Antagonistic binding of substrates to 3-phosphoglycerate kinase monitored by the fluorescent analogue 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate*

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The analogue of ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), binds tightly to pig muscle 3-phosphoglycerate kinase. A dissociation constant K_d of 0.0095 ± 0.0015 mM was determined by fluorimetric titration on the basis of 1:1 stoichiometry. TNP-ATP is a strong competitive inhibitor towards MgATP and MgADP with a K_i of 0.008 ± 0.001 mM for both substrates. It is also a mixed-type inhibitor towards 3-phosphoglycerate with similar inhibition constants. Binding of TNP-ATP to 3-phosphoglycerate kinase is accompanied by a tenfold intensity increase and a blue shift of about 20 nm in its fluorescence emission spectrum and a shift of the pK of its trinitrophenyl group towards a more acidic pH. These findings suggest that the negatively charged trinitrophenyl group of TNP-ATP significantly contributes to the binding of the analogue. By stepwise replacement of the fluorescent TNP-ATP, the dissociation constants (K_d) for ADP and MgADP binding were determined and found to be 0.78 ± 0.08 and 0.048 ± 0.006 mM respectively, which are consistent with the values previously determined by equilibrium dialysis [Molnár and Vas (1993) Biochem J. **293**, 595–599]. In similar competitive-titration experiments, ATP and MgATP did not completely substitute for TNP-ATP. For the fraction of the analogue that could be substituted, the dissociation constants for MgATP and ATP were estimated to be 0.27 ± 0.09 and 0.33 ± 0.15 mM respectively, close to the values determined by equilibrium dialysis. Using the same method, a significant weakening of binding of both (Mg)ADP and (Mg)ATP could be detected in the presence of 3-phosphoglycerate: their respective K_d values became 0.34 ± 0.04 and 0.51 ± 0.22 mM. The reciprocal effect, i.e. weakening of 3-phosphoglycerate binding in the presence of the nucleotide substrates, has been observed previously [Vas and Batke (1984) Eur. J. Biochem. **139**, 115–123]. Similarly, a much weaker binding of (Mg)ATP could be observed in the presence of 1,3-bisphosphoglycerate ($K_d = 2.30 \pm 0.68$ mM). The possible reason for the mutual weakening of substrate binding is discussed in the light of the available structural data.

INTRODUCTION

The interaction of the two substrates in the reversible reaction catalysed by 3-phosphoglycerate kinase is far from being understood. Numerous studies of enzyme kinetics [1–4] and equilibrium binding [5–11] indicate random substrate binding, but the influence of each substrate on the binding of the other is still an open question.

A significant weakening of 3-phosphoglycerate binding to 3-phosphoglycerate kinase in the presence of nucleotides has been reported [7,8]. It has been suggested that the negatively charged phosphates of the nucleotide are important in this phenomenon since AMP has a much lower effect and adenosine has no effect at all [8]. This weakening effect might be a typical property of the active form of 3-phosphoglycerate kinase, as the binding of 3-phosphoglycerate to enzyme previously inactivated by specific chemical modification of the two reactive cysteines is not affected by the presence of the nucleotide substrate [12]. On the other hand, data on nucleotide binding in the presence of 3-phosphoglycerate were not conclusive because the differences in the dissociation constants were of the same order of magnitude as the large experimental errors [8].

The structure of the ternary complex formed by 3-phosphoglycerate kinase with the two substrates has not yet been elucidated. The structures determined for the two binary complexes (with either the nucleotide substrate [13–16] or 3-phosphoglycerate [17]) do not allow the steric mechanism of their interaction to be explained (see also [18,19] and the review in [20]). Some uncertainties still exist even of the details of nucleotide binding to the enzyme. For example, the position of the phosphate chain and the location and role of bound Mg^{2+} has not yet been clarified by either crystallographic [13–15] or solution-affinity-labelling [21,22] studies.

To monitor the binding of the nucleotide substrates to 3-phosphoglycerate kinase and to extend the investigation to the binding of substrates to single crystals of the enzyme by microspectrophotometry (see the review in [23]), we have now used a coloured and fluorescent analogue of ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) [24], which has been successfully used either as a substrate for certain ATP-dependent enzymes, including adenylate kinase and myosin ATPase [25], or as an affinity label for other ATP-dependent enzymes [25–29]. We have determined by competitive titration the binding constants of ADP, MgADP, ATP and MgATP to 3-phosphoglycerate kinase, in both the presence and absence of either 3-phosphoglycerate or the unstable and extremely tightly bound substrate 1,3-bisphosphoglycerate.

MATERIALS AND METHODS

Enzymes and chemicals

3-Phosphoglycerate kinase was isolated from pig muscle as

* Dedicated to Professor F. B. Straub on the occasion of his 80th birthday.
Abbreviation used: TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate.
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described by Harlos et al. [17] and stored in a microcrystalline suspension in the presence of dithiothreitol. Its molar activity varied between 500 and 700 kat/mol with 3-phospho-D-glycerate and MgATP as substrates.

