# INHIBITION OF YEAST PHOSPHOGLYCERATE KINASE BY LANTHANIDE–ATP COMPLEXES

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## 1. Introduction

The use of lanthanide cations as probes is assuming increasing importance in high resolution nuclear magnetic resonance structural studies of proteins [1] and nucleotides [2], both with and without metal cofactor requirements. We describe accordingly in this report steady state kinetic investigations into the binding of lanthanide (Ln)–ATP to yeast phosphoglycerate kinase (PGK) (3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3.) in presence of the substrates Mg–ATP and 3-phosphoglycerate. These studies are intended as a complement to our n.m.r. investigations of this enzyme and together with earlier results [3], will be necessary for the correct interpretation of lanthanide shifts and broadenings in the n.m.r. spectra of PGK–substrate complexes [4].

In this work La–$ATP^-$ was found to be an inhibitor competing with Mg–$ATP^{2-}$, similar results being obtained when Eu, Pr, and Yb were substituted for La. Direct binding determinations using Sephadex, and also an n.m.r. spectral titration, provided information about the number of binding sites for metal–ATP.

## 2. Materials and methods

Selected batches of yeast PGK were purchased from Boehringer–Mannheim Ltd. They are the result of an improved preparation without foreign protein contamination found in earlier commercial samples [5]. Polyacrylamide disc-gel electrophoresis experiments showed over 97% of the protein in a single band, both for the native enzyme and in sodium dodecyl sulfate solution. The specific activity was 580 U/mg. Centrifugation and dialysis were employed immediately prior to a kinetic run to remove ammonium sulphate from the kinase and from the coupling enzyme used in the assay, glyceraldehyde 3-phosphate dehydrogenase.

NADH, ATP, and D-3-phosphoglycerate (3-PGA) were obtained from the Sigma Corp. as their sodium salts, since monovalent cations at low concentrations do not affect the reaction [6]. Tritium-labelled ADP and ATP were obtained from New England Nuclear Corp. and certified better than 98% pure. Rare-earth solutions were prepared as the chlorides by roasting the oxides (Koch–Light Ltd.) at 900°C for 3 hr (to remove moisture and to decompose carbonates), dissolving with a minimum amount of Analar HCl and titrating to near neutral pH with NaOH solution. Magnesium was added as the acetate salt. Lanthanide–$ATP^-$ complexes were made up at pH 7,8 immediately before an assay. At this pH, binding is sufficiently strong to enable soluble complexes of up to about 25 mM concentration to be prepared. The standard coupled assay technique was utilized for activity measurements [7], the initial velocities being expressed as $\Delta A_{340}$ per min for NADH. The spectrometer used was a Pye Unicam SP8000 thermostated at 25 ± 0.02°C. The assay mixture contained 0.25 mM NADH, 5 mM 3-phosphoglycerate and 50 mM Tris–HCl buffer at pH 7.8, unless otherwise stated. 0.25 μg of PGK and 300 μg glyceraldehyde phosphate dehydrogenase were used per ml of substrate. 3 ml × 1 cm cells were

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employed to minimize volumetric errors. The concentration of the complex Mg–$ATP^{2-}$ was calculated using a dissociation constant of 0.05 mM [8], and 8 mM for MgPGA [3].

Binding constants of ATP complexes to PGK were determined by gelfiltration of G25 (fine) Sephadex using a column of dimensions 1 cm by 14 cm. Protein was determined by the micro biuret method [9]: ATP and ADP were determined by scintillation counting of the radioactive nucleotide after standardization of the protein-free solution spectro-photometrically ($A_{260}^{mM} \approx 15.4$) [10]. Proton n.m.r. spectral titration were carried out using the 270 MHz Fourier Transform instrumentation described previously [4] and under similar conditions.

## 3. Results and discussion

### 3.1. *Inhibition by ATP–La⁻*

Double reciprocal plots showing the effect on the reaction velocity of different concentrations of La–$ATP^{-}$ are shown in fig. 1. The concentration of Mg–ATP was varied by changing the concentrations of $Mg^{2+}$ and ATP simultaneously to maintain their concentrations in a 1:1 ratio. Due to dissociation, a certain amount of free ATP is inevitably present, varying from 0.033 mM at 0.2 $K_m$ to 0.1 mM at 2 $K_m$. It has been shown that the Mg–ATP complex is the true substrate [3]. The $K_m$ obtained for Mg–$ATP^{2-}$ was 0.073 ± 0.002 mM (the mean of three determinations), considerably lower than the values previously reported [3,11]. Moreover, in contrast to previously published results [11], this value was found to depend on the concentration of 3-phosphoglycerate (as did the Ln–ATP inhibition constants), dropping from the above value at 5 mM PGA to 0.013 ± 0.001 mM at 1 mM PGA, although the type of inhibition by Ln–ATP did not change. Since the conditions and substrate concentrations we have used are similar to those of Larsson–Raznikiewicz, the difference in results is striking. It may result from our thorough removal of sulfate ion from the assay solution, allowing the PGA to compete for sites otherwise occupied by sulfate. This could explain both our lower $K_m$ for ATP and the competition by PGA.

