Ligand-induced conformational changes in wild-type and mutant yeast pyruvate kinase

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Abstract

A mutant form of pyruvate kinase in which serine 384 has been mutated to proline has been engineered in the yeast Saccharomyces cerevisiae. Residue 384 is located in a helix in a subunit interface of the tetrameric enzyme, and the mutation was anticipated to alter the conformation of the helix and hence destabilize the interface. Previous results indicate that the mutant favours the T quaternary conformation over the R conformation, and this is confirmed by the results presented here. Addition of phosphoenolpyruvate (PEP), ADP and fructose-1,6-bisphosphate (Fru-1,6-P2) singly to the wild-type and mutant enzymes results in a significant quenching of tryptophan fluorescence (12–44%), and for Fru-1,6-P2, a red shift of 15 nm in the emission maximum. Fluorescence titration experiments showed that PEP, ADP and Fru-1,6-P2 induce conformational changes which have similar ligand-binding properties in the wild-type and mutant enzymes. However, the Fru-1,6-P2 induced conformation is demonstrably different from those induced by either ADP or PEP. The enzymes differ in their susceptibility to trypsin digestion and N-ethylmaleimide inhibition. The thermal stability of the enzyme is unaltered by the mutation. Far-UV CD spectra show that both enzymes adopt a similar overall secondary structure in solution. Taken together, the results suggest that the Ser384-Pro mutation causes the enzyme to adopt a different tertiary and/or quaternary structure from the wild-type enzyme and affects the type and extent of the conformational changes induced in the enzyme upon ligand binding. A simplified minimal reaction mechanism is proposed in which the R and T states differ in both affinity and kcat. Thus, in terms of the models of cooperativity and allosteric interaction, pyruvate kinase is both a K and a V system.

Keywords: mutant/NEM/proteolysis/pyruvate kinase/R–T state

Introduction

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase; EC 2.7.1.40) is a glycolytic enzyme that plays an important role in regulating the flux from fructose-1,6-bisphosphate (Fru-1,6-P2) to pyruvate. The enzymes from several different organisms have been isolated and characterized. In most cases the enzyme is a tetramer of identical subunits each about 500 amino acid residues in length. The enzyme requires both divalent and bivalent cations for activity (see Muirhead, 1987, for a review). The reaction catalysed is

\[
\text{PEP + Mg-ADP} \rightarrow \text{pyruvate + Mg-ATP}
\]

Ligand-induced conformational changes in pyruvate kinase have been demonstrated by a number of techniques, e.g. fluorescence quenching (Kuczenki and Suelter, 1970), UV CD spectroscopy (Wildes et al., 1971), sedimentation velocity and optical rotatory dispersion (Kayne and Suelter, 1968), electron paramagnetic resonance spectroscopy (Reed and Cohn, 1972), and chemical modification (Wieker and Hess, 1972; Plaxton et al., 1990).

Limited proteolysis has been used as an effective tool to study the conformational properties of globular proteins, as well as to probe conformational transitions such as occur upon ligand binding (McClymont and Markus, 1968; Mort and Sanwal, 1978; Arnone et al., 1992). Trypsin is a useful reagent for these types of studies because it has wide substrate specificity, is tolerant of a variety of incubation conditions and can be specifically and irreversibly inhibited.

The thiol-modifying compound N-ethylmaleimide (NEM) can also be used to investigate protein conformation. NEM reacts specifically with the cysteine residues of proteins, often leading to progressive loss of enzymic activity. This compound has been used previously with the pyruvate kinase enzyme from Saccharomyces carlsbergensis (Wieker and Hess, 1972). Thus, trypsin digestion and NEM inhibition of variously ligated enzymes can be used to probe the subtleties of the ligand-induced conformational changes. It was anticipated that differences in the susceptibility to these two treatments might provide useful indications of the structural adjustments provoked by ligand binding, especially in terms of the accessibility of target basic and thiol residues.