Glyceraldehyde-3-phosphate dehydrogenase (350 kat/mol) was prepared from pig muscle [30]. A crystalline mixture of hexokinase and glucose-6-phosphate dehydrogenase (3 mg/ml) was purchased from Boehringer.

Sodium salts of ADP, ATP, 3-phospho-D-glycerate and $NADP^+$ were also Boehringer products. On the basis of enzymic determination (see below), ADP and ATP contained at most 1% contamination of each other. The disodium salt of TNP-ATP was purchased from Molecular Probes. NADH, $MgCl_2$ and D-glucose were Sigma products. The substrate 1,3-bisphosphoglycerate was prepared as described previously [31]. All other chemicals were reagent-grade commercial preparations.

Preparation of enzyme and nucleotide solutions

Microcrystals of 3-phosphoglycerate kinase were dissolved in 50 mM Tris/HCl buffer, pH 7.0, dialysed against the same buffer to remove $(NH_4)_2SO_4$ and dithiothreitol. Although thiol compounds, such as dithiothreitol, were found to protect the enzyme against inactivation [31], the present experiments required their absence, as these compounds caused decomposition of TNP-ATP. Concentration and activity of 3-phosphoglycerate kinase solution were determined as described previously (e.g. [9,31]). The dialysed enzyme was completely active and its activity did not decrease during the time of experiment.

All other solutions were prepared in the above buffer. The concentrations of ADP and ATP were measured both spectrophotometrically (using values of $\epsilon_{260} = 15000\ M^{-1}\cdot cm^{-1}$ for ADP and $15400\ M^{-1}\cdot cm^{-1}$ for ATP) and enzymically (using the appropriate assay mixtures described previously [9]). The concentration of TNP-ATP was checked spectrophotometrically using a value of $\epsilon_{408} = 26400\ M^{-1}\cdot cm^{-1}$ [24].

Enzyme kinetic measurements

The activity of 3-phosphoglycerate kinase was assayed spectrophotometrically at 340 nm either with 3-phospho-D-glycerate and MgATP as substrates (as described in [9]) or in the reverse direction with 1,3-bisphosphoglycerate and MgADP as substrates (as described in [32]), at pH 7.0 and at 20 °C. Enzyme-inhibition studies were carried out in the presence of excess (10 mM) $MgCl_2$, so that all ATP or ADP was complexed with Mg^{2+} on the basis of their reported dissociation constants [33–35].

Spectroscopic measurements

Absorption spectra and kinetic parameters were determined using an Opton DMR 21 double-beam spectrophotometer equipped with a cell compartment thermostatically controlled at 20 °C.

Fluorescence-emission spectral measurements were recorded by using either a Perkin–Elmer MPF 3L or a Jasco FP-777 spectrofluorimeter, both equipped with a cell compartment thermostatically controlled at 20 °C.

Fluorimetric titrations were carried out using an excitation wavelength of 408 nm and measuring emission at 535 nm. In preliminary titrations, a concentration range of TNP-ATP was selected to obtain a linear response in emission intensity. All titrations were carried out with increasing concentrations of 3-phosphoglycerate kinase, which does not absorb at either the excitation or the emission wavelength, while the concentration of TNP-ATP was kept constant. In competitive titrations, various, but in each case constant, concentrations of nucleotide substrates were additionally present.

To investigate the pH-dependence of the spectrum of TNP-ATP in both the presence and absence of 3-phosphoglycerate kinase, we used either 50 mM Tris/HCl or 50 mM acetate/NaOH buffers, containing 1 mM EDTA. No loss of enzyme activity occurred even around pH 4 during the time of measurement. All other experiments were carried out in 50 mM Tris/HCl buffer, pH 7.0, containing 1 mM EDTA.

Evaluation of the kinetic and equilibrium data

Quantitative evaluation of the data was achieved partly by non-linear regression analysis by the Grafit program (R. J. Leatherbarrow, 1989/1990, Erithhacus Software Limited, supplied by Sigma) and partly by introducing the equations (see below) to the software provided by Sigma Plot (Jandel). Both programs were operated with the aid of an IBM Varyter AT computer connected to a LaserJet IIP printer.

Equations used to analyse the results of fluorimetric titrations

A fixed concentration of TNP-ATP ([A]), usually 0.0125 mM, was titrated with increasing amounts of 3-phosphoglycerate kinase ([E] = total enzyme concentration). The concentration of enzyme-bound TNP-ATP ([EA]) was determined on the basis of the fluorescence increase (ΔI) occurring when TNP-ATP binds to the protein. The maximum fluorescence increase ($\Delta I_{max.}$) was determined by extrapolation at the infinite enzyme concentration using a double-reciprocal plot as previously reported by others (e.g. [36]).

Although this procedure would be essentially correct only in case of $[E] \gg [EA]$, we could use it with good approximation on the basis of the following observations: (1) straight lines with good correlation coefficients were obtained; (2) in the titrations of TNP-ATP with the enzyme, the extrapolated $\Delta I_{max.}$ was proportional to the concentration of TNP-ATP used; (3) in the titration of the enzyme with TNP-ATP, the extrapolated $\Delta I_{max.}$ was proportional to the enzyme concentration. The proportionality was verified only below certain concentrations of either enzyme or TNP-ATP and therefore the experiments were carried out only within this range of concentrations.

