YEAST PHOSPHOGLYCERATE KINASE – EVIDENCE FROM AFFINITY ELUTION STUDIES FOR CONFORMATIONAL CHANGES ON BINDING OF SUBSTRATES

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1. Introduction

The structure and kinetics of phosphoglycerate kinase (EC 2.7.2.3) have been under intensive investigation for several years. Recent papers have presented the X-ray crystallographic structure of the horse muscle enzyme [1,2] and the yeast enzyme [3], the complete amino acid sequence of the horse enzyme [2], and kinetic [4] and ligand binding studies [5,6]. Interpretation of these results has led to suggestions that conformational bending about the 'neck' region connecting the two domains of the enzyme may be involved in the catalytic activity, and direct evidence of a decrease in the radius of gyration of the ternary enzyme–substrate complex, compared with free enzyme, is to hand [7]. Studies we have carried out on the adsorption of this enzyme to cation-exchangers, and its subsequent elution by ligands (affinity elution [8,9]) have led to similar conclusions concerning conformational changes with this enzyme. A change of the shape of a protein molecule can be expected to affect the strength of its adsorption to materials such as ion-exchangers; consequently a study of the latter can indicate conformational changes under different conditions. The results presented below are taken from a more extensive study on the binding of many different enzymes to ion-exchangers in the presence and absence of ligands (in preparation); a preliminary report has been presented [10].

2. Materials and methods

Yeast phosphoglycerate kinase was purified by the procedure in [9,11], and was recrystallised twice; its specific activity was 700 units $.mg^{-1}$ at 25°C in the standard assay conditions [12]. CM-cellulose (CM 52) from Whatman was suspended in buffer (see below) equilibrated at pH ~9.0, filtered by suction and stored damp. Of this 5 g was added to 13 ml buffer plus enzyme; the effective total volume of liquid (determined by protein exclusion) was 17 ml, and the equivalent packed-column volume of CM-cellulose was 8 cm^3. Using these figures, the partition coefficient between adsorbed and non-adsorbed protein could be calculated from the protein concentration in the liquid phase.

The slurry of CM-cellulose, buffer and protein was stirred, sampled (0.10 ml $.min^{-1}$) through a low dead-volume filter, and mixed with Lowry protein reagents with a Technicon Auto Analyser. The starting buffer consisted of 10 mM KOH adjusted to pH 9.0 with Tris (hydroxymethyl) methyl-aminopropane sulphonic acid (TAPS). The slurry was continuously titrated with 0.10 ml $.min^{-1}$ of 0.05 M of the appropriate acid (*N*-Tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES) to pH 7.5, (*N*-morpholino)-ethane sulphonic acid (MES) to pH 6.2, acetic acid for lower pH values); this system ensured that the ionic strength remained at 0.01 M. 1,3-Bis-phosphoglycerate was purified as in [5]; other ligands were sodium salts obtained from Sigma Chem. Co.

3. Results

The pH/protein concentration curve for yeast phosphoglycerate not adsorbed to CM-cellulose is

shown in fig.1(a); the protein concentration has been offset to correspond with the pH at the time of sampling. Other experiments with faster or slower titration rates indicated that the limiting factor was the dead space in the sampling system; equilibration of protein with the CM-cellulose was relatively rapid in comparison. Figure 1(b) shows these results calculated in terms of partition coefficient against pH, where the partition coefficient is defined as that proportion of the total protein that would be adsorbed in a given volume of packed CM-cellulose. The protein concentration in these experiments was between 0.5 and 1 mg .cm^{-3}, only 1–2% of the total adsorptive capacity of the CM-cellulose for phosphoglycerate kinase (100 mg .cm^{-3}).