Pyruvate kinase from Saccharomyces cerevisiae is an excellent subject for such studies as its amino acid sequence is known, and tertiary and quaternary structures are available from detailed modelling studies. From an analysis of its three-dimensional structure, the enzyme is a globular protein composed of distinct domains (Muirhead, 1987). It is known to undergo conformational changes upon ligand binding, and the allosteric transition upon effector binding is amenable to study. The yeast pyruvate kinase also contains a single tryptophan residue (Trp451) and previous studies indicate that tryptophan fluorescence gives useful information on protein conformation. For example, studies on the wild-type enzyme have indicated that tryptophan fluorescence measurements can be used to monitor the binding of Fru-1,6-P2 (Muncott et al., 1992). These results showed that yeast pyruvate kinase binds four molecules of Fru-1,6-P2 per tetramer and that the observed fluorescence quench follows the binding of the ligand and not the cooperative T to R state transition. Additionally, it showed that the binding of Fru-1,6-P2 to yeast pyruvate kinase is compatible with a model of cooperativity that incorporates an intermediate state, R', with properties between those of the T and R states.

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Serine 384 is located in an α-helix (Cα2) known from the crystal structure (Muirhead, 1987) to be important in forming inter-subunit contacts (Figure 1), and was replaced by proline by site-directed mutagenesis (McNally, 1989; Collins et al., 1995). This substitution would have a pronounced effect on the α helix and on the packing of the two symmetry-related helices in the subunit interface. Previous studies have demonstrated that this substitution causes the enzyme to adopt the T quaternary conformation over the R conformation (Collins et al., 1995). Other studies have suggested that point mutations introduced into subunit interface regions have pronounced effects on the subsequent activity of the enzyme. For example, a subunit interface mutation (Gly418Trp) in the dimeric enzyme glutathione reductase from Escherichia coli, resulted in a reduction in thermal stability, a 50% decrease in $k_{\text{cat}}$ and introduced apparent cooperativity in substrate binding at non-saturating NADPH (Scrutton et al., 1992). A pronounced destabilization of yeast phosphoglycerate mutase has also been produced in a subunit interface region by a Lys168Pro substitution (White et al., 1993).

This paper extends studies on the R and T states of pyruvate kinase and helps to define the differences in the kinetic and physical properties of the R and T states.

Materials and methods

Reagents

Rabbit muscle lactate dehydrogenase (EC 1.1.1.21) and pyruvate kinase were obtained from Boehringer Mannheim UK (Lewes, East Sussex, UK). The di(monocyclohexylammonium) salt of ADP, the mono(cyclohexylammonium) salt of phosphoenolpyruvate (PEP), the disodium salt of NADH, the trisodium salt of Fru-1,6-P$_2$, NEM, phenylmethylsulphonyl fluoride (PMSF) and TPCK-treated trypsin were obtained from Sigma Chemical (Poole, Dorset, UK). All other reagents were of reagent grade and obtained from either Sigma Chemical or BDH (Poole, Dorset, UK). Plasmid pMA91pyk was a generous gift from Dr J.Mellor, Department of Biochemistry, University of Oxford, UK.

Transformation of yeast

The yeast strains used and the method of transformation with plasmid DNA have been described earlier (Collins et al., 1995).

Mutagenesis of pyruvate kinase gene

The Ser384 residue was mutated to a proline with oligonucleotide-mediated site-directed mutagenesis (Kunkel, 1985). This procedure has been described for yeast pyruvate kinase in detail elsewhere (McNally, 1989).

Purification of wild-type and mutant pyruvate kinases

The purification of the wild-type and mutant enzymes has been described in detail elsewhere (Collins, 1993; Collins et al., 1995).