Therefore the enzyme-bound TNP-ATP ([EA]) is calculated as:

$$[EA] = (\Delta I/\Delta I_{max.})[A] \quad (1)$$

The dissociation constant of TNP-ATP binding (K_{d1}) was calculated using eqn. (2) from each point of the titration curve and the values obtained were averaged.

$$K_{d1} = ([E]-[EA])([A]-[EA])/[EA] \quad (2)$$

For competitive titrations in the presence of a fixed concentration of (Mg)ATP or (Mg)ADP (i.e. [N]), the dissociation constant for the nucleotide substrate (K_{d2}) was expressed as:

$$K_{d2} = [E_{free}][N_{free}]/[EN] \quad (3)$$

By substituting for [EN] the concentration of the enzyme complexed with the nucleotide substrate, $[EN] = [E]-[EA]-[E_{free}]$ and, from eqn. (2), $[E_{free}] = K_{d1}m$ {where $m = [EA]/([A]-[EA])$ is the ratio between bound and free TNP-ATP}:

$$K_{d2} = K_{d1}m\{[N]-([E]-[EA]-K_{d1}m)\}/([E]-[EA]-K_{d1}m) \quad (4)$$

Knowing the value of K_{d1}, K_{d2} was calculated using eqn. (4) for each point of the titration curve and the resulting values were averaged.

In competitive-titration experiments in the presence of (Mg)ATP, an additional phenomenon had to be taken into account in determining [EA]: (Mg)ATP could not completely displace the analogue even at very high concentrations, although competitive inhibition of TNP-ATP with respect to MgATP was observed (see the Results section). Therefore to estimate the dissociation constant of ATP binding from fluorimetric competitive titrations, the fluorescence increase measured at each enzyme concentration at high ATP levels (around 20 mM) was subtracted from the fluorescence increase observed at lower levels of ATP. The difference was then normalized to the maximum obtained in the absence of ATP and used in eqn. (1) to calculate the concentration of bound TNP-ATP.

Using the above determined K_d values, theoretical curves were calculated and compared with the experimental data (in this case only ADP binding was considered) on the basis of the following relationship:

$$[E] = [EA] + [E_{free}] + [EN] \quad (5)$$

By substituting for $[E_{free}]$ and [EN] in eqn. (5) the expressions obtained respectively by eqn. (2) and eqn. (3), the equation that expresses [E] as a function of [EA] is obtained:

$$[E] = ([EA] + K_{d1}m + K_{d1}m[N])/(K_{d2} + K_{d1}m) \quad (6)$$

Molecular modelling studies

X-ray co-ordinates of pig muscle 3-phosphoglycerate kinase (kindly provided by Dr. K. Harlos, Laboratory of Molecular Biophysics, University of Oxford) were used to envisage the enzyme structure by the aid of the software Insight II. 2.1.0 (Biosym Technologies, Inc.) operated with a Silicon Graphics Personal Iris 4D25TG computer. An approximate model of TNP-ATP was constructed from the co-ordinates of ATP and trinitrophenol (Cambridge Data Bank), connecting 02′ and 03′ of the ATP molecule with the C1 of trinitrophenol with two bonds having the normal length of an ether bond.

RESULTS AND DISCUSSION

Is TNP-ATP a substrate of 3-phosphoglycerate kinase?

As TNP-ATP has previously been shown to substitute for ATP as a substrate in certain enzyme-catalysed reactions [23], we verified whether it could be recognized by 3-phosphoglycerate kinase. When MgTNP-ATP was added to the assay mixture in place of MgATP, even at concentrations of 3-phosphoglycerate kinase four orders of magnitude higher than required under usual assay conditions, no formation of 1,3-bisphosphoglycerate could be detected. This finding implies that the large trinitrophenyl group attached to the ribose does not allow productive binding and/or transfer of the γ-phosphate of the modified nucleotide.

Inhibitory properties of TNP-ATP

When the physiological reaction catalysed by 3-phosphoglycerate kinase was studied in the presence of MgTNP-ATP, competitive inhibition towards both MgATP (Figure 1a) and and MgADP (results not shown) was observed. The inhibition constant (K_i) is 0.008 ± 0.001 mM in both cases. In contrast, the inhibition against 3-phosphoglycerate is a mixed type, having characteristics close to the non-competitive type, and the inhibition constants, K_i and K_i', are again of the order of 0.01 mM (Figure 1b).

