a release of lysine during the treatment of yeast PGK with trypsin, occurring over the first 20 min (Fig. 1). After 20 min of digestion, which gives the maximal activation, the molar free lysine concentration is 70% of the total PGK concentration. However, at the same time as the removal of Lys-415, digestion will occur at other sites, resulting in inactivation. Because of this, the maximal activity observed is lower than that of 100% de-Lys-415 PGK. The kinetics of release of lysine are seen to parallel those of activation. This suggests that the observed activation of the protein is due to the release of a single lysine from the C-terminus yeast PGK.

Table 1. Michaelis constants of both native and trypsin treated PGK for ATP and 3-phosphoglycerate (3PG)

	K_m (ATP) (mM)	K_m (3-PG) (mM)	Specific activity (U/mg)
Native	0.32	0.43	850
Activated	0.41	0.55	3250

K_m values and specific activities were determined as described (Sherman et al. [18]) except that for specific activity measurements, ATP and 3-PG were present at 5 mM and 15 mM respectively. Native and activated refers to phosphoglycerate kinase untreated and treated with trypsin for 20 min respectively, as described in the text.

3.4. There is no change in the K_m

The effect of trypsin treatment on the Michaelis constants for ATP and 3-phosphoglycerate of yeast PGK was determined (Table 1). On digestion with trypsin for 20 min there is a fourfold increase in activity but only a marginally significant increase in K_m. The K_m of the native protein is consistent with published data [18].

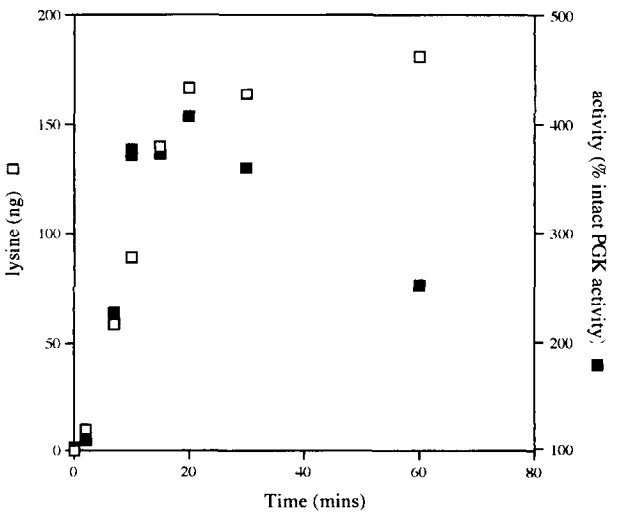

Fig. 1. The activation of, and release of free lysine from, yeast phosphoglycerate kinase (PGK) by trypsin. PGK at 4 mg/ml in 100 mM Tris, 10 mM $CaCl_2$ was incubated at 37°C with trypsin at 10 μg/ml. Samples were take at various times and the trypsin inactivated with PMSF. Samples were assayed for PGK activity by the method of Scopes [21], and the activity normalised to the activity of untreated PGK. Free lysine was determined by the method of Scalia and Massaccesi [22].

Fig. 2. The structure of the hinTge region of yeast phosphoglycerate kinase. Based on molecular coordinates from the crystal structure of Watson et al. [2], displayed on the QUANTA molecular graphics package. Glutamate-413 is in yellow and lysine-415 (to the right of glutamate-413) and lysine-5 (to the left of glutamate-413) are in red; other residues are in pink.

4. Discussion

Treatment of yeast PGK with trypsin results in a dramatic increase in its specific activity. That there is no accompanying change in the mobility of this enzyme on polyacrylamide gel electrophoresis shows that trypsin is not removing more that a short peptide from either the C- or N-terminal end of the protein. Inspection of the primary sequence of yeast PGK [2] shows that likely peptides to be released are a pentapeptide from the N-terminus, a 12 amino acid peptide from the C-terminus, or a single C-terminal lysine. In the light of the results of Ritco-Vonsovici et al. [10] it is highly improbable that removal of the C-terminal 12 amino acids is involved. They showed that a truncated protein lacking these 12 amino acids, although able to fold, exhibits only 0.1% of the activity of the wild-type enzyme.

Attempts to detect any peptide using the gel system of Schagger and Vonjagow [20], designed to visualise small peptides, showed no evidence for the release of any larger peptides during the activation of PGK by trypsin. When either native or activated phosphoglycerate kinase was subjected to N-terminal sequence analysis, no clear sequence was obtained. The trace residues detected on sequencing reflect the gradual digestion of PGK in regions not including the N- or C-termini. Clearly no peptide has been removed from the N-terminus. If a peptide had been hydrolysed from the C-terminus, then the sequence of this peptide should have been detected. It is concluded that the activation of yeast PGK is not caused by the cleavage of a peptide from either terminus of PGK. The only other possible cause for the tryptic activation of yeast PGK is the removal of the C-terminal lysine residue.

An HPLC method [22] was used to detect free lysine. This showed that during the tryptic activation of PGK 1 mol of lysine was liberated per mol of PGK. This leads to the conclusion that the activation is caused by the removal of the C-terminal lysine.

Upon activation of PGK there is no significant effect on the K_m for either substrate. This is not surprising as in the three-dimensional structure lysine-415 is well separated from either substrate binding site [2]. It is therefore improbable that the activation of PGK by trypsin is mediated by a change in the immediate conformation of the substrate binding sites.

The C-terminal helix is important in the interaction between the two domains. In the crystal structure [2] it folds over the N-terminal domain. Truncated PGK, lacking the 15 C-terminal amino acids, has been characterised [10]. This protein, although able to fold to a native-like structure and bind substrates, exhibits only 0.1% of the catalytic activity of the wild-type enzyme. This has been interpreted as showing that interactions between the C-terminal helix and the N-terminal domain have an essential role in mediating the conformational change during catalysis.

It is therefore reasonable to conclude that the removal of the C-terminal lysine residue activates PGK by modifying the interactions of the C-terminal loop with the N-terminal domain. Observation of the three-dimensional structure shows that this lysine, Lys-415, lies on the surface of the protein, 5.5 Å from glutamate-413. Glutamate-413 is also 6.5 Å from lysine-5 (Fig. 2). The linear array of these three residues suggests that glutamate-413 can make interactions with both of these lysine residues. Removal of lysine-415 will therefore be expected to strengthen the interaction between lysine-5 and glutamate-413. Lysine-5 is part of the N-terminal sequence which folds over and contacts the C-terminal domain. Strengthening the interactions between these two inter-domain spanning regions appears to rigidify the hinge in the active orientation. The present results suggest that the C-terminal lysine acts as a sensitive moderator of the energy barrier to hinge bending, resulting in the native PGK having a specific activity that is less than it could otherwise have, if hinge bending were not an important feature of this enzyme.

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