Figure 2 shows the effects of the presence of 0.1 mM ADP, ATP or 3-phosphoglycerate; this concentration is ~10-times the K_d for each of these substrates [5]. A substantial decrease in binding is

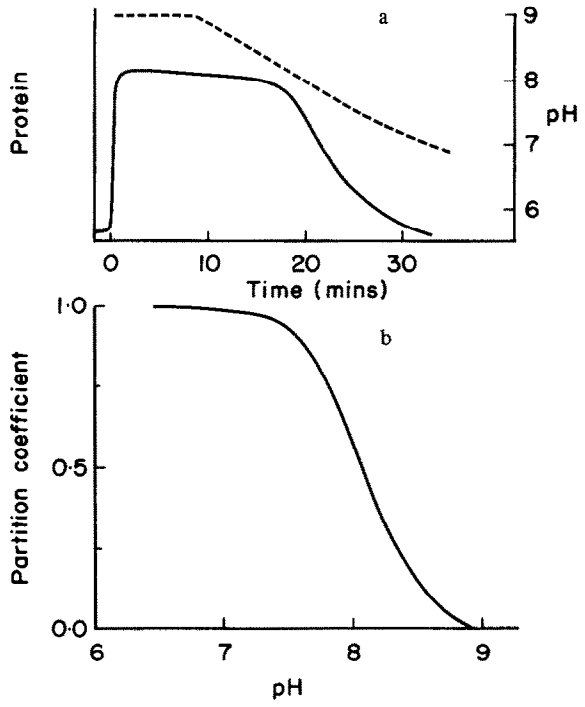

Fig.1. Adsorption of yeast phosphoglycerate kinase to CM-cellulose: variation with pH. (a) Recorder traces of protein concentration in liquid phase (———) and pH (· ·) continuously monitored. (b) Results from (a) plotted as partition coefficient against pH.

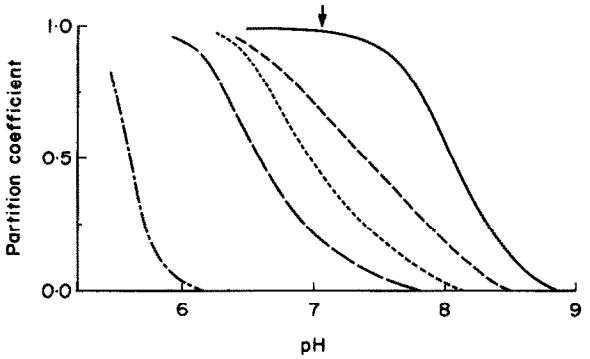

Fig.2. Adsorption of phosphoglycerate kinase to CM-cellulose in the presence of substrates; partition coefficient plotted against pH. No ligand (———); (– –) + 0.1 mM ADP (±1 mM Mg-acetate); (· ·) + 0.1 mM 3-phosphoglycerate; (— —) + 0.1 mM ATP (±1 mM Mg-acetate); (·–·) + 0.1 mM 1,3-bisphosphoglycerate. The arrow indicates the isoelectric point of the free enzyme.

observed with ATP (± Mg^{2+}), somewhat less with 3-phosphoglycerate, and a relatively small decrease with ADP. Curves for both 0.5 mM sulphate and 0.1 mM citrate were very similar to that for ADP. Also in fig.2 the effect of 0.1 mM 1,3-bisphosphoglycerate is shown. Although perhaps 10^4-times greater than the K_d [5], 0.1 mM was used, since protein was 0.01 mM, and an excess of ligand must be used to displace the protein from adsorbent. 1,3-Bisphosphoglycerate had a far greater effect than any of the other ligands, displacing the partition coefficient/pH curve by >2 pH units. Similar results have been obtained at sub-μM levels of 1,3-bisphosphoglycerate using 1 μg .cm^{-3} protein, and measuring it by enzymic activity.

A pH–titration curve for yeast phosphoglycerate kinase was obtained experimentally, which agreed well with a theoretical one using appropriate values for the pK_a values of titrating amino acids. The results were then plotted as partition coefficient against total charge of protein–ligand complex, shown in fig.3. With the exception of ADP, it is clear that more than just charge addition is involved in these affinity elution effects. At these concentrations of ligand there is no significant contribution by the weaker adenine nucleotide binding site [5], nor does the ligand presence significantly increase the ionic strength of the buffer.

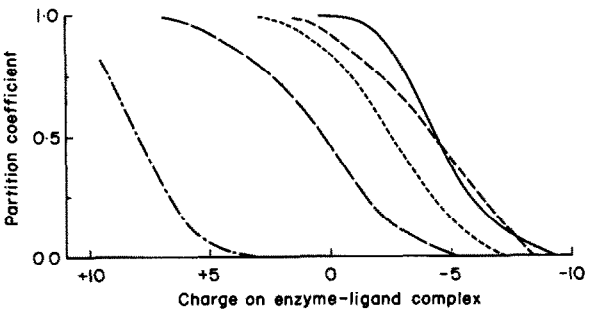

Fig.3. Results from fig.2 plotted as partition coefficient against total ionic charge on the protein–ligand complex. Symbols as for fig.2.

