Mg²⁺ affects the binding of ADP but not ATP to 3-phosphoglycerate kinase

Correlation between equilibrium dialysis binding and enzyme kinetic data

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The role of Mg²⁺ in the binding of ADP and ATP to pig muscle and yeast 3-phosphoglycerate kinases has been studied by equilibrium dialysis. Whereas the *K_a* of ATP binding varies between 0.17 and 0.23 mM (S.E.M. 0.03 mM) for both enzymes, independently of the presence of Mg²⁺, the *K_a* values for ADP and MgADP binding are in the range 0.18–0.27 mM (S.E.M. 0.04 mM) and 0.05–0.06 mM (S.E.M. 0.01 mM) respectively. Thus Mg²⁺ exclusively tightens the interaction of ADP, but not of ATP, with the protein molecule. Although the equilibrium dialysis data are consistent with a model possessing a single site for nucleotides, the existence of a much weaker secondary site (with a *K_a* value at least two orders of magnitude larger) cannot be excluded. The binding of AMP and adenosine to pig muscle 3-phosphoglycerate kinase is weaker than binding of MgATP; the respective *K_a* values are 0.36 ± 0.05 mM and 0.65 ± 0.05 mM. Thus, in addition to the interaction of the α-phosphate that is detectable by crystallography [Banks, Blake, Evans, Haser, Rice,

Hardy, Merrett and Phillips (1979) *Nature* (London) **279**, 773–777], the β- and/or γ-phosphate(s) of MgATP may also interact with the enzyme molecule. The fact that MgADP binds more tightly than ADP is consistent with its stronger inhibition of the reaction catalysed by the enzyme between 3-phosphoglycerate and MgATP. MgADP is a product of this reaction, and inhibits it competitively with both substrates; as an inhibitor its *K_i* is comparable with the *K_a* found in binding studies. At the same time, the *K_m* value for MgADP in the reverse reaction (0.18 ± 0.05 mM; mean ± S.E.M.) is higher than these constants; this may be due either to a different kinetic mechanism in this direction of the enzymic reaction, or to different binding modes of MgADP as inhibitor and as substrate. The reason why inhibition by MgADP is competitive with 3-phosphoglycerate may be that its binding prevents the specific change in conformation that the enzyme undergoes [Harlos, Vas and Blake (1992) *Proteins* **12**, 133–144] when it binds 3-phosphoglycerate.

INTRODUCTION

There are uncertainties about the binding of the nucleotide substrates, MgADP and MgATP, to 3-phosphoglycerate kinase (EC 2.7.2.3), a possible ‘hinge-binding’ [1,2] enzyme. A single nucleotide site has been determined by X-ray crystallography in the structure of both muscle [1] and yeast [2] 3-phosphoglycerate kinases. Studies in solution, however, by equilibrium dialysis [3], gel filtration [4,5] and chemical modification [6] have been claimed to indicate the existence of an additional secondary site. Some studies by n.m.r. [4,7] showed only the primary site for nucleotide binding, whereas others [8–12] required a secondary site to be postulated. Spectrophotometric [13] and fluorimetric [14,15] titration data, as well as studies of thiol reactivity [16], could be satisfactorily fitted to a single-site model.

The *K_a* values of nucleotide binding also show large variability. For example, with the single-site assumption the following *K_a* values were determined: 0.015 mM [13], 0.08 mM [14] and 0.15 mM [4,16] for MgATP binding; and 0.04 mM [16], 0.19 mM [14] and 0.25 mM [4,13] for MgADP binding. Similar variation is also found for the values presented for the two-site model.

The role of Mg²⁺ in the binding of nucleotide substrates to 3-phosphoglycerate kinase is still an open question. Although a critical role for the metal ion in the tight binding of nucleotides has been reported [17], later studies [9,13–15] failed to detect an effect of Mg²⁺ on the binding of either ADP or ATP. A recent investigation by n.m.r. again revealed an effect of Mg²⁺ in strengthening the binding of ADP to one of the two sites [12], while for ATP binding a less pronounced reverse effect could be observed [11].