Pyruvate kinase assay

Pyruvate kinase activity was measured using a coupled enzyme assay (Collins et al., 1995). For assays in which the pH was varied, Tris–HCl buffers were used for pH values above 7.0 and Mes-TPA [2-(N-morpholino)ethanesulphonatotetrapropylammonium] buffers below pH 7.0. For assays in which the activating cations were studied, 100 mM monovalent or 15 mM bivalent chloride salts were added in place of the physiological cations. Barium was added as the acetate salt. The reaction was followed by monitoring the decrease in absorbance at 340 nm in a Philips PU8400 spectrophotometer. One unit of pyruvate kinase is defined as the amount of enzyme that results in the utilization of 1 μmol NADH/min under the conditions described. The results shown are the averages of duplicate measurements. The variation between replicates was less than 5%.

Protein concentration determination

The concentration of the purified pyruvate kinase enzymes was determined spectrophotometrically using an absorbance of 0.51 at 280 nm for a 1 mg/ml solution (Yun et al., 1976).

Circular dichroism spectroscopy

Far-UV CD spectra of the wild-type and mutant enzymes were obtained as described in detail earlier (Collins et al., 1995). The buffer comprised 20 mM Tris–HCl (pH 8.5), 3 mM MgCl$_2$, 20% (v/v) glycerol and 1 mM dithiothreitol (DTT). The protein concentration was in the range 0.1–0.25 mg/ml.

Fluorimetric measurements

Fluorimetric measurements were performed with a Perkin-Elmer LS50 spectrofluorimeter. The excitation and emission slit widths were both 5 nm. Analyses were made in a 1 ml capacity quartz cuvette equilibrated at 25°C. Excitation of the sample was at 295 nm and emission was measured between 300 and 400 nm. Fluorescence titrations to determine $K_S$ for the ligands were performed at 340 nm. The buffer comprised 20 mM Tris–HCl (pH 8.5), 20% (v/v) glycerol, 3 mM MgCl$_2$ and 1 mM DTT. Potassium was not included in the assay buffer. The protein concentration was in the range 0.1–0.25 mg/ml. Ligands were added singly to each enzyme solution. Reported values are the averages of duplicate measurements. Fluorescence titration measurements of the wild-type and Ser384 Pro mutant forms of pyruvate kinase allow dissociation constants ($K_S$) to be determined in the absence of other ligands. Kinetic measurements give $K_{0.5}$, or the substrate concentration which gives $V_{\text{max}}/2$ with all substrates and metal ions present in excess.
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Intensity

Wavelength (nm)

Fig. 2. Effect of various ligands on fluorescence emission of wild-type pyruvate kinase. Wild-type pyruvate kinase (0.2 mg/ml) was incubated with (a) 5 mM PEP, (b) 2 mM ADP and (c) increasing concentrations of Fru-1,6-P₂. Upper trace in each plot is the unligated enzyme. Lower trace is the spectrum obtained in the presence of ligand. Titration of enzyme with Fru-1,6-P₂ was as follows: 27, 54, 78, 110 and 133 mM, with the increase in concentration corresponding to the progression to lower traces. For more complete details, see Materials and methods.

Intensity

Wavelength (nm)

Fig. 3. Effect of various ligands on the fluorescence emission of mutant pyruvate kinase. Mutant pyruvate kinase (0.2 mg/ml) was incubated with (a) 5 mM PEP, (b) 2 mM ADP and (c) increasing concentrations of Fru-1,6-P₂. Upper trace in each plot is the unligated enzyme. Lower trace is the spectra obtained in the presence of ligand. Titration with Fru-1,6-P₂ is identical with that in Figure 2. For more complete details, see Materials and methods.

