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Studies with Inhibitors of the Glycolytic Enzyme Phosphoglycerate Kinase for Potential Treatment of Cardiovascular and Respiratory Disorders

J. MCHARG AND J. A. LITTLECHILD

Departments of Chemistry and Biological Sciences, University of Exeter, Exeter EX4 4QD, UK

Abstract

Inhibition of the glycolytic enzyme phosphoglycerate kinase (PGK) in erythrocyte cells could provide a method of treatment for cardiovascular and respiratory disorders. The product of the reaction catalysed by PGK, 1,3-diphosphoglycerate, is converted by another enzyme in erythrocytes to 2,3-diphosphoglycerate, which is an allosteric effector of haemoglobin. For this reason, a series of fluoro-phosphonate inhibitors have been tested for their potency in detailed inhibition kinetic experiments with yeast PGK.

The results were analysed by Lineweaver-Burk and Dixon plots and K_i values obtained. Two fluorophosphonates were found to be inhibitory and both have an electron rich mid-chain functionality, which is thought to provide electrons for hydrogen bonding to residues in the triose binding site of the enzyme.

It is postulated that either the fluorine or mid-chain moieties of the analogues are binding to Asp23 and Asn25 residues in the so called 'basic patch' area of the triose site. These residues are shown to bind to the D-hydroxyl moiety on the C2 of the true substrate, 3-phosphoglycerate, in the high-resolution crystal structure of pig muscle PGK co-crystallized with 3-phosphoglycerate.

Phosphoglycerate kinase (PGK) is a glycolytic enzyme which catalyses the reaction:

$$\text{1,3-diphosphoglycerate} + \text{Mg.ADP} \leftrightarrow \text{3-phosphoglycerate} + \text{Mg.ATP}$$

A bilobal monomeric protein of 45 kDa, PGK has been purified and sequenced from 39 sources, and their sequences aligned (Watson & Littlechild 1990). Although only approximately 11% of the protein is totally conserved, (44 residues), most of these residues line the cleft between the two lobes where the substrate binding sites are located. High resolution X-ray crystallographic structures have been determined for PGK from five species and show the secondary and tertiary structures to be very similar (Banks et al 1979; Watson et al 1982; Littlechild et al 1987; Harlos et al 1992; Davies et al 1994). All of the structures represent an open conformation of the enzyme and both the nucleotide and triose substrate binding sites have been determined by co-crystallization and soaking of substrate analogues in this form of the protein. The nucleotide binds in a shallow depression on the *C*-terminal domain of the enzyme opposite the triose binding site. This is an area of highly conserved basic residues, known as the basic patch located on the *N*-terminal domain. This region consists of five arginine residues (21, 38, 65, 121 and 168; yeast numbering) and three histidine residues (62, 167 and 170) as shown in Fig. 1. When both substrates are bound in their crystallographically determined positions the attacking oxygen atom of the triose substrate is 12–15 Å away from the γ-phosphate of the nucleotide. It was therefore proposed that a hinge-bending mechanism occurs, which brings the substrates together allowing phosphoryl transfer to occur in a water-free, low dielectric environment (Banks et al 1979).

Correspondence: J. McHarg, Departments of Chemistry and Biological Sciences, University of Exeter, Exeter EX4 4QD, UK.

Inhibitors of PGK are being investigated since the product of this enzyme reaction, 1,3-diphosphoglycerate (1,3-DPG) is converted by a mutase in erythrocyte cells to 2,3-diphosphoglycerate which is an allosteric effector of haemoglobin, affecting the ability of haemoglobin to take up oxygen (Perutz 1980). This makes inhibitors of PGK potential candidates in the treatment of cardiovascular and respiratory disorders.

This paper reports the inhibition kinetics of a series of fluoro-phosphonate analogues of 1,3-DPG with yeast PGK, and their potential interaction with the conserved residues of the basic-patch region of the enzyme.

Materials and Methods

The fluoro-phosphonate analogues of varying chain length and mid-chain functionality, were designed and synthesized by Professor G. M. Blackburn and his group at the University of Sheffield (Blackburn et al 1994). The CF_2 moiety was incorporated into these analogues as a non-scissile, isosteric and isoelectronic replacement for an oxygen atom, which improves the physicochemical parameters associated with a phosphonate, especially the state of ionization of a phosphonic acid. The analogues used are shown in Fig. 2.

