A subunit interface mutant of yeast pyruvate kinase requires the allosteric activator fructose 1,6-bisphosphate for activity

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A variant form of yeast pyruvate kinase (EC 2.7.1.40) with Ser-384 mutated to proline has been engineered in order to study the allosteric properties of this enzyme. Both the mutant and wild-type enzymes were overexpressed in a strain of yeast in which the genomic copy of the pyruvate kinase gene had been disrupted by an insertion of the *Ura3* gene. Both enzymes were purified to homogeneity and their kinetic properties characterized. The wild-type enzyme displays sigmoid kinetics with respect to phosphoenolpyruvate (PEP) concentration, and is activated by the allosteric effector fructose 1,6-bisphosphate with concomitant reduction in co-operativity. In contrast, the mutant was found to be dependent on the presence of the effector for catalytic activity and was inactive in its absence. The fully activated mutant enzyme had a k_{cat} 1.6 times greater than that of the wild-type enzyme. The mutation introduced into the enzyme is in an intersubunit contact which is known to be critical for the allosteric properties of the enzyme, and is far removed from the active site.

The major effect of the mutation seems to be to stabilize the low-affinity T state of the apoenzyme, although k_{cat} is also affected. The $S_{0.5}$ for PEP and $S_{0.5}$ for ADP of the wild-type enzyme were 0.22 ± 0.004 and 0.15 ± 0.01 mM respectively (means $\pm$ S.E.M.). In the activated mutant enzyme, these kinetic parameters increased to 0.67 ± 0.03 and 0.43 ± 0.03 mM respectively. The co-operativity between ADP-binding sites was altered in the mutant enzyme, with the Hill coefficient (h) for ADP increasing to 1.65 ± 0.07 in the presence of the effector, compared with a value of 1.01 ± 0.07 for the wild-type enzyme under the same conditions. CD spectroscopy revealed the secondary structure of the mutant enzyme to be little different from that of the wild-type enzyme, indicating that the two enzymes have similar secondary structures in solution. Precise tertiary and quaternary structures such as intersubunit and interdomain interactions may be modified. An improved purification procedure has been devised that allows large quantities of enzyme to be rapidly prepared.

INTRODUCTION

Pyruvate kinase (EC 2.7.1.40; ATP–pyruvate 2-*O*-phosphotransferase) is a glycolytic enzyme that plays an important role in regulating the flux from Fru-1,6- P_2 to pyruvate. The enzyme has been isolated and characterized from a number of organisms, and is usually found as a homotetramer, with a subunit M_r of 55000–60000. It catalyses the essentially irreversible reaction:

where PEP is phosphoenolpyruvate.

In vertebrates the enzyme exists in four distinct isoenzymic forms which are expressed in a tissue-specific manner reflecting the different metabolic requirements of the tissues. The M1 isoenzyme is found predominantly in skeletal muscle, is essentially unregulated and displays hyperbolic saturation kinetics with respect to the substrate PEP. The M2 isoenzyme is widely distributed in vertebrate tissues. The R and L isoenzymes are found in erythrocytes and liver respectively. All these isoenzymes display sigmoidal kinetics with respect to the substrate PEP. The R and L isoenzymes can also be phosphorylated, with concomitant loss of activity, at a specific serine residue near the N-terminus of the polypeptide. The isoenzymes M2, R and L are all allosterically regulated by a number of non-substrate molecules, the most important of which is Fru-1,6- P_2 . All forms of the enzyme require both univalent and bivalent cations for activity. The former is normally K^+ , whereas a number of different

bivalent cations will suffice. Two bivalent cations are required per active site. One is primarily associated with the nucleotide and the other is more closely associated with the enzyme (see ref. [1] for a review).

The three-dimensional structure of the cat muscle M1 isoenzyme has been solved to a resolution of 0.26 nm [2]. Each subunit of the enzyme consists of four domains, N, A, B and C. Domain A is an eight-stranded α - β barrel, typical of a number of enzymes [3]. The active site lies in a cleft between domains A and B. A putative effector site has been identified and is situated between domains A and C.