Competitive inhibition with respect to the nucleotide substrates is expected by structural analogy. Thus one can assume that TNP-ATP binds to the well-defined nucleotide site [13–15] on the C-terminal domain of the enzyme. The mixed-type inhibition towards 3-phosphoglycerate, although plausible, is somewhat surprising in the light of previous studies showing competitive product inhibition by MgADP [2,32] and by the ATP analogue, 5′-adenylyl imidodiphosphate [37] with respect to 3-phosphoglycerate (M. Vas, A. Merli and G. L. Rossi, unpublished work), as well as competitive inhibition of certain analogues of 3-phosphoglycerate with respect to MgATP [9]. At present, double-inhibition kinetic studies are underway in order to obtain information about the possible sites of those inhibitors that are competitive towards both substrates.

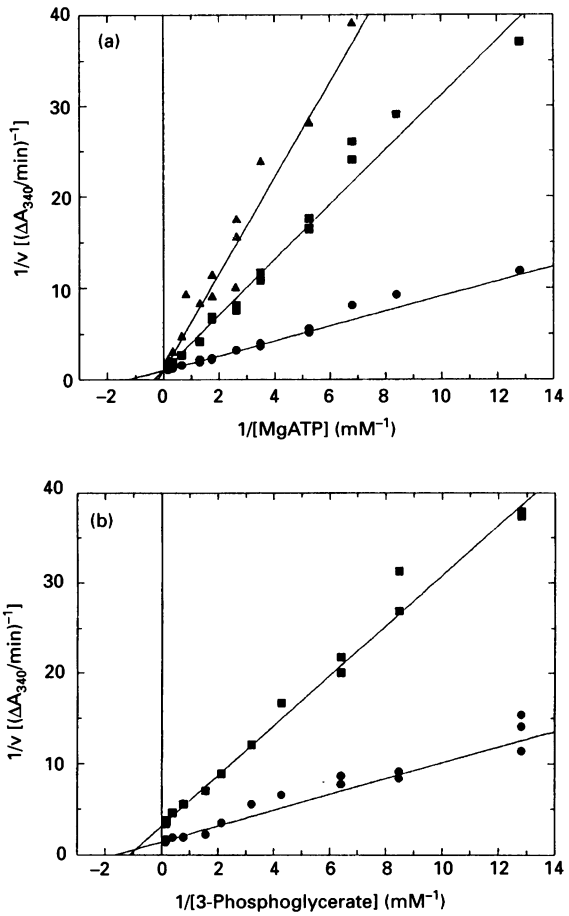

Figure 1 Inhibitory effect of TNP-ATP in 3-phosphoglycerate kinase-catalysed reaction against MgATP (a) and 3-phosphoglycerate (b): Lineweaver–Burke plots

The activity of 5.6 nM 3-phosphoglycerate kinase was measured at a constant concentration (1.26 mM) of 3-phosphoglycerate and various concentrations of MgATP (**a**) and at a constant concentration (1.23 mM) of MgATP and various concentrations of 3-phosphoglycerate (**b**). The measurements were carried out in both the absence (●) and presence of 0.02 (■) and 0.04 (▲) mM TNP-ATP. A K_i value of 0.008 ± 0.001 mM was derived from non-linear regression analysis of the non-transformed data of (**a**), and $K_i = 0.009$ mM and $K_i' = 0.020$ mM were obtained from the data in (**b**).

In conclusion, the effects of TNP-ATP on the kinetics of 3-phosphoglycerate kinase-catalysed reactions indicate a mode of binding somewhat different from that of ADP, ATP and other nucleotide analogues.

pH-dependence of the absorption spectrum of enzyme-bound TNP-ATP

The characteristic visible-absorption spectrum of TNP-ATP and its typical sigmoidal pH-dependence with a pK of 5.1 have been reported. The pH-dependence has been attributed to the ionization of the free hydroxy group of the ribose with the formation of a Meisenheimer complex or trinitrophenylidene anion [24].

Binding of TNP-ATP to 3-phosphoglycerate kinase does not cause a readily detectable change in its absorption spectrum at pH 7.0. However, the pH-dependence of the absorption spectrum of enzyme-bound TNP-ATP exhibits a large (more than 1 pH unit) shift of the pK value towards a more acidic pH (Figure 2). This shift is not further influenced by the presence of 3-phosphoglycerate (not shown).

This observation suggests the presence of a positively charged residue close to the trinitrophenyl ring of bound TNP-ATP which would stabilize the ionized form of the chromophore. On the basis of the co-ordinates of the muscle enzyme determined by X-ray crystallography [13,17] Lys-215, which is close to the nucleotide site but apparently does not interact with the nucleotide substrate, might be a possible candidate for this role.