GAPDH assay

The reaction catalysed by GAPDH in which glyceraldehyde-3-phosphate is converted to 1,3-DPG using the co-factor NAD^+, was driven in the forward direction and the increase in NADH monitored spectrophotometrically at

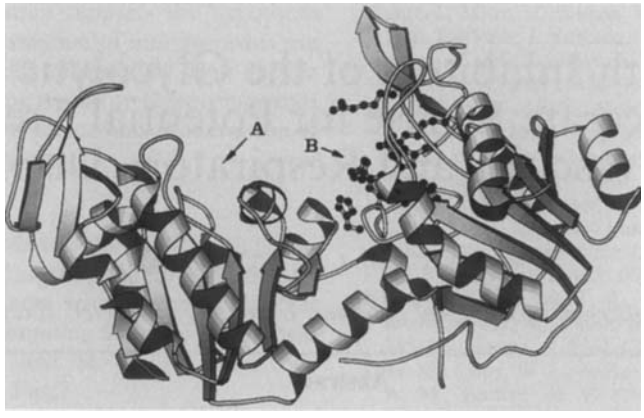

FIG. 1. Diagrammatic representation of the structure of yeast PGK, created using MOLSCRIPT (Kraulis 1991). The molecule is shown in ribbon representation with the β-sheets as arrows and the amino acid residues of the basic patch in ball and stick. The nucleotide binding site is indicated at A and the triose binding site at B.

340 nm (Fig. 3). Assays were conducted at 22°C and the assay mixture contained 4 mM NAD^+ and 0.03-0.21 mM glyceraldehyde-3-phosphate in a freshly-prepared buffer of 1 mM EDTA, 50 mM disodium hydrogen orthophosphate, 40 mM triethanolamine, pH 8·6.

PGK assay

A linked-assay system was used, in which the reaction catalysed by PGK is driven in reverse and linked to the previous reaction in the glycolytic pathway, as shown in Fig. 3. The decrease in extinction of NADH at 340 nm is followed spectrophotometrically. Assays were carried out as above in a buffer of 30 mM triethanolamine pH 7·5, 50 mM KCl, 0·2 mM EDTA. The assay mixture also contained 0·15 mM NADH, a saturating level of GAPDH (4 μg mL^{-1}) and 40 mM $(NH_4)_2SO_4$. A saturating level of 50 mM $MgCl_2$, 40 mM ATP was used with a range of 3-PGA concentrations, 0·2–1·0 mM, and a saturating level of 5 mM 3-PGA with 1–5 mM Mg.ATP. All reagents were obtained from Sigma Chemical Company (Poole, UK) or Boehringer Mannheim GmbH, (Lewis, UK), apart from yeast PGK which was purified from an over-expressed system in *Saccharomyces cerevisiae* (Minard et al 1990) to single-band purity on SDS-PAGE gel (Laemmli & Favre 1973) stained with Coomassie blue.

Results

None of the analogues significantly inhibited the binding of Mg.ATP to PGK, nor was any inhibitory effect on GAPDH detected.

Initial assays showed analogues **1** and **2** to be active as inhibitors whilst **3**–**6** showed no significant inhibition. The results of the kinetic studies were analysed by the use of Lineweaver-Burk and by Dixon plots (Figs 4, 5). Although these models are designed for one-substrate, one-product reactions, they were deemed acceptable for analysis of this system when one substrate, 3-PGA, was present in excess and the other, Mg.ATP, was a limiting factor. The intersection of the Lineweaver-Burk plots above the 1/S axis and to the left of the 1/V axis identifies the type of inhibition exhibited by analogues **1** and **2** as the linear mixed type. This is the system in which the enzyme-inhibitor complex has a lower affinity than the enzyme for the substrate, and the enzyme substrate-inhibitor complex is non-productive. The equilibria describing this system are shown in Fig 6.

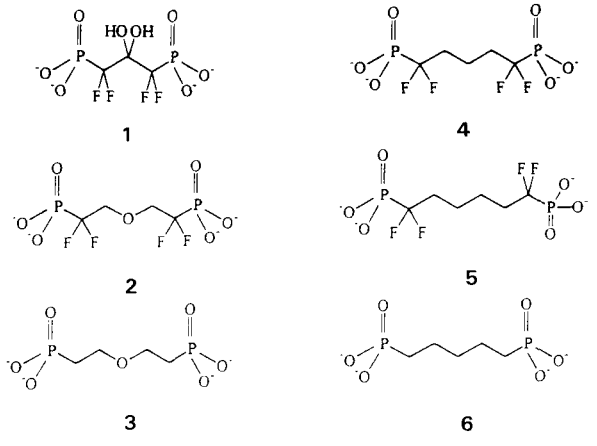

FIG. 2. Phosphonate analogues of 1,3-DPG.