Table I. Comparison of enzymically and fluorimetrically derived parameters for wild-type and mutant pyruvate kinases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₀.₅ PEP (- FBP)</td>
<td>2.76 ± 0.08 mM</td>
<td>-⁵</td>
</tr>
<tr>
<td>nₜ</td>
<td>2.93 ± 0.07</td>
<td>-</td>
</tr>
<tr>
<td>K₀.₅ PEP (+ FBP)</td>
<td>0.22 ± 0.004 mM</td>
<td>0.67 ± 0.03 mM</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.72 ± 0.05</td>
<td>1.95 ± 0.04</td>
</tr>
<tr>
<td>Kₛ PEP (- FBP)</td>
<td>0.093 ± 0.01 mM</td>
<td>0.17 ± 0.02 mM</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.06 ± 0.03</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>K₀.₅ ADP (- FBP)</td>
<td>0.25 ± 0.006 mM</td>
<td>-</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.53 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>K₀.₅ ADP (+ FBP)</td>
<td>0.15 ± 0.01 mM</td>
<td>0.43 ± 0.03 mM</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.01 ± 0.07</td>
<td>1.65 ± 0.07</td>
</tr>
<tr>
<td>Kₛ ADP (- FBP)</td>
<td>3.02 ± 0.03 mM</td>
<td>2.49 ± 0.06 mM</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.06 ± 0.02</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>Kₛ FBP</td>
<td>0.0493 ± 0.002 mM</td>
<td>0.0156 ± 0.001 mM</td>
</tr>
<tr>
<td>nₜ</td>
<td>1.23 ± 0.05</td>
<td>1.80 ± 0.06</td>
</tr>
<tr>
<td>kₓ (- FBP)</td>
<td>188 ± 5 s⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>kₓ (+ FBP)</td>
<td>195 ± 2 s⁻¹</td>
<td>314 ± 5 s⁻¹</td>
</tr>
</tbody>
</table>

*K₀.₅ determined by enzyme assay.

FBP = fructose-1,6-bisphosphate.

Dashes indicate not obtained owing to inactivity of mutant in absence of effector.

Kₛ determined by fluorescence titration.

Trypsin digestion

Wild-type and mutant pyruvate kinase (0.5 mg/ml) were treated with TPCK-trypsin in pyruvate kinase assay buffer [50 mM Tris-HCl (pH 6.5), 100 mM KCl, 15 mM MgCl₂] at 37°C. The mass ratio of pyruvate kinase to trypsin was 4:1. The total reaction volume was 50 ml. The wild-type and mutant enzymes were allowed to interact with the added ligands (PEP 9.4 mM, ADP 8.0 mM and Fru-1,6-P₂ 1.2 mM) for 5 min at 25°C before the addition of trypsin. At indicated time points, aliquots (5 ml) were removed and added to 1 ml pre-equilibrated assay mixture containing 0.1 mM PMSF. The dilution and specific inhibition of trypsin effectively prevented further proteolysis. PMSF, or the other reaction components, did not interfere with the activity of the coupling enzyme, lactate dehydrogenase. The pyruvate kinase reaction was started by the addition of excess PEP after the partially digested enzyme had equilibrated in assay mixture for 2 min. Assays for the mutant enzyme also contained 5 mM Fru-1,6-P₂. The residual activity of the partially digested forms is expressed as a percentage of the initial activity of the appropriate ligated form. Experiments were performed in duplicate.

NEM inhibition

Wild-type and mutant pyruvate kinases (0.5 mg/ml) were incubated in assay buffer. The total reaction volume was
The enzymes were allowed to interact with ligands for 5 min before the addition of NEM to 2 mM. The concentration of ligands was identical with the trypsin digestion experiment. At the indicated times, aliquots (5 ml) were removed and added to 1 ml of pre-equilibrated assay mixture and incubated at 25°C for 2 min, and the reaction was started by the addition of excess PEP. The mutant enzyme was assayed in the presence of 5 mM Fru-1,6-P_2. Residual NEM did not interfere with the activity of the coupling enzyme. The residual activity of the wild-type and mutant enzymes was expressed as a percentage of the initial activity of the appropriate liganded forms.

Thermal denaturation
Samples (0.05 mg) of purified wild-type, Ser384 Pro mutant and commercially available rabbit muscle pyruvate kinase were incubated in assay buffer and treated as described previously (Plaxton et al., 1990). Experiments were performed in duplicate.