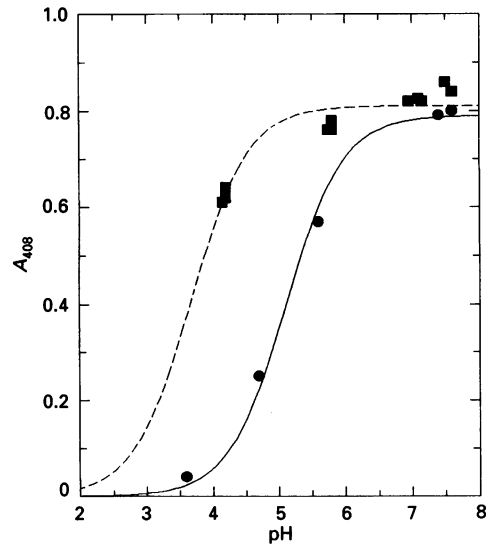

Figure 2 pH-dependence of the absorption spectrum of the enzyme-bound TNP-ATP

Absorption spectra of 0.032 mM TNP-ATP were recorded at different pH values in either 50 mM Tris/HCl buffer (between pH 5.8 and 7.7) or 50 mM sodium acetate buffer (between pH 3.6 and 5.8) in both the absence (●) and presence (■) of 0.12 mM 3-phosphoglycerate kinase. All solutions contained 1 mM EDTA and 10 mM $MgCl_2$. The absorption maxima at 408 nm are plotted as a function of pH and were fitted by theoretical dissociation curves (solid and dashed lines respectively) with pK values of 5.09 ± 0.06 (●) and 3.60 (■) in the absence and presence of the enzyme. The latter value is only estimated because of the absence of experimental points below pH 4.

Fluorescence spectrum of TNP-ATP bound to 3-phosphoglycerate kinase and determination of the dissociation constant

A large (at least tenfold) increase in intensity and a blue shift of about 20 nm were observed in the emission maximum of TNP-ATP on binding to the enzyme (Figure 3). These findings indicate that the ligand is bound within a relatively hydrophobic microenvironment. The presence of the substrate 3-phosphoglycerate or 1,3-bisphosphoglycerate has negligible effect on the fluorescence spectrum.

Taking advantage of the large fluorescence enhancement caused by binding to the protein, the dissociation constant, K_d, for TNP-ATP binding was determined by fluorimetric titration (see the curve in the absence of MgADP in Figure 4). This curve can be satisfactorily fitted by a value of $K_d = 0.0095 \pm 0.0015$ mM assuming a single binding site. The single-site model is supported by Scatchard analysis of the data (not shown) as well as by equilibrium-dialysis data on nucleotide binding to pig muscle 3-phosphoglycerate kinase [32]. (According to the latter data, if a secondary site does exist its K_d should be at least two orders of magnitude higher than that of the primary site.) The substrates 3-phosphoglycerate and 1,3-bisphosphoglycerate apparently do not affect the K_d of TNP-ATP.

The K_d value is very close to the value of the inhibition constants. It is noteworthy that binding of TNP-ATP to 3-phosphoglycerate kinase is much tighter than the binding of a nucleotide substrate (see Table 1) in agreement with the assumed additional electrostatic interaction of the trinitrophenyl ring with the protein.

By computer modelling, possible modes of binding of TNP-ATP have been investigated assuming that the adenine moiety occupies the same position as that occupied by the ATP adenine, which has been detected by X-ray crystallography [13–15]. It is not possible to superimpose all the other corresponding TNP-

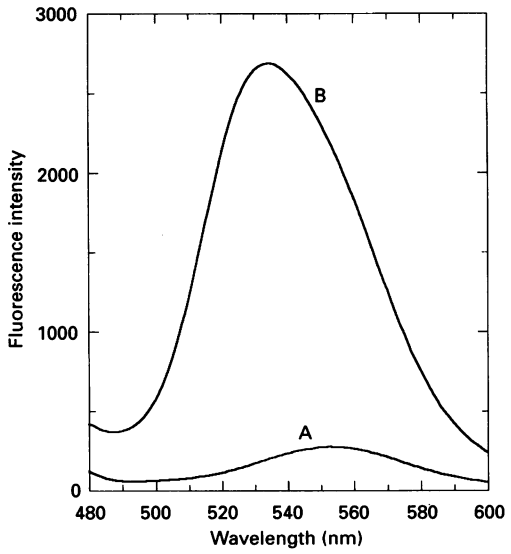

Figure 3 Change in fluorescence emission of TNP-ATP on binding to 3-phosphoglycerate kinase

The emission spectra of 0.01 mM TNP-ATP were recorded at pH 7.0 in 50 mM Tris/HCl buffer, containing 1 mM EDTA and 10 mM $MgCl_2$, on excitation at 408 nm in the absence (A) and presence (B) of 0.03 mM 3-phosphoglycerate kinase.

ATP atoms on the ATP atoms because of steric hindrance by protein residues 340–350. However, it is possible to fit the trinitrophenylidene ring close to the βG strand (i.e. residues 207–212 according to the numbering of the horse muscle enzyme

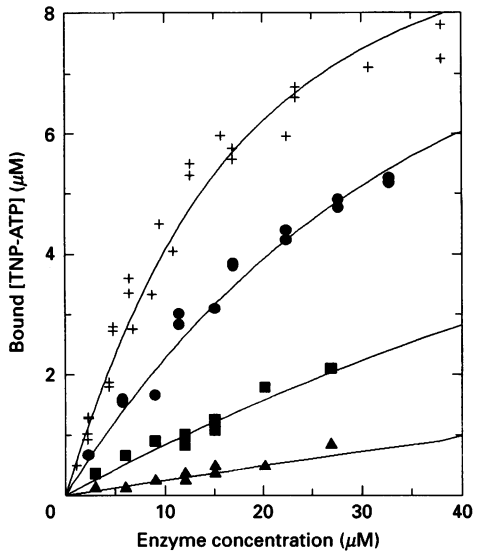

Figure 4 Fluorimetric titration of TNP-ATP with 3-phosphoglycerate kinase at various concentrations of MgADP