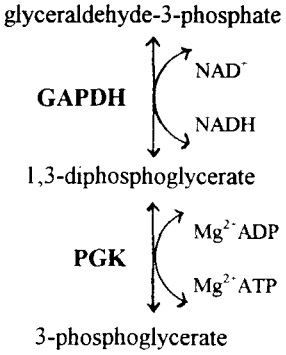

FIG. 3. Reactions of glycolytic pathway used in the assays for PGK and GAPDH. The PGK assay uses the two reactions shown driven in reverse (up the page), whilst GAPDH is assayed in the forward direction. (down the page).

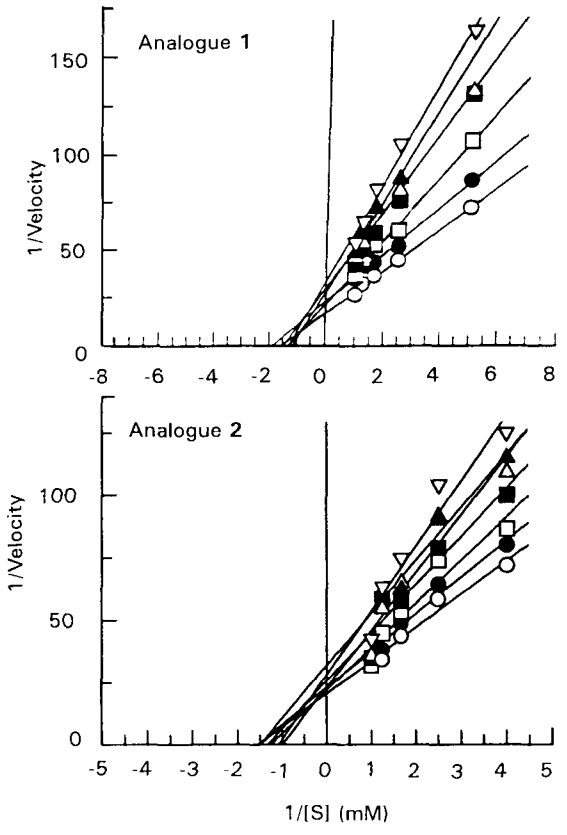

FIG. 4. Lineweaver-Burk plots of analogues **1** and **2** showing linear mixed inhibition of PGK by analogues **1** and **2**.

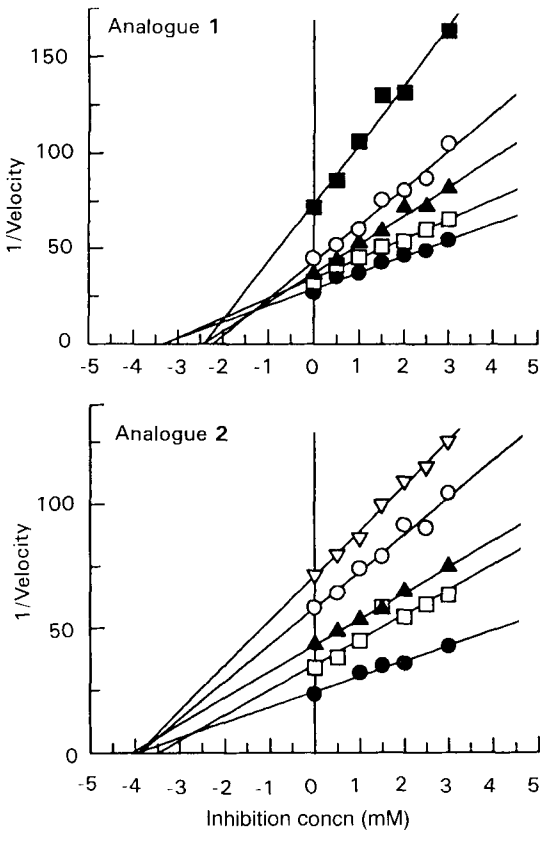

FIG. 5. Dixon plots of analogues **1** and **2** showing inhibition constants (K_i) of 1·5 mM and 3·3 mM respectively.