The plots in fig. 1 show inhibition by La·$ATP^{-}$ that is competitive with Mg·ATP. A value for the apparent inhibition constant was obtained from the secondary plot (fig. 2) of the apparent $K_m$ ($K_m'$) vs. inhibitor concentration, assuming one mole of inhibitor competing with one mole of substrate:

$$V_o = V_{max}[1 + (K_m/s)(1 + I/K_I)]^{-1}$$

where the apparent $K_m$ at any value of I would be $K_m' = K_m(1 + I/K_I)$. From fig. 2 we calculate $K_I = 0.04 \pm 0.01$ mM. This value is somewhat less than the Michaelis constant of 0.073 mM for Mg·ATP, at a PGA concentration of 5 mM. From the form of

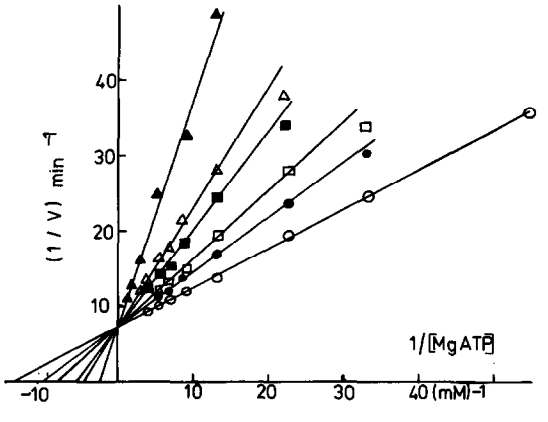


Fig. 1. The effect of various concentrations of Lanthanum–$ATP^{-}$ on the phosphoglycerate kinase reaction: ○ zero; ● 0.033 mM; □ 0.05 mM; ■ 0.083 mM; △ 0.10 mM; ▲ 0.167 mM.

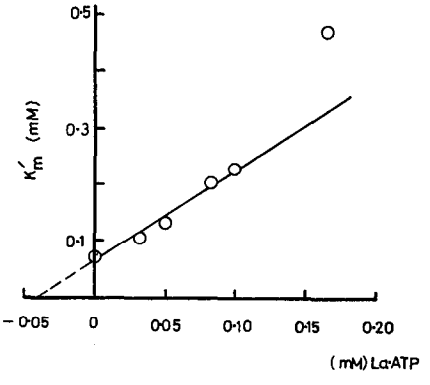


Fig. 2. Secondary plot (derived from fig. 1) showing apparent $K_m$ ($K_m'$) as a function of La·$ATP^{-}$ concentration.

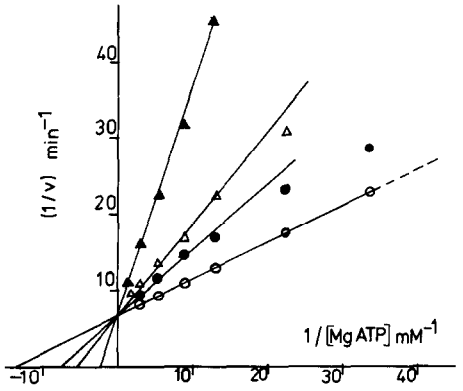


Fig. 3. The effect of various concentrations of Eu·ATP$^-$ on the phosphoglycerate kinase reaction: ○ zero; ● 0.05 mM; □ 0.10 mM; ■ 0.167 mM. Identical behaviour is shown by Pr·ATP$^-$ and Yb·ATP$^-$.

the secondary plot it may be the case that some form of higher power inhibition is operative.

### 3.2. *Inhibition by Eu–, Pr–, and Yb–ATP$^-$ complexes.*

Competitive inhibition by these complexes was also observed, yielding apparent $K_I$ values equal to that of La–ATP$^-$ within the experimental error. The plots of $V^{-1}$ vs. [Mg–ATP]$^{-1}$ showed in these cases a concave-downward tendency; fig. 3 shows the behaviour of Eu–ATP$^-$, identical results being obtained with Pr–ATP$^-$ and Yb–ATP$^-$.

Explanations consistent with the shape of these curves are (i) that Mg·ATP competes more effectively with the Ln–ATP$^-$ site to relieve the inhibition, varying with Ln, and (ii) that Ln·ATP$^-$ tends to function as an activator at very low substrate and inhibitor concentrations. The effect cannot be due to free lanthanide, since the dissociation constant of Ln·ATP$^-$ is very low (approx. $10^{-6}$ M), [15]. Our n.m.r. results show that the constant for dissociation of PGA from (Ln·ATP$^-$) is sufficiently large (0.077 M) that the formation of the species Ln·ATP·PGA, which could be a kinetic complexity, is negligible under these condition, [13].