The increase in fluorescence emission of 0.0095 mM TNP-ATP, on addition of increasing concentration of the enzyme, was measured at 535 nm in the absence (+) and in the presence of 0.10 (●), 0.50 (■) and 2.0 (▲) mM MgADP. The $MgCl_2$ concentration was kept constant (10 mM). The continuous lines are theoretical curves calculated using the equations given in the Materials and methods section by taking $K_{d1} = 0.0095$ mM for TNP-ATP binding and $K_{d2} = 0.048$ mM for MgADP binding.

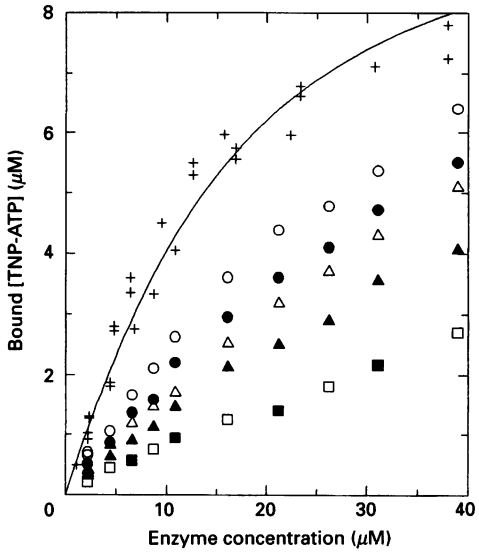

Figure 5 Effect of 3-phosphoglycerate on binding of MgATP to 3-phosphoglycerate kinase measured by monitoring fluorescent TNP-ATP replacement

Fluorimetric titrations similar to those in Figure 4 were carried out under the same conditions in the absence (+) and presence of 2.5 (●, ○), 5.0 (▲, △), 25 mM (■) and 50 (□) mM MgATP. The filled and open symbols refer to the absence and presence respectively of 10 mM 3-phosphoglycerate.

Table 1 Dissociation constants (K_d) of binding of nucleotide substrates to 3-phosphoglycerate kinase as determined by competitive titration using TNP-ATP

The values given in parentheses are equilibrium-dialysis data [32].

Nucleotide	Additive	K_d (mM) No Mg^{2+}	K_d (mM) 10 mM Mg^{2+}
ADP	——	0.78 ± 0.08 (0.27 ± 0.04)	0.048 ± 0.006 (0.060 ± 0.01)
	3-Phosphoglycerate (10 mM)	1.52 ± 0.15	0.34 ± 0.04
ATP	——	0.33 ± 0.15* (0.21 ± 0.03)	0.27 ± 0.09* (0.23 ± 0.03)
	3-Phosphoglycerate (10 mM)	1.02 ± 0.40*	0.51 ± 0.22*
	1,3-Bisphosphoglycerate (1 mM)	2.19 ± 0.54*	2.39 ± 0.68*

* Tentative values because of incomplete replacement of TNP-ATP by ATP (see the Materials and methods section).

[13]), without any changes in the protein structure, if the ribose is rotated by 180° around the glycosidic bond. In this position the chromophoric ring is surrounded by the apolar residues 207–214 and one of the nitro group oxygens is about 0.3 nm (3 Å) from the ε-amino group nitrogen of Lys-215. In other possible orientations that TNP-ATP can assume without perturbation of the protein structure, the trinitrophenylidene ring is exposed to the solvent. The tight binding, the pH-dependence of the emission spectrum and the fluorescence properties of enzyme-bound TNP-ATP all argue in favour of the orientation suggested first.

Dissociation constants of nucleotide substrates determined by replacement of TNP-ATP

K_d values for the binding of nucleotide substrates were determined from enzyme-titration curves similar to those shown in Figure 4 in the presence of both the analogue and the nucleotide substrate. As the concentration of the substrate increased relative to that of the analogue, less and less of the bound analogue could be detected at the same enzyme concentration because of its stepwise replacement by the substrate. Knowing the independently determined K_d value for TNP-ATP, the K_d for the nucleotide substrate could be calculated (see the Materials and methods section).

This method was satisfactorily used in the case of ADP and MgADP binding. The respective K_d values are reported in Table 1 and are found to be quite similar to the values previously determined by equilibrium dialysis [32]. The continuous lines in Figure 4 are theoretical curves obtained by using the above determined value for MgADP binding.

Similar experiments with ATP (in either the absence or presence of Mg^{2+}) were complicated by the fact that even at saturating concentration (i.e. at concentrations two orders of magnitude higher than the binding constant determined by equilibrium dialysis [32]), ATP could not completely replace TNP-ATP (Figure 5).