The M1 and M2 isoenzymes are produced from the same gene transcript by alternative mRNA splicing [4]. The M gene contains two adjacent exons, one coding for residues 380–435 in M1, and the other coding for the corresponding residues in M2. Apart from this region coded by alternative exons, the sequences in M1 and M2 are identical. The differences between M1 and M2 are located solely in the C domain, and more specifically, the differences occur within two α -helices ($\alpha 1$ and $\alpha 2$) that form important intersubunit contacts (Figure 1a). The M1 and M2 isoenzymes differ strikingly with regard to allosteric properties, M1 being predominantly in the high-affinity R state whereas M2 shows sigmoidal kinetics suggesting that the unligated protein is predominantly in the T state [5]. The two states could well differ in the packing of the subunits at this interface.

The enzyme from *Saccharomyces cerevisiae* has been isolated and characterized by a number of workers, and its kinetic and regulatory behaviour are well documented [6–8]. The yeast

Abbreviations used: DCIC, 3,4-dichloroisocoumarin; DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane; Fru-1,6- P_2 , fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate.

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Figure 1 Structure of yeast pyruvate kinase

The structure was obtained by modelling the yeast pyruvate kinase sequence on the three-dimensional co-ordinates determined for the cat muscle enzyme [2]. (a) Ribbon representation of the yeast pyruvate kinase tetramer with the important C-domain α -helices and the active sites indicated in two subunits. (b) Close-up diagram of the $C\alpha 1/C\alpha 2$ interface with space-filling representation of the two symmetry-related Ser-384 side chains on helix $C\alpha 2$. Helix $C\alpha 1$ lies nearly anti-parallel to helix $C\alpha 2$.

pyruvate kinase gene has been cloned and sequenced [9,10], the wild-type and site-directed mutant forms of the enzyme have been overexpressed, purified and characterized from a yeast strain in which the genomic copy of the gene had been disrupted by an insertion of the *Ura3* gene, thus yielding an enzyme preparation uncontaminated with constitutive wild-type enzyme [11].

Yeast pyruvate kinase appears to be kinetically most similar to the vertebrate M2 isoenzyme. The sequence of the yeast enzyme has been modelled on the co-ordinates of the cat muscle M1 enzyme obtained from X-ray crystallographic studies [12]. From an analysis of this computer model it was observed that Ser-384, present in the middle of helix $C\alpha 2$, contributed significantly to the important intersubunit contact zone. The observed kinetic behaviour is compatible with a mechanism whereby the pyruvate kinase molecule acts as a dimer of dimers with two concerted steps in a heterotropic co-operative transition [13,14], where Ser-384 of one subunit lies in apposition to the equivalent residue of its neighbouring subunit (Figure 1b). Thus the effect of any mutation involving Ser-384 would affect the relative stability of the R and T quaternary structures and the effect would be enhanced as a result of the proximity of equivalent residues.

Ser-384 was replaced by a proline by site-directed mutagenesis [15,16]. This substitution was predicted to have a pronounced effect on the α -helix into which it was introduced, and was hoped to have measurable effects on the interaction of the enzyme with its substrates and allosteric effectors. This paper describes the

purification and characterization of this mutant form of yeast pyruvate kinase. The wild-type enzyme was used to optimize the purification protocol, and its physical and kinetic properties are compared with those of the mutant enzyme prepared under identical conditions.