The structural details of MgADP and MgATP binding also remain unclear. X-ray data on both the horse muscle [1] and

yeast [2] enzymes, as well as n.m.r. studies in solution [18,19], agree that the location of the adenosine site of the nucleotides is on the inner surface of the C-terminal domain. However, the position of the nucleotide phosphate chain and that of the bound Mg²⁺ are equivocal. In the structure of the crystalline horse muscle 3-phosphoglycerate kinase, the bound Mg²⁺ interacts with both the α- and β-phosphates of ADP and with the side chain Asp-374 (residue 372 in the yeast enzyme), but the β- and γ-phosphates of MgATP apparently do not interact with any protein side chain, and the metal ion is located close to the γ-phosphate [1]. In contrast, X-ray data on the crystalline yeast enzyme using an ATP analogue revealed an interaction of Mg²⁺ both with the nucleotide γ-phosphate and with the carboxy group of Asp-372 [2,20].

As the γ-phosphate is transferred in the enzymic reaction, its interaction with the protein may be crucial for catalysis. Data from n.m.r. studies in solution indicate that sulphate ion, present in the crystal, may interfere with Mg²⁺ chelation of the enzyme-bound nucleotides [9]. N.m.r. studies also revealed a direct co-ordination of the cation to all the phosphate groups of the nucleotide in both complexes with the enzyme [21,22], but interaction with protein side chains is still questionable. Thiol reactivity studies indicate differences in the mode of interaction of MgADP and MgATP with the enzyme molecule [16,23].

In the present work we have used equilibrium dialysis, as the most direct method for ligand binding studies, to characterize quantitatively the interaction of ADP, ATP, MgADP and MgATP with 3-phosphoglycerate kinase. Comparative studies with AMP and adenosine were also carried out to determine how much of the affinity of their phosphates for the enzyme was due to their phosphate groups. In most of the experiments the pig muscle enzyme was used, as in our previous work [e.g.

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15,16,23,24], but comparative measurements were also made with the yeast enzyme.

Kinetic studies were also conducted with both pig muscle and yeast 3-phosphoglycerate kinases in the presence of MgADP as an inhibitory product, and as a substrate in the reverse reaction. The correlation of the equilibrium and kinetic results is discussed in the light of the known structural data.

MATERIALS AND METHODS

Enzymes and chemicals

3-Phosphoglycerate kinase was isolated from pig muscle [24] or yeast [25] and stored as a crystalline suspension or as a freeze-dried product respectively. The yeast enzyme was prepared in the Institute of Protein Research (Russian Academy of Sciences) and was kindly donated by Dr. G. V. Semisotnov (142292 Pushchino, Moscow Region, Russia). The molar activity of both enzymes varied between 500 and 700 kat/mol using D-3-phosphoglycerate and MgATP as substrates.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (350 kat/mol) and lactate dehydrogenase (EC 1.1.1.27) (800 kat/mol) were prepared from pig muscle [26,27]. Crystalline pyruvate kinase (EC 2.7.1.40) (1100 kat/mol) and a crystalline mixture of hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (3 mg/ml) were purchased from Boehringer. BSA was a Sigma product.

Adenosine, sodium salts of ATP, ADP and AMP, 3-phospho-D-glycerate, phosphoenolpyruvate and NADP⁺ were also Boehringer products. It was determined enzymically (see below) that the ADP and ATP contained at most about 1% contamination by the other nucleotide. NADH was obtained from Reanal. MgCl₂ and D-glucose were Sigma products. The substrate 1,3-bisphosphoglycerate was prepared as described previously [23]. All other chemicals were reagent grade commercial preparations.

Preparation of enzyme solutions

Crystals of pig muscle 3-phosphoglycerate kinase were dissolved in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and were dialysed against the same buffer to remove ammonium sulphate. The final dialysis buffer also contained 2 mM dithiothreitol.

The freeze-dried yeast 3-phosphoglycerate kinase was dissolved in the above buffer containing 2 mM dithiothreitol, and insoluble material was removed by centrifugation.

Crystals of glyceraldehyde-3-phosphate dehydrogenase were dissolved and dialysed in the same way as for pig muscle 3-phosphoglycerate kinase.