This is a rather unexpected result in the light of the ability of ADP to displace TNP-ATP fully and the structural information suggesting the presence of a single binding site for MgADP and MgATP [13–15]. However, different modes of binding of the two nucleotides in the active site cannot be excluded, as it is known

that they cause markedly different effects on the reactivity of the two fast-reacting cysteines [9,31] and, furthermore, that their binding to the enzyme is affected differently by Mg^{2+} [32].

In agreement with this, different locations of Mg^{2+} with respect to the phosphate chain of the bound nucleotide substrates have been suggested by X-ray structural data [13]. Therefore it is possible that the TNP-ATP site overlaps the MgADP site more than the MgATP site. An alternative explanation might be the presence of a second binding site for TNP-ATP that is affected differently by ATP and ADP binding. The possibility that a second binding site for TNP-ATP exists with a much larger dissociation constant cannot be excluded by the equilibrium-dialysis studies (results not shown). However, binding to this site is not detectable at the concentrations of TNP-ATP employed in the experiments with ADP. Thus one can assume that the dissociation constant for this second TNP-ATP site is lowered by the presence of ATP at the active site and that binding of TNP-ATP at this second site becomes detectable only in the presence of (Mg)ATP.

By subtracting from all the titration curves the signal derived from the amount of analogue that remains bound even in the presence of 20 mM ATP (which, in the absence of TNP-ATP, saturates the enzyme active sites up to 98%, as judged by equilibrium-dialysis data [32]) and by normalizing the differences obtained (see the Materials and methods section), K_d values for ATP and MgATP could be evaluated, although with a much larger error than for ADP or MgADP (Table 1). These values fall close to the range of K_d values determined by equilibrium dialysis [32], confirming that the presence of Mg^{2+} strengthens ADP binding but has no effect on ATP binding.

Effect of 3-phosphoglycerate and 1,3-bisphosphoglycerate on the K_d values of binding of (Mg)ADP and (Mg)ATP

The competitive titrations described above allow one to overcome the limitations of equilibrium-dialysis binding studies, in particular the relatively long time required for the measurements and the requirement of comparable concentrations of ligand and enzyme. Thus we could conveniently use this technique to investigate the effect on the nucleotide dissociation constants not only of 3-phosphoglycerate but also of the unstable substrate, 1,3-bisphosphoglycerate. Table 1 summarizes the results.

The K_d values for both ADP and MgADP obtained in the presence of saturating concentrations of 3-phosphoglycerate were higher than those obtained in its absence. This weakening effect of 3-phosphoglycerate on (Mg)ADP binding has also been observed in equilibrium-dialysis experiments (M. Vas, A. Merli and G. L. Rossi, unpublished work). X-ray-crystallographic studies on the binary complex formed by the enzyme with 3-phosphoglycerate have shown that this substrate induces order in helix 13 [17]. It is possible that this conformational change causes an unfavourable interaction between MgADP and Asp-374 of helix 13. Such an interaction has been previously proposed to cause the competitive inhibition of MgADP with respect to 3-phosphoglycerate [32].

According to the data shown in Table 1, weakening of ADP binding occurs even in the absence of Mg^{2+}, although to a smaller extent. Thus other, still unidentified, conformational changes caused by 3-phosphoglycerate might also affect ADP binding.

Similarly, when the K_d for ATP was determined in the presence of a saturating concentration of 1,3-bisphosphoglycerate, a much weaker binding of ATP was observed. (It should be noted that, in the presence of 1,3-bisphosphoglycerate, Mg^{2+} does not significantly affect the binding of ATP.) This finding, which could not be obtained by equilibrium-dialysis measurement because of the instability of 1,3-bisphosphoglycerate, was expected, as the transferable phosphate of ATP and that of 1,3-bisphosphoglycerate should compete for the same binding site. In accordance, a competitive inhibitory effect of 1,3-bisphosphoglycerate with respect to MgATP was observed in kinetic studies of both yeast [38] and pig muscle (M. Vas, A. Merli and G. L. Rossi, unpublished work) enzymes.

However, the K_d for MgATP in the presence of 1,3-bisphosphoglycerate indicates an interaction at least fivefold weaker than that of AMP and about threefold weaker than that of the phosphate-lacking adenosine (the latter data were obtained by equilibrium dialysis [32]). Therefore an effect of 1,3-bisphosphoglycerate on ATP binding, possibly a protein conformational change in addition to the interactions of the transferable phosphates, must be considered.

By the same fluorimetric technique we were able to investigate the effect of 3-phosphoglycerate on the binding of MgATP in the productive ternary complex. In contrast with equilibrium dialysis, this technique can be conveniently used at enzyme concentrations that are negligible with respect to ligand concentrations. Under these conditions, the equilibrium of the 3-phosphoglycerate kinase-catalysed reaction is largely shifted towards the production of 3-phosphoglycerate and MgATP [39]. Thus the concentrations of the two other substrates are negligible. Therefore measurements could be carried out without serious interference by the tightly bound 1,3-bisphosphoglycerate. The data in Table 1 demonstrate a significant weakening effect of 3-phosphoglycerate on MgATP binding.