The kinetic parameters K_s, K_i and α were obtained both by direct replots of the Lineweaver Burk intercepts and slopes and by analysis of the Dixon plots (Segel 1993). The results for analogues **1** and **2** are shown in Table 1. The pairs of values obtained, one from Lineweaver Burk analysis and one from Dixon analysis, correlate well. The K_s values compare well with the K_m value of 0·7 mM for 3-PGA obtained from the uninhibited enzyme kinetic data, suggesting the correctness of the model. The kinetic results suggest that the affinity of PGK for analogue **1** is twice that of analogue **2**.

Discussion

Comparison of analogues **2** and **3**, and **2** and **4** suggests that two components are important for binding in the compounds assayed: the presence of the fluorine atoms and an additional electron-rich functionality in the middle of the carbon chain available for hydrogen bonding.

Analogue **1** was found to bind the tightest of the group assayed. It is a shorter molecule than any of the others with only a 3-carbon chain. The fluorine atoms on C1 and C3, and the two hydroxyl moieties on C2, provide a sheath of electron-rich orbitals for hydrogen bonding. It is interesting to note that one of the C3 fluorines is in the same position as the C2 D-hydroxyl group of the substrate, which has been implicated as important for binding in other studies of short analogues. Tompa et al (1986) found DL-glycerol-3-phosphate to bind strongly to pig muscle PGK with a K_i value of 0·7 mM, but the L-stereo isomer showed no inhibition, suggesting the importance of the D-C2 hydroxyl group. Vas (1990) reported DL-2-hydroxy-3-propionate to be a weak inhibitor with a K_i value of 17 mM, which is explained by the reduction in length of the molecule such that the D-C2 hydroxyl group does not occupy the same spatial position as the hydroxyl of the substrate. In analogue **1**, since one of the C2 hydroxyl moieties is in almost exactly the same position as the hydroxyl of the much weaker inhibitor DL-2-hydroxy-3-phosphonopropionate, it may be that one of the fluorines at C1 is fulfilling the D-C2 hydroxyl binding role. On the other hand, as the chain is very flexible, and electron-rich, it may be that two or three different binding interactions occur with this molecule.

Table 1. K_s, K_i and α values for analogues **1** and **2**, derived from Lineweaver Burk and Dixon plots. The K_m value for the substrate, 3-PGA is 0·7 mM (see text).

	K_s (mM)	K_i (mM)	α
Analogue 1			
Lineweaver-Burk direct replot	0·60	1·44	1·65
Dixon plot	0·60	1·50	2·30
Analogue 2			
Lineweaver Burk direct replot	0·67	3·30	2·05
Dixon plot	0·67	3·25	1·29

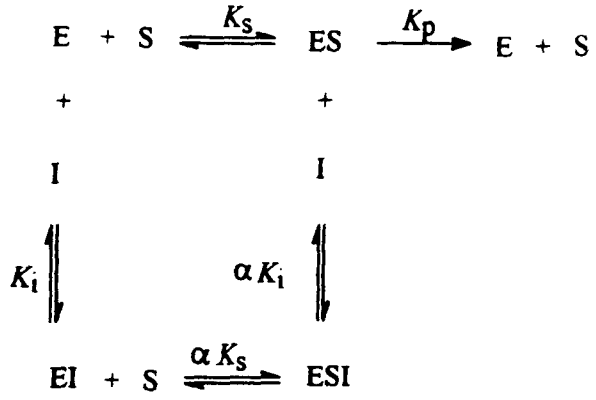

FIG. 6. Equilibria describing the linear mixed type of inhibition.

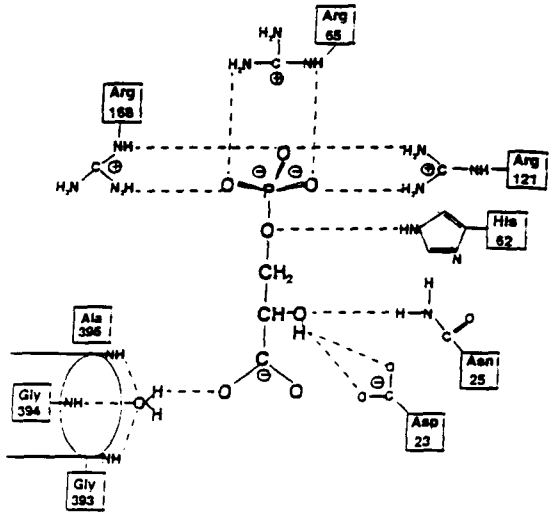

FIG. 7. The triose substrate binding site observed in pig muscle PGK. A schematic representation of the hydrogen bond (broken lines) and electrostatic interactions observed in the binary complex between pig muscle PGK and 3-PGA using yeast numbering (Harlos et al 1992).