Results and discussion
Fluorescence studies
The fluorescence emission spectra of the wild-type enzyme obtained in the presence and absence of various ligands are shown in Figure 2. The changes in emission intensity can be used to determine the dissociation constants (K_S) of the ligands. In the absence of ligand, the emission spectrum is a broad peak with an emission maximum at 340 nm. PEP and ADP at saturating concentrations reduce the intensity of the emission at 340 nm by 16 and 12%, respectively, and are accompanied by a 2–3 nm red shift in emission maximum. A similar result is observed with the mutant enzyme (Figure 3). Addition of PEP and ADP to the mutant enzyme, to saturating concentrations, causes a 34 and 15% reduction in emission intensity at 340 nm, respectively. The emission spectra of both the wild-type and mutant enzymes indicate that the single tryptophan residue (Trp451) is moderately exposed to the solvent in the non-ligated T structure (Eftink and Ghiron, 1976). An analysis of a three-dimensional model of the yeast enzyme shows that Trp451 is located in the first turn of helix Ca5. The environment of this residue differs in the T and R structures (Mattevi et al., 1995). When the allosteric effector Fru-1,6-P_2 is added to the wild-type enzyme to saturating concentrations the emission at 340 nm is reduced by 40% and the emission maximum is shifted to 355 nm. A similar addition of effector to the mutant enzyme results in a 44% decrease in emission intensity at 340 nm and a shift in emission maximum to 355 nm. This indicates that Trp451 has become almost completely exposed to the solvent, implying that the transition to an R state has taken place. This conformational change is fundamentally different from that induced by the substrates. These data also reveal that the inactivity of the mutant enzyme described previously (Collins et al., 1995) is not a result of the absence of substrate binding, but is due to catalytic inactivity of the enzyme. The effector binding site lies between domains A and C, whilst the active site is in a cleft located between domains A and B. Domains A and B close over the domains A and C, whilst the active site is in a cleft located between domains A and B. Ligand binding causes a rotation in all 12 domains of the tetramer, leading to changes in intersubunit contacts (Mattevi et al., 1995). The Ser384Pro mutation lies well away from either the substrate or effector binding sites and so must affect protein conformation in order to influence such parameters.

Kinetic studies
The kinetic and equilibrium binding parameters for the wild-type and the mutant enzyme, determined by enzymic assay and fluorimetric titration, are shown in Table I. The mutant enzyme is dependent upon the presence of the allosteric effector Fru-1,6-P_2 for activity (Collins, 1993; Collins et al., 1995) and so kinetic parameters in the absence of this effector could not be calculated from enzymic assay data.

The Hill constant of the wild-type and mutant enzymes for both ADP and PEP when determined by fluorescence titration is approximately 1.0 (Table I). These fluorescent data reflect non-cooperative substrate binding and not the cooperative effect. This suggests that a cooperative conformational change occurs after the binding of both substrates and that a subsequent catalytic step must be rate-limiting. The differences between K_S and K_H for Fru-1,6-P_2 in the wild-type and mutant enzymes indicate that the mutant may adopt a conformation such that effector binding can influence the other subunits in the tetramer without the requirement for structural changes to be induced by other ligands. It should also be noted that the fluorescence measurements (K_S) were done in the absence of potassium, which is known to play a role in the orientation and ligation of substrates at the active site. The true K_S values in the presence of potassium may differ. The equilibrium data suggest that the type and extent of inter- and intra-molecular rearrangements necessary for the T to R transition are different in the mutant and wild-type. From a comparison of the structures of E.coli pyruvate kinase in the T state (Mattevi et al., 1995) and M1 pyruvate kinase in the R state (Larsen et al., 1994; Allen and Muirhead, 1996), it can be seen that the Ca2 helices are further apart in the T structure than in the R structure. The Ser384Pro mutation would distort the Ca2 helices. This distortion would destabilize the R state but could be accommodated in the T state.

It should be noted that K_{0.5} is a complex parameter depending on several interactions with and between ligands, some of which may involve significant conformational changes in the enzyme. It is dependent upon the complete reaction mechanism, and the relationship between K_{0.5} and K_S will depend upon the rate constants in the complex reaction mechanism.