The reverse effect, i.e. the weakening of 3-phosphoglycerate binding by bound MgATP, has been previously detected [8]. Thus, taken together, the previous and the present data suggest a mutual weakening effect of the two substrates of this enzyme.

It is notable that the K_d values of substrates measured in the presence of each other resemble the respective K_m values [1–4,8,9]. This is consistent with a rapid-equilibrium random-kinetic mechanism, in which the substrates influence the binding of each other. Antagonistic substrate binding might also have some general relevance in the functioning of kinases, as a similar phenomenon has been observed for phosphofructokinase [40–42].

Interpretation of substrate antagonism in the light of crystallographic data

In the absence of structures for the ternary complexes, one can only speculate about the possible structural basis of substrate antagonism. Our previous kinetic [9,43] and equilibrium [8,32] studies indicate that the carboxy group of 3-phosphoglycerate and the phosphate(s) of the nucleotides are mainly responsible for the mutual weakening of binding. In the structure of the binary complex with 3-phosphoglycerate, the carboxy group of this substrate interacts via a water molecule with the N-terminus of helix 14 [17]. The interaction of the transferable γ-phosphate of ATP with the protein is still poorly understood [13].

One possibility is that in the ternary complex the β- and/or γ-phosphate(s) of ATP also interact with helix 14 and thereby directly influence the binding of the other substrate through its carboxy group. As illustrated in Figure 6, an oxygen atom of ATP γ-phosphate is only 0.445 nm (4.45 Å) away from the N-terminal glycine residue of helix 14. Although this distance is a bit too large for hydrogen-bond formation, a small conformational change might make it possible. Furthermore, the negatively charged phosphate might interact electrostatically with the positive N-terminus of the helix dipole, as suggested for other enzymes [44].

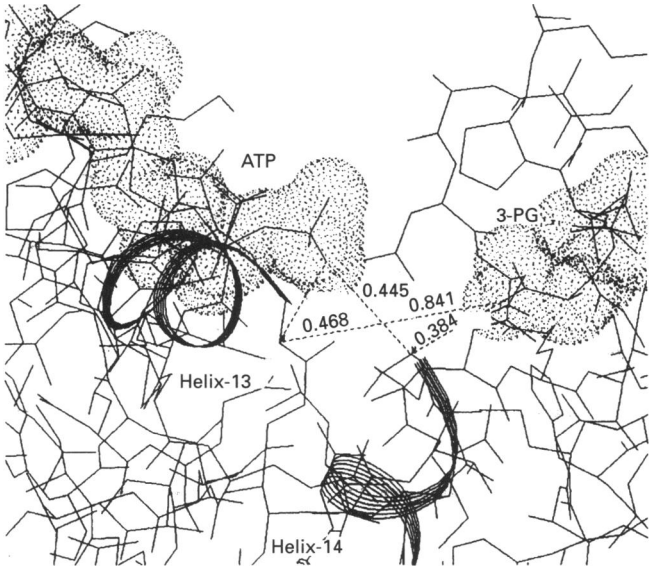

Figure 6 Stereo drawing of the binding mode of the substrates in the active site of muscle 3-phosphoglycerate kinase based on available X-ray-crystallographic-data

The Figure was prepared by using the co-ordinates of the binary complex of pig muscle enzyme with 3-phosphoglycerate [17], and ATP was positioned in this complex as determined for the horse muscle enzyme [13]. The numbers indicate the distances (in nm) of both the oxygen OP8 of ATP γ-phosphate and the oxygen O12 of the 3-phosphoglycerate (3-PG) carboxy group from either the peptide N of Gly-396 at the N-terminus of helix 14 or that of Gly-373 of helix 13.

Alternatively, a similar interaction between the γ-phosphate of ATP and the N-terminus of helix 13 might be possible, if one considers that the distance between the same oxygen of ATP γ-phosphate and the N-terminus of helix 13 is only 0.468 nm (4.68 Å) (Figure 6). Although the carboxy group of 3-phosphoglycerate is 0.841 nm (8.41 Å) away from this end of helix 13, ordering of this helix as a result of the presence of 3-phosphoglycerate has been detected [17]. This conformational change might interfere with the assumed interaction with ATP γ-phosphate, as suggested above for MgADP.

In either case, additional conformational changes occurring only in the ternary enzyme–substrate complex have to be assumed in order to interpret the antagonistic substrate binding. It is possible that the same conformational changes may also orient the substrate reactive groups correctly for catalysis. Thus it might be interesting to determine whether the substrate antagonism and the accompanying protein structural changes can also occur in the crystalline state of the enzyme, i.e. in the ternary complex formed on diffusion of the nucleotide substrate into the pregrown crystal of the binary complex with 3-phosphoglycerate. We therefore intend to extend these studies to include single-crystal microspectrophotometric measurements of crystals of the binary complex, the structure of which has already been determined at high resolution.

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