Analogue **2** has a K_i value of 3·3 mM, which is more than a 2-fold increase over analogue **1**. This can be accounted for by the diminished electron density along the chain. The ethyl oxygen is not positioned spatially for such efficient binding as either a fluorine or hydroxyl moiety.

Analogue **6** showed no significant inhibition in this study; however, Yaw-Kuen Li & Byers (1993) have reported this compound to inhibit PGK with a K_i value of 1·19 mM at pH 8·5 and 27°C. The assay conditions described for that study are different from those employed here. It has been found that PGK does not always conform to Michaelis-Menten kinetics (Scopes 1978; Khamis & Larsson-Raznikiewicz 1981). When both substrates are present at concentrations below 5 mM, sulphate ions have been found to be activatory up to a concentration of approximately 20 mM. As the sulphate ion concentration increases in excess of 20 mM, the activatory effect is reduced until in excess of 40 mM, the effect becomes inhibitory. For this reason, the experiments described here were performed in 40 mM $(NH_4)_2SO_4$, where the enzyme behaves in a Michaelis-Menten fashion. Sulphate anions were not included in the assay system used by Yaw-Kuen Li & Byers, and in view of the non-Michaelis-Menten kinetics under these conditions, the K_i value cannot be calculated accurately. However, when assays were conducted without sulphate for analogue **6**, no inhibition was observed.

The results can be interpreted in the light of the high-resolution crystal structures of yeast PGK (Gamblin et al unpublished) and pig muscle PGK (Harlos et al 1992), which was co-crystallized with 3-PGA. Both of these structures are in the open form of the enzyme. In the closed catalytically-active form, the triose binding site may not be in the same conformational state. Bearing this in mind, some tentative attempts have been made to explain the results in terms of the geometry of the 3-PGA site in the open conformer.

The pig muscle PGK-3-PGA crystal structure shows that the sugar substrate binds in the highly conserved area, known as the basic patch. Three arginine residues Arg 65, 121 and 168 (yeast numbering) each forms two bonds with the oxygens of the non-transferable phosphate head, and His 62 bonds with the linking oxygen of the phosphate (Fig. 7). It is interesting to note that the D-alcohol moiety of the substrate binds to two additional totally conserved residues, Asp 23 and Asn 25. This finding explains the strong inhibition by DL-glycerol-3-phosphate and the absence of inhibition by the L-stereoisomer. It appears that the interaction between the D-alcohol and those two totally conserved residues is important for good enzyme-substrate binding.

A series of site-directed mutants has been made of the yeast PGK enzyme (Walker et al 1989; Sherman et al 1990; Fairbrother et al 1989a; Barber et al 1993). They have confirmed the importance of 'basic patch' residues His 62, Args 65, 121 and 168 and Asn 25 in the catalytic mechanism of the enzyme. These studies will be extended to include the mutation of Asp 23. Asn 25, when mutated, was found to completely abolish activity, (Bowen et al; unpublished results) demonstrating its role in substrate binding, and adding to the evidence that the stereochemistry of the hydroxyl moiety on the substrate is important.

Co-crystallisation and crystal soaking experiments of the inhibitors with both yeast and *Bacillus stearothermophilus* PGK are in progress. Results from these experiments will determine the exact mode of binding of the inhibitors at the triose binding site of the enzyme.

This study is part of a multidisciplinary approach correlating the crystal studies above with solution studies. The K_i values described here are compared with K_d measurements obtained from ^{1}H-NMR studies, which monitor the resonance shifts of the basic patch histidine residues in response to inhibitor binding (Fairbrother et al 1989b). Kinetic and ^{1}H-NMR experiments have been extended to study inhibition of other fluoro-phosphonate analogues and K_i and K_d values are being correlated (Jakeman et al manuscript in preparation).

The studies reported here are helping our understanding of the enzymatic mechanism of PGK and developing the potential use of 1,3-DPG analogues as inhibitors of this glycolytic enzyme.