Protein concentrations were determined using $A_{280}^{1\text{mg/ml}}$ values of 0.69 for muscle 3-phosphoglycerate kinase, 0.49 for yeast 3-phosphoglycerate kinase [28] and 1.0 for glyceraldehyde-3-phosphate dehydrogenase [29]. The molecular mass of 3-phosphoglycerate kinase was taken to be 47 kDa ([30] and references therein).

Equilibrium dialysis experiments

Dialysis was carried out in a series of Micro D200 cells of a Dianorm (MSE) equilibrium dialysis apparatus. Sheets of the Spectropor 2 dialysis membrane (cut-off 12–14 kDa) were pre-washed in boiling distilled water containing about 1 mM EDTA. On each side of the membrane, 0.15 ml of solution was poured into the chambers: on one side 0.5–2.0 mM 3-phosphoglycerate kinase was present in all cells, while on the other side the ligand concentration was varied from cell to cell. Control experiments

were always carried out with cells containing buffer in place of either the enzyme or the ligand.

We also checked whether, at the high protein concentrations used, the Donnan effect can influence the equilibrium distribution of the ligand. The usual means of eliminating such an effect, i.e. using buffer containing NaCl, could not be employed with 3-phosphoglycerate kinase, because chloride (and other anions) has a large effect on the activity of the enzyme [31]. Instead, control equilibrium dialysis was carried out with a non-nucleotide-binding protein, BSA (90 mg/ml), in place of 3-phosphoglycerate kinase. Within experimental error, the same equilibrium distribution of MgADP was observed in the presence and absence of BSA, excluding the possibility of the operation of a substantial Donnan effect.

The cells were placed into a thermostatted water bath (20 °C), with a rotation speed of 12 rev./min. In this system equilibrium was attained within about 2 h; during this time no inactivation of the enzyme and no decomposition of the ligands could be detected. At equilibrium, the concentrations of both the enzyme and the ligand were determined in samples withdrawn from each side of the membrane.

The concentrations of ATP, ADP, AMP and adenosine were measured spectrophotometrically both in the initial solutions and after equilibration (using ϵ_{280} values of 15400 M⁻¹·cm⁻¹ for ATP and 15000 M⁻¹·cm⁻¹ for ADP, AMP and adenosine). Absorbances of the nucleotides and of 3-phosphoglycerate kinase were found to be additive, as also observed by Scopes [5]. The concentrations of ATP and ADP were also determined enzymically using the appropriate assay mixtures, as described earlier [16], containing either 3-phosphoglycerate kinase plus glyceraldehyde-3-phosphate dehydrogenase, or pyruvate kinase plus lactate dehydrogenase.

In nucleotide binding experiments, 10 mM MgCl₂ was added to ensure almost complete complex formation of the nucleotides with Mg²⁺, taking into account their reported dissociation constants [32–34].

Measurement of enzyme kinetics

The activity of 3-phosphoglycerate kinase was assayed spectrophotometrically at 340 nm with 3-phospho-D-glycerate and MgATP (as described in [16]) as substrates, or in the reverse direction using 1,3-bisphosphoglycerate and MgADP as substrates (according to Lavoigne et al. [35]). The latter reaction mixture contained 0.2 mM 1,3-bisphosphoglycerate, 2.5 mM ADP, 5 mM MgCl₂, 10 mM D-glucose, 0.5 mM NADP⁺, about 0.03 mg/ml hexokinase/glucose-6-phosphate dehydrogenase mixture and 0.5–1.0 nM 3-phosphoglycerate kinase.

Enzyme inhibition studies were carried out either in the presence of excess (10 mM) MgCl₂ or at Mg²⁺ concentrations equivalent to the applied ATP concentrations. In the latter case the concentration of MgATP was calculated by using a dissociation constant of 0.1 mM, which was obtained by averaging the data in the literature [32–34].

The quantitative evaluation of the dissociation and kinetic constants from the experimental data was carried out by non-linear regression analysis using the Grit program (R. J. Leatherbarrow, 1989/1990; Erithacus Software Limited, supplied by Sigma) with the aid of an IBM Varyter AT computer connected to a LaserJet IIP printer.