Using phosphoglycerate kinases from other sources (carp, rabbit, trout, muscle enzymes) similar results to those presented in fig.1 and 2 have been obtained.

4. Discussion

The presence of a ligand in the buffer during ion-exchange chromatography may cause 'affinity elution' (specific elution from the column of the protein binding that ligand). The magnitude of this effect may depend on at least 3 factors.

1. The addition of a charged ligand (usually negatively charged) changes the net charge on the protein so that it is less strongly held to the adsorbent.
2. This binding of ligand may mask an area which was specifically involved in the adsorption process, for instance a localised cluster of charges of opposite sign to the adsorbent.
3. There may be a conformational change in the shape of the protein molecule which makes it less (or more) strongly adsorbed.

All of these factors are probably involved in the process of affinity elution of phosphoglycerate kinase from CM-cellulose. This enzyme anomalously adsorbs at pH values above its isoelectric point (a negatively charged protein adsorbing to a negatively charged ion-exchanger). The isoelectric point of 7.1 [13,14] has been confirmed by us using isoelectric focussing on an LKB PAG-plate. This anomaly could be due to non-ionic interactions, but the lack of similar behaviour by other proteins investigated by us seems to indicate this as unlikely. Moreover, the enzyme does not adsorb to DEAE-cellulose at pH <7.0 at this ionic strength. It appears more likely that the anomalous adsorption is due to the localised cluster of positive charges on the N-domain, including 5 arginine and 3 histidine residues [2]. If this area interacts specifically with CM-cellulose, the net repulsive effect of the remaining protein may be weaker than the specific attraction, because of longer distances of interaction. Also, the lysine residues around the adenine nucleotide binding site on the C-domain may strengthen this specific effect.

When negatively charged ligands are present, the effective isoelectric point is lowered. With non-substrate ligands such as sulphate and citrate, the shift in the partition coefficient/pH curve has been found to be slight, about that expected for the charges added. ADP also causes little shift (fig.3); ATP and 3-phosphoglycerate each cause a much greater shift. It is interesting that in the absence of Mg^{2+}, when ATP has 3–4 negative charges, the shift is the same as in the presence of Mg^{2+}, when the ligand MgATP has only 2 charges. In these cases it is difficult to decide to what extent a conformational change is involved, or just a masking effect; still the overall adsorption to CM-cellulose remains anomalous in that negatively charged protein–ligand complex binds to the adsorbent. With 1,3-bisphosphoglycerate however, there can be little doubt that a conformational change is involved; the extra one negative charge compared with 3-phosphoglycerate would neither make a substantial difference to the effective isoelectric point, nor mask much more of the protein surface. A conformational change of the type suggested [2] would result in a burial of all the positively charged surface, so eliminating the specific adsorption to the CM-cellulose. In fact when plotted against charge (fig.3), it is seen that the enzyme–1,3-bisphosphoglycerate complex behaves as would be expected, with no binding to CM-cellulose when its net charge is negative.

The conclusion drawn from these experiments is that whereas there appears to be no conformational change that affects the interaction with CM-cellulose when phosphoglycerate kinase binds ADP, sulphate or citrate, there may be a small change with 3-phosphoglycerate, and a somewhat larger change with ATP. However, neither of the latter shape

changes can be as large as that occurring when 1,3-bisphosphoglycerate binds to the enzyme. It has been suggested [2,7] that binding of some substrates causes a closing of the structure, such that the two domains meet, allowing interaction between the two substrates during enzymic activity. From these results, and those on the measurement of dissociation constants [5], it appears that only 1,3-bisphosphoglycerate causes the equilibrium between open and closed forms of the enzyme to favour the closed form. This substrate binds very tightly (K_d <0.1 μM) which can be explained by presuming that the compound gets trapped, and can only dissociate when the enzyme opens up again. This results in a low rate of dissociation of 1,3-bisphosphoglycerate, since only a small proportion of the complex at any given moment is in the open form. Indeed there is reason to suppose that 1,3-bisphosphoglycerate bridges the two domains, and so holds the closed form together.

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