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References

Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merret, M., Phillips, A. W. (1979) Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme. Nature 279: 773–777

Barber, M. D., Gamblin, S. J., Watson, H. C., Littlechild, J. A. (1993) Site-directed mutagenesis of yeast phosphoglycerate kinase Arginines 65, 121 and 168. FEBS Lett. 320: 193–197

Blackburn, G. M., Jakeman, D. L., Ivory, A., Williamson, M. P. (1994) Synthesis of phosphonate analogues of 1,3-bisphosphoglyceric acid and their binding to yeast phosphoglycerate kinase. Bioorganic and Medicinal Chemistry Letters 4: 2573–2578

Davies, G. J., Gamblin, S. J., Littlechild, J. A., Dauter, Z., Wilson, K. S., Watson, H. C. (1994) Structure of the ADP Complex of the 3-phosphoglycerate kinase from *Bacillus stearothermophilus* at 1. 65Å. Acta Crystallogr. D50: 202–209

Fairbrother, W. J., Hall, L., Littlechild, J. A., Walker, P. A., Watson, H. C., Williams, R. J. P. (1989a) Site-directed mutagenesis of histidine 62 in the basic patch region of yeast phosphoglycerate kinase. FEBS Lett. 258: 247–250

Fairbrother, W. J., Walker, P. A., Minard, P., Littlechild, J. A., Watson, H. C., Williams, R. J. P. (1989b) NMR analysis of site-specific mutants of yeast phosphoglycerate kinase. Eur. J. Biochem. 189: 57–67

Gamblin, S. J., Davies, G. J., Holden, P., Littlechild, J. A., Watson, H. C. (1996) The structure of yeast phosphoglycerate kinase refined to 2Å resolution. Acta Crystallogr., in press

Harlos, K., Vas, M., Blake, C. F. (1992) Crystal structure of the binary complex of pig muscle phosphoglycerate kinase and its substrate 3-phosphoglycerate. Proteins: Structure, Function and Genetics 12: 133–144

Jakeman, D., M^{c}Harg, J., Ivory, A., Littlechild, J. A., Blackburn, G. M., Eur. J. Biochem. manuscript in preparation

Khamis, M. M., Larsson-Raznikiewicz, M. (1981) Anion effects on the kinetics of yeast PGK. Biochimica et Biophysica Acta 657: 190–194

Kraulis, P. J. (1991) MOLSCRIPT—a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallogr. 24: 946

Laemmli, U. K., Favre, M. (1973) Maturation of the head of bacteriophage T4. J. Mol. Biol. 80: 575–599

Littlechild, J. A., Davies, G. J., Gamblin, S. J., Watson H. C. (1987) Phosphoglycerate kinase from the extreme thermophile *Thermus thermophilus*. FEBS Letters 225: 123–126

Minard, P., Bowen, D. J., Hall, L., Littlechild, J. A., Watson, H. C. (1990) Site-directed mutagenesis of aspartic acid 372 at the ATP binding site of yeast phosphoglycerate kinase: over-expression and characterisation of the mutant enzyme. Protein Engineering 3: 515–521

Perutz, M. F. (1980) Proc. Roy. Soc., London 208: 135–162

Scopes, R. K. (1978) The steady-state kinetics of yeast phosphoglycerate kinase. Eur. J. Biochem. 85: 503–516

Segel, I. H. (1993) Rapid Equilibrium Partial and Mixed Type Inhibition. In: Segel, I. H., Enzyme Kinetics. Wiley Classics Library Edn, Wiley, USA pp 170–176

Sherman, M. A., Szipkowska, B. K., Dean, S. A., Mathiowetz, A. M., M^{c}Queen, N. L., Mas, M. T. (1990) Probing the role of arginines and histidines in the catalytic function and activation of yeast 3-phosphoglycerate kinase by site-directed mutagenesis. J. Biol. Chem. 265: 10659–10665

Tompa, P., Hong, P. T., Vas, M. (1986) The phosphate group of 3-phosphoglycerate accounts for conformational changes occurring on binding to 3-phosphoglycerate kinase. Eur. J. Biochem. 154: 643–649

Vas, M. (1990) Modelling of substrate binding to 3-phosphoglycerate kinase with analogues of 3-phosphoglycerate. Eur. J. Biochem. 194: 639–645

Walker, P. A., Littlechild, J. A., Hall, L., Watson, H. C.(1989) Site-directed mutagenesis of yeast phosphoglycerate kinase: The basic patch residue arginine 168. Eur. J. Biochem. 183: 49–55

Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., Kingsman, S. M. (1982) Sequence and structure of yeast phosphoglycerate kinase. EMBO J. 1: 1635–1640

Watson, H. C., Littlechild, J. A. (1990) Isoenzymes of phosphoglycerate kinase: Evolutionary conservation of the structure of this glycolytic enzyme. Biochem. Soc. Trans. 18: 187–190

Yaw-Kuen, Li, Byers, L. D. (1993) Phosphonate inhibitors of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. Biochim. Biophys. Acta 1164: 17–21