RESULTS

Effect of Mg²⁺ on ADP and ATP binding

Figure 1 illustrates the binding of nucleotide substrates to pig

Figure 1 Effect of Mg^{2+} on binding of ADP (a) and ATP (b) to pig muscle 3-phosphoglycerate kinase

In equilibrium dialysis cells, one half of each cell contained 0.76 mM enzyme and the other half contained different initial concentrations (from 0.5 to 2.0 mM) of ADP (a) or ATP (b), with (●) or without (+) 10 mM $MgCl_2$. The lines are theoretical binding curves giving the best fit to the experimental points by assuming a single binding site. The respective K_d values are given in Table 1.

Table 1 Dissociation constants of binding of the nucleotide substrates and their analogues to 3-phosphoglycerate kinase

The K_d values were determined by equilibrium dialysis, as described in the Materials and methods section. The data refer to pig muscle (*) and yeast (†) enzymes respectively. Results are means $\pm$ S.E.M.

Ligand	K_d (mM)	
	Mg^{2+} ... None	5 mM
ADP	$0.27 \pm 0.04^*$	$0.060 \pm 0.01^*$
	$0.18 \pm 0.04^\dagger$	$0.051 \pm 0.01^\dagger$
ATP	$0.21 \pm 0.03^*$	$0.23 \pm 0.03^*$
	$0.17 \pm 0.03^\dagger$	$0.19 \pm 0.03^\dagger$
AMP	—	$0.36 \pm 0.05^*$
Adenosine	$0.65 \pm 0.05^*$	—

muscle 3-phosphoglycerate kinase, both in the absence and in the presence of Mg^{2+} . While Mg^{2+} clearly strengthened the binding of ADP (Figure 1a), it had no detectable effect on the binding of

Figure 2 Fitting of the MgADP binding data by assuming two binding sites per mol of enzyme

The binding experiment was carried out with 2.2 mM pig muscle 3-phosphoglycerate kinase on one side of the membrane and various initial concentrations (from 1.5 to 8.0 mM) of MgADP on the other. Curve fitting was carried out according to non-linear regression analysis by assuming the sum of two rectangular hyperbola. $K_1 = 0.06$ mM (first site) was taken as a non-variant parameter and K_2 (second site) was fitted, yielding a value of 19.1 ± 5.4 mM (mean $\pm$ S.E.M.) (—). As a comparison, theoretical curves with K_2 values of 0.6 mM (---) and of 6.0 mM (.....) have also been plotted ($K_1 = 0.06$ mM in both cases).

ATP (Figure 1b). Assuming a 1:1 stoichiometry of binding as the simplest model, the K_d values giving the best fit to the experimental points are summarized in Table 1. In the absence of Mg^{2+} the binding constants of ADP and ATP are very similar to each other and only Mg^{2+} , possibly by creating the real nucleotide substrates, causes significant differences.

The results obtained with the yeast enzyme are also shown in Table 1. It is remarkable that there are no appreciable differences between the nucleotide binding properties of the 3-phosphoglycerate kinases from these two different sources.

Stoichiometry of nucleotide binding

To investigate the existence of a second nucleotide binding site, an experiment with MgADP (i.e. the most strongly bound nucleotide) was repeated at higher enzyme and MgADP concentrations, so that the site previously titrated (Figure 1) was almost completely saturated. Figure 2 shows the results obtained with the pig muscle enzyme. Assuming the existence of two independent binding sites and fixing K_1 at 0.06 mM for the first site, as determined above, yielded a K_2 of 19.1 ± 5.4 mM (mean $\pm$ S.E.M.) for the second site. Similar results were obtained even if both dissociation constants were fitted simultaneously: $K_1 = 0.062 \pm 0.007$ mM and $K_2 = 18.6 \pm 5.7$ mM. The relatively large error in the K_d for the second loose binding site would be reduced if higher protein and ligand concentrations were used. However, as this type of experiment would consume too large an amount of enzyme relative to the additional information obtained, our purpose remained only the estimation, and not the exact determination, of the K_d for a possible secondary site.

Binding experiments, similar to the one presented in Figure 2, were not carried out in the absence of Mg^{2+} or with ATP, but all the equilibrium dialysis data given in Table 1 could be satisfactorily fitted to a single-site model.