Effects of cations and pH
The activity of the wild-type and mutant pyruvate kinases in the presence of different monovalent and bivalent cations was examined. The effect of Fru-1,6-P_2 on the activity of the enzyme stimulated by various cations was tested. In the absence of Fru-1,6-P_2, the order of activation of the wild-type enzyme by monovalent cations was NH_4^+ > K^+ = Rb^+ >> Cs^+ > Na^+ = Li^+. In the presence of saturating Fru-1,6-P_2 the order of activation was changed to K^+ > Rb^+ = NH_4^+ > Cs^+ > Na^+ = Li^+. The order of bivalent cation activation of the wild-type enzyme in the absence of Fru-1,6-P_2 was
Conformational changes in pyruvate kinase

Table III. Effect of ligands on the susceptibility of wild-type and mutant pyruvate kinase to inhibition by NEM

<table>
<thead>
<tr>
<th>Addition</th>
<th>Wild-type</th>
<th></th>
<th>Mutant</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{50} ) (min)</td>
<td>Fold</td>
<td>( t_{50} ) (min)</td>
<td>Fold</td>
</tr>
<tr>
<td>None</td>
<td>8.8</td>
<td>1.0</td>
<td>6.9</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP</td>
<td>23.1</td>
<td>2.6</td>
<td>14.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Fru-1,6-P₂</td>
<td>32.3</td>
<td>3.7</td>
<td>8.0</td>
<td>1.2</td>
</tr>
<tr>
<td>PEP</td>
<td>60.0</td>
<td>6.8</td>
<td>15.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\( \text{Mg}^{2+} = \text{Mn}^{2+} > \text{Co}^{2+} >> \text{Ca}^{2+} = \text{Ba}^{2+} \). In the presence of saturating concentrations of Fru-1,6-P₂ the order of activation became \( \text{Mg}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} >> \text{Ca}^{2+} = \text{Ba}^{2+} \). In the absence of the allosteric effector monovalent or bivalent cations could not stimulate the mutant enzyme. In the presence of saturating concentrations of Fru-1,6-P₂ the ability of monovalent cations to stimulate the enzyme was \( \text{K}^+ = \text{NH}_4^+ > \text{Rb}^+ >> \text{Cs}^+ = \text{Na}^+ = \text{Li}^+ \). Similarly, in the presence of effector the ability of the bivalent cations to stimulate the mutant enzyme decreased in the order \( \text{Mn}^{2+} = \text{Mg}^{2+} = \text{Co}^{2+} >> \text{Ca}^{2+} = \text{Ba}^{2+} \).

The effect of pH on the activity of the wild-type and

![Fig. 4. Effect of pH on the activity of the wild-type and mutant pyruvate kinase enzymes in the presence and absence of Fru-1,6-P₂. (a) Wild-type and (b) mutant enzymes were assayed as described in Materials and methods. Above pH 7.0 Tris-HCl buffers were used. Below pH 7.0, Mes-TPA buffers were used. Measurements were done in the absence (■) or presence (●) of Fru-1,6-P₂.](https://academic.oup.com/peds/article-abstract/9/12/1203/1601461)

![Fig. 5. Effect of trypsin on various ligated forms of pyruvate kinase. (a) Wild-type and (b) mutant pyruvate kinase. (■) No trypsin; (●) ADP; (▲) PEP; (○) unligated; (▼) FBP. Full experimental details are described in Materials and methods.](https://academic.oup.com/peds/article-abstract/9/12/1203/1601461)
mutant enzymes in the presence and absence of saturating concentrations of Fru-1,6-P_2 is shown in Figure 4. The effect of Fru-1,6-P_2 on the wild-type enzyme is to increase the activity of the enzyme at higher pH. In the absence of the effector the mutant enzyme displays only 8% of the activity of the wild-type enzyme at pH 6.5. The effector-activated mutant shows a 61% higher activity than the wild-type under identical conditions. Both effector-activated enzymes show maximum activity at pH 6.5. The effect of Fru-1,6-P_2 on the mutant enzyme is more profound and has been discussed more fully elsewhere (Collins et al., 1995).