### 3.3. *Binding of metal–nucleotide complexes to phosphoglycerate kinase*

The possibility that there may be more than one nucleotide–metal binding site on the enzyme was explored by direct binding experiments and n.m.r. spectral titration. Larsson–Razniekiewicz has recently shown that ATP binding of the enzyme is not saturated when 1 mole of ATP is bound [12]. Our gel-filtration studies were conducted under the following conditions: 23°C, 1.0 mM $Mg(CH_3COO)_2$ and 0.05 M Hepes buffer, pH 7.8.

Hepes buffer was used in place of Tris, which interfered with the biuret assay for protein (as did TES buffer). The nucleotide–metal complexes bind to the Sephadex column, so it was found necessary to equilibrate the Sephadex by passing several column volumes of each solution over the Sephadex before the protein (about 2 mg at 10 mg/ml) was applied to the column.

At 1 mM total ATP (pH 7.8) two moles of nucleotide were bound per mole of enzyme. This is much higher than found by Larsson–Razniekiewicz [12] under similar conditions but in the absence of divalent cation. Half-maximal binding of ATP·Mg at pH 7.8. occurred at about 0.1 mM.

Two molecules of ATP were bound to the enzyme in pH 7.8 Hepes buffer containing 1 mM $Mg^{2+}$ with 0.12 mM ATP plus 0.44 mM $LaCl_3$; with 0.22 mM ATP plus 0.089 mM $LaCl_3$; and with 0.49 mM ATP plus 0.089 mM $LaCl_3$. At 0.12 mM ATP in the absence of $LaCl_3$ just over one mole of ATP is bound, showing that the lanthanide complex is bound appreciably tighter than the magnesium complex.

A 270 MHz n.m.r. spectral titration was carried out at pH 6.3 (80 mg/ml yeast PGK, 100 mM [$^2$H] sodium acetate buffer) in order to investigate binding under identical conditions to our other n.m.r. studies [4,13]. On addition of ADP and 8 mM [$^2$H] magnesium acetate, peaks at 7.33/7.16 ppm ($X$) and 7.00 ppm ($Y$) appear in the difference spectrum [4]. Identical peaks were observed on addition of Mg·ATP, and of La·ATP$^-$. Titration of the amplitudes of peaks $X$ and $Y$ with increasing Mg·ATP concentration showed a single binding site for this species, fig. 4, with a dissociation constant $K = 0.15 \pm 0.07$ mM. Single binding sites were also shown by titrations for Mg·ADP ($K = 0.25 \pm 0.13$ mM) and for La·ATP$^-$ (binding too tight for evaluation of a dissociation constant). These n.m.r. results are in agreement with X-ray data [16,17] and U.V. spectrophotometric results [18] which also show a single metal–nucleotide binding site, at neutral pH. The disagreement

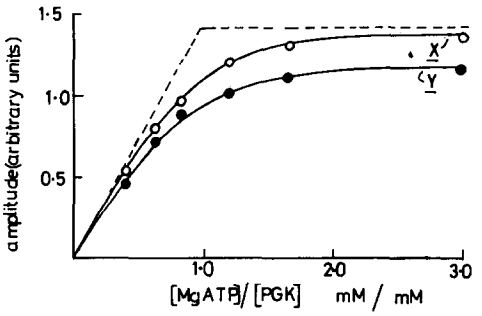


Fig. 4. Binding curve of Mg·ATP to PGK. The amplitudes of peaks X and Y in the difference spectrum, 40°C, pH 6.30 (see text and ref. [4] are plotted as a function of Mg·ATP concentration. [PGK] = 1.7 mM. A similar curve (showing one binding site) is obtained for La·ATP.

with the above and other [12] gel-filtration measurements may mean that one site is inoperative or unobserved under the experimental conditions of spectrometric determinations.

## 4. Conclusions

We can conclude from this kinetic study that the Ln·ATP$^-$complex binds at the Mg·ATP site(s) of phosphoglycerate kinase at pH 7.8, as an inhibitor. Our n.m.r. data further supports this result at pH 6.3. where Mg·ATP and La·ATP cause very similar perturbations of the enzyme resonances, and binding of the relaxation probe Gd·ATP$^-$causes identical line-broadenings to those introduced by Mn·ATP$^{2-}$ [13]. (Mn·ATP forms as active a complex with the enzyme as does Mg·ATP [12]). These observations suggest that the replacement of a native group IIA metal ion by a trivalent lanthanide may be of general applicability in ATP–phosphotransferase systems. The inhibition by the lanthanides may be attributable to the increased size and charge relative to $Mg^{2+}$, although they are generally preferable to the transition metals as probes for group IIA metals for chemical [4] and other reasons [2].

It is apparent from our results and from comparable results in the literature that the number of binding sites detected for ATP depends on the pH, the metal ions present and the method used for the determination. The observation of a single site binding both Mg·ATP and Ln·ATP$^-$ at pH 6.3, and the competitive inhibition by Ln·ATP$^-$at pH 7.8 validates the use of Ln·ATP$^-$complexes as shift and broadening probes of the resonances of nuclei in the active site region in n.m.r. studies. Data from this technique has been obtained [4,13] enabling the mapping of the geometry of the metal/ATP/PGK active site complex.

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