Figure 3 Inhibitory effect of ADP and MgADP against MgATP (a) and 3-phosphoglycerate (b): Lineweaver-Burk plots

The activity of 5 nM pig muscle 3-phosphoglycerate kinase was measured both at a constant concentration (1.4 mM) of 3-phosphoglycerate and a varying concentration of MgATP (a), and at a constant concentration (1.25 mM) of ATP and a varying concentration of 3-phosphoglycerate (b). The concentration of MgCl_2 was either equivalent to that of ATP (-----) or 10 mM (—). Both types of experiments were repeated in the absence (+) and in the presence of 0.1 mM (Δ and Δ) and 0.5 mM ($\circ$ and $\bullet$) ADP. The filled symbols refer to the excess of Mg^{2+} . Dixon plots of the data also gave straight lines (results not shown). K_i values of 0.042 ± 0.015 and 0.22 ± 0.03 mM (a) and of 0.050 ± 0.015 and 0.21 ± 0.03 mM (b) were derived from non-linear regression analysis of the non-transformed data. K_i values depend only slightly on the concentrations of the non-variable substrates; e.g. at 10 mM 3-phosphoglycerate, a value of 0.07 ± 0.02 mM was obtained (a) and at 10 mM MgATP a value of 0.14 ± 0.05 mM (b) was determined (results not shown).

Binding of AMP and adenosine to 3-phosphoglycerate kinase

Comparison of the dissociation constants of adenosine and (Mg)AMP binding with that of MgATP binding can reveal the contribution to binding of the phosphate groups of MgATP. The K_d values obtained by equilibrium dialysis (Table 1) clearly demonstrate that the adenosine and MgAMP bind more weakly than does MgATP.

Enzyme kinetic data with MgADP

Inhibitory studies with pig muscle 3-phosphoglycerate kinase revealed, as for the yeast enzyme [37], that MgADP inhibits the catalysed reaction between 3-phosphoglycerate and MgATP much more effectively than does free ADP (Figure 3). The competitive K_i values for MgADP versus MgATP (Figure 3a) and 3-phosphoglycerate (Figure 3b) are very similar to each

Figure 4 Double-reciprocal plot for the reaction catalysed by 3-phosphoglycerate kinase, starting from 1,3-bisphosphoglycerate, as the MgADP saturation is varied

The activity of pig muscle enzyme (0.4 nM) was measured in the presence of 10 mM MgCl_2 , in the presence of 0.05 (+) and 0.16 (●) mM 1,3-bisphosphoglycerate at various concentrations of ADP. A K_m of 0.18 ± 0.05 mM was obtained for MgADP from non-linear fitting of the original data.

other and to the K_d value determined for MgADP binding in equilibrium dialysis experiments (Table 1), and depend only slightly on the concentration of the other substrate (results not shown). On the other hand, a larger value (0.18 ± 0.05 mM) has been obtained for the K_m of MgADP in the reverse enzymic reaction using both the pig muscle (Figure 4) and the yeast (results not shown) enzymes, in agreement with previous data obtained with the rat liver enzyme [35].

DISCUSSION

The role of Mg^{2+} in ADP and ATP binding

The selective effect of Mg^{2+} in strengthening ADP binding (Figure 1 and Table 1) suggests a different type of metal complex formation by the enzyme-bound ADP and ATP. This is consistent with the considerable protection given by MgADP, but not by MgATP, against thiol reactivity to pig muscle 3-phosphoglycerate kinase [16], and also with the X-ray data on the horse muscle enzyme [1] indicating different binding modes of MgADP and MgATP. The present results also agree, at least in part, with the recent n.m.r. titration data on yeast 3-phosphoglycerate kinase [12], which reflect a stronger binding of MgADP relative to ADP. On the other hand, our data do not confirm an effect of Mg^{2+} in weakening ATP binding that was concluded from other n.m.r. data [11].