The pH profile and cation activation data for the wild-type and mutant enzymes in the presence and absence of Fru-1,6-P_2 suggest that the active site conformations of the two enzymes are different. This is supported on comparing the k_cat values (Table I). Previous studies indicated that the Ser384Pro mutation stabilized the T quaternary structure (or destabilized the R state), which is consistent with the observed differences (Collins et al., 1995).

Trypsin digestion studies

The effect of the various ligands on the protection of the wild-type and mutant forms of pyruvate kinase from trypsin digestion are shown in Figure 5. The times required for the wild-type and mutant enzymes to lose 50% of their activities (t_50) during trypsin digestion are given in Table II. These data show that ADP and PEP, binding alone to the wild-type enzyme, protect the wild-type enzyme to comparable extents (Table II), suggesting that the proximity of substrate binding in the active site induces similar conformational changes and hence comparable levels of protection from trypsin. ADP and PEP also protect the mutant enzyme from trypsin to similar extents (Table II), although the degree of protection is not as great as with the wild-type enzyme, indicating that these singly-ligated enzymes have slightly different conformations. The protective effect indicates that the T state is more susceptible than the R state. The effector Fru-1,6-P_2 offers no protection at all, and may even enhance the degradation of the enzyme (Table II), indicating that the effector-ligated enzymes have significantly different conformations from the substrate-ligated enzymes. This supports the fluorescence data (Figures 2 and 3) and the kinetic and equilibrium parameters (Table I). Fru-1,6-P_2 causes a large conformational change as measured by tryptophan environment (Kuczenski and Sueltzer, 1970; Fell et al., 1974; Kapoor, 1975).

The amino acid sequence of yeast pyruvate kinase (Burke et al., 1983; McNally et al., 1989) shows that there are 62 trypsin cleavage sites per subunit. Not all sites are equally accessible, so cleavage at just a few critical sites would be all that is required to inactivate the enzyme. SDS-PAGE analysis of the resulting digest mixtures showed no clear bands and was thus inconclusive in identifying critical residues from the size of the peptides produced (data not shown).

Inhibition by NEM

The effect of the various ligands on the sensitivity of the wild-type and mutant pyruvate kinase enzymes to inhibition by NEM is shown in Figure 6. The times required for the two enzyme forms to lose 50% of their activities (t_50) in the presence of NEM is given in Table III. The ligands PEP, Fru-1,6-P_2 and ADP binding alone to the the wild-type enzyme all enhance resistance to NEM inhibition (6.8-, 3.7- and 2.6-fold increase, respectively) compared with the unliganded enzyme. ADP binding induces similar small conformational changes in both the wild-type and mutant enzymes. This is seen in the comparable level of protection of both enzymes from NEM compared with the unliganded enzyme (Table III), and the similar maximum fluorescence quenching observed in the presence of ADP (Figures 2 and 3). PEP binding induces different, probably more widespread conformational changes in the wild-type than in the mutant, as seen in the increased protection from NEM in the PEP-ligated wild-type enzyme and the decrease in maximum fluorescence quenching compared with the mutant (Figures 2 and 3). By contrast, these same ligands have little or no effect in protecting the mutant enzyme from NEM inhibition (2.2-, 1.2- and 2.1-fold increase in t_50, respectively).

Thermal denaturation

The effect of heating on the wild-type, mutant and rabbit muscle enzymes is shown in Figure 7. The temperature at which the enzymes lose 50% of their activity upon heating (t_50) was determined. The rabbit muscle enzyme was found to be highly resistant to thermal denaturation (t_50 = 58 ± 2°C). The wild-type yeast enzyme had a t_50 of 42 ± 1°C, whilst the mutant yeast enzyme had a t_50 of 37 ± 1°C. Hence the mutation has only a slight effect on the thermal stability of the enzyme. Thermal denaturation is likely a multi-step phenomenon that is poorly understood. The conformational changes induced by only small alterations in structure may well be sufficient to account for the observed differences in catalytic activity and susceptibility to trypsin and NEM.