The role of the phosphate chain in MgATP binding

The tighter binding of AMP relative to adenosine (Table 1) is in line with crystallographic evidence for a role of the α -phosphate in MgATP binding [1]. In addition, the weaker binding of AMP compared with MgATP (Table 1) unequivocally revealed some contribution of the β - and/or γ -phosphates of MgATP to its binding, although this has not been observed in X-ray studies on the muscle enzyme [1]. The absence of X-ray crystallographic evidence might be due to an interference of binding by the sulphate ions present in the crystallization medium. An effect of sulphate on nucleotide binding has indeed been noted previously [5,9].

In fact, more extensive interaction(s) of the MgATP phosphate chain have been deduced from structural studies on the yeast enzyme [2,20], but the results of affinity labelling with a reactive ATP analogue [36] raise the possibility that other types of interaction(s) might occur. Further crystallographic work is needed in order to explore the details of the interaction of the phosphate chain of MgATP with the enzyme.

Binding stoichiometry of nucleotide substrates

The present equilibrium dialysis data are consistent with the existence of a single nucleotide binding site on 3-phosphoglycerate kinase, in agreement with structural observations [1,2]. However, the binding results with MgADP (Figure 2) might also be explained by assuming the existence of a very weakly binding secondary site, similar to the one observed by n.m.r. in the yeast enzyme at high Mg^{2+} concentrations [12]. On the other hand, as illustrated by the theoretical curves in Figure 2, we were unable to confirm the existence of a relatively strong secondary MgADP binding site, although its presence has been detected in direct gel filtration binding studies with the yeast enzyme [5]. We also failed to observe the secondary sites postulated on the basis of n.m.r. titrations for ADP [12] and ATP [11] to have affinities comparable with their respective primary sites.

Correlation between equilibrium and kinetic data

The stronger product inhibition by MgADP than by ADP, observed with pig muscle 3-phosphoglycerate kinase (Figure 3), agrees fairly well with previous data on the yeast enzyme [37] and correlates with the present equilibrium binding data. The close agreement between the K_i and K_d values argues in favour of a rapid equilibrium kinetic mechanism. On the other hand, the different K_m of MgADP in the reverse direction of the enzymic reaction (Figure 4), similar to previous data on the rat liver enzyme [35], raises the possibility that either the reaction mechanisms are different in the reverse direction or the binding modes of MgADP as an inhibitor and as a substrate are different. This question remains open, as enzyme kinetic data [e.g. 35, 37–40] have not led to an unequivocal conclusion about the reaction mechanism.

Very little is as yet known about the mode of binding of the nucleotide substrates during the enzymic reaction. Therefore it is rather difficult to give a structural basis for the competitive inhibition by MgADP against both MgATP and 3-phosphoglycerate. Considering the model with a single binding site for nucleotides, it is possible that MgATP cannot completely prevent the binding of MgADP, simply because of the different binding modes of MgATP and MgADP. In particular, the phosphates of MgADP may bind, as indicated by X-ray studies [1,2], through electrostatic interactions (enhanced by Mg^{2+}) to Asp-374 (Asp-372 in the yeast enzyme), even in the presence of MgATP. During the enzymic reaction, i.e. when 3-phosphoglycerate is also bound, a protein conformational change might occur which can prevent the binding of MgADP. Thus both substrates together (MgATP and 3-phosphoglycerate) would be required to abolish completely the inhibition by MgADP, as in fact we found in the kinetic experiments.

The recently published high-resolution X-ray structure of the binary complex of the pig muscle enzyme with 3-phosphoglycerate [24] is fully consistent with the above assumption. These workers found that the distant α -helix that contains Asp-

374 (the residue that interacts with the phosphate groups of MgADP) undergoes local ordering when 3-phosphoglycerate is bound. We assume that this conformational change is responsible for preventing the binding of MgADP through its phosphates or, conversely, if MgADP is bound, it prevents this substrate-dependent conformational change and therefore inhibits the enzymic reaction competitively with 3-phosphoglycerate also.

We are very grateful to Ms. Susan Földi and to Ms. Christina Garaczi for their excellent technical assistance. The critical reading of the manuscript by Dr. H. B. F. Dixon (Department of Biochemistry, University of Cambridge) is gratefully acknowledged. Preparation of the figures by Ms. Tünde Apatini is gratefully acknowledged. The work has been supported by grant OTKA(I/3, no. 1366).

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