Circular dichroism spectroscopy

The far-UV CD spectra of the effector-activated wild-type and mutant pyruvate kinase enzymes are shown in Figure 8. The amount of secondary structure of the enzymes, as determined by spectral analysis, is virtually identical (data not shown), indicating that the two enzymes adopt a similar overall structure in solution. Addition of PEP and ADP to the wild-type or mutant enzyme did not affect the CD spectra compared with the unliganded enzymes (data not shown).

Conclusions

The determination of K_S and K_{0.5} values, and the other data presented, allow a simplified minimum reaction mechanism for the enzyme to be proposed. The important point is that the T state has both a lower affinity and lower V_max than the R state, i.e. it is both a V and a K system, where the T state is catalytically incompetent. Binding of substrates to the T state is non-cooperative (n_H = 1) and gives a modified T structure. This is followed by a cooperative conformational change, which is pH sensitive, to a ligand-bound R state. Catalysis then occurs with one of the catalytic steps, perhaps product release, being rate-limiting.

Binding of Fru-1,6-P_2 is cooperative and induces a structural transition to an R state which is kinetically identical with the R-state induced by substrate binding but which has a slightly different conformation.

The mutant is locked in the T state in the absence of Fru-1,6-P_2. Fluorescence data show that the mutant enzyme is capable of binding substrates with high affinity but remains catalytically incompetent. This suggests that the T and R states differ both in their affinity for substrates and in their k_cat values.
Conformational changes in pyruvate kinase

Fig. 6. Effect of NEM on various ligated forms of pyruvate kinase. (a) Wild-type and (b) mutant pyruvate kinase. (□) No NEM; (•) ADP; (▲) PEP; (●) unligated; (▼) FBP. Full experimental details are described in Materials and methods.

Fig. 7. Thermal denaturation of pyruvate kinase enzymes. Yeast wild-type (■, WT), Ser384Pro mutant (●, S384P) and rabbit muscle (▲, PKrb) pyruvate kinase were heated at the indicated temperatures. The residual activity of each enzyme is indicated as a percentage of its activity at 28°C. Full assay details are given in Materials and methods.

Fig. 8. Far-UV CD spectra of wild-type (WT) and mutant (S384P) pyruvate kinase in the presence of 5 mM Fru-1,6-P₂. Wild-type (0.137 mg/ml, continuous line) or mutant (0.20 mg/ml, dashed line) protein in 20 mM Tris-HCl (pH 8.5), 20% (v/v) glycerol, 3 mM MgCl₂, 1 mM DTT and 5 mM Fru-1,6-P₂. The mean ellipticity (θ) (degrees cm²/dmol) as a function of wavelength (nm) is recorded.

Taken together, the fluorescence, NEM inhibition, trypsin digestion and CD spectra data support the case that substrate binding causes conformational changes resulting in the exposure or shielding of residues susceptible to modification. Fru-1,6-P₂-induced changes are demonstrably different from substrate-induced changes and indicate a fundamental difference in the conformation of the enzymes when this ligand is bound. Conformational equilibrium is influenced by intersubunit mutations far from the active site. In yeast pyruvate kinase changes in both $K_s$ and $V_{max}$ accompany conformational changes, i.e. it is both a $V$ and a $K$ system.

Acknowledgements

The authors thank Dr G.L. Atkins, Dr D.J. Rigden and Dr A.R. Clarke for advice and assistance with the interpretation of kinetic data. R.A.C. was funded by SERC/Faculty of Medicine, University of Edinburgh, and was in receipt of a William Thynne Centenary Fellowship award.

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Received January 16, 1996; revised April 10, 1996; accepted April 18, 1996