EXPERIMENTAL

Materials

Amino acids were obtained from BDH Laboratory Supplies, Lutterworth, Leics., U.K. Rabbit muscle lactate dehydrogenase and pyruvate kinase were obtained from Boehringer-Mannheim UK, Lewes, East Sussex, U.K. The di(monocyclohexylammonium) salt of ADP, the mono(cyclohexylammonium) salt of PEP, the disodium salt of NADH and the trisodium salt of Fru-1,6- P_2 were obtained from Sigma, Poole, Dorset, U.K. Sephacryl S200 was from Pharmacia Biosystems, Milton Keynes, Beds, U.K. QA-52 anion exchanger was from Whatman Laboratory Division, Maidstone, Kent, U.K. Benzamidine hydrochloride, PMSF, 3,4-dichloroisocoumarin (DCIC) and *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E64) were from Sigma. 1,10-Phenanthroline was from Aldrich. All other reagents were reagent grade and obtained from either Sigma or BDH. Plasmid pMA91pyk was generously given by Dr. J. Mellor, Department of Biochemistry, University of Oxford, Oxford, U.K.

Yeast strains

The yeast strains used were *S. cerevisiae* SF747: *leu2, ura3, trp1, gal10, mata* and *S. cerevisiae* PYK1: :URA3 which is the same as SF747, but with disruption of the *PYK1* gene by the insertion of *URA3*, thus abolishing expression of the *PYK1* gene.

Transformation with plasmid DNA

Transformation of yeast with supercoiled plasmid DNA was by the lithium acetate pretreatment method of Ito et al. [17], with the following modifications. The yeast growth medium contained 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, East Molesey, Surrey, U.K.), 5.0 g/l $(\text{NH}_4)_2\text{SO}_4$, 20 g/l carbon source [D-glucose or 2% (v/v) ethanol plus 20 g/l glycerol], 20 mg/l L-tryptophan, 24 mg/l L-histidine, L-arginine and L-methionine, 36 mg/l L-tyrosine and L-lysine, 50 mg/l adenine, 60 mg/l L-leucine and L-phenylalanine, 120 mg/l L-aspartate and L-glutamate, 180 mg/l L-valine, 240 mg/l L-threonine and 450 mg/l L-serine. The amino acids were in the form of the free acid or base. Glycerol and D-glucose were autoclaved with the other components. The ethanol was filter-sterilized and added aseptically after the medium had cooled. The amino acids were stored as 100× concentrated stock at -20°C . The pyruvate kinase-deficient yeast strain was grown in uracil-deficient selection medium with ethanol and glycerol as carbon sources. Transformed yeast was grown in uracil- and leucine-deficient medium with D-glucose as the carbon source for the wild-type and mutant pyruvate kinases. The pyruvate kinase-deficient yeast strain was prepared as described previously [11,15]. For the purposes of expression of pyruvate kinase mutants, it would have been preferable to produce a deleted rather than a disrupted pyruvate kinase-deficient yeast strain in order to prevent unwanted recombination. However, repeated attempts at producing such a mutant have failed [11]. The pyruvate kinase gene (*PYK1*) has also been identified as the locus *cdc19*, and so may have some function in growth and cell division, which helps explain the difficulty of producing viable strains deleted in *PYK1*.

Mutagenesis of yeast pyruvate kinase

The Ser-384 residue was mutated to a proline by oligonucleotide-directed site-directed mutagenesis using the method of Kunkel [18], as described previously [10]. The mutated gene was sequenced in its entirety to ensure that no spurious mutations had been introduced.

Yeast pyruvate kinase assay

Pyruvate kinase was assayed spectrophotometrically by the method of Bücher and Pfeleiderer [19] with the following modifications. Assays were carried out in a 1-cm-pathlength quartz cuvette in a total volume of 1 ml at 25°C , and mixtures contained 0.5 pmol of pyruvate kinase, 1.1 units of lactate dehydrogenase, 0.15 μmol of NADH, 15 μmol of MgCl_2 and 50 μmol of Mes/tetrapropylammonium, pH 6.5. The standard assay mixture contained 2.4 μmol of ADP, 5.6 μmol of PEP and 100 μmol of KCl. The $S_{0.5}$ value for PEP was determined by varying the amount of PEP between 0 and 5.5 μmol , and maintaining ADP constant at 2.4 μmol and KCl at 100 μmol . The reaction was started by adding ADP to pyruvate kinase and the other assay components after equilibration for 3 min. The $S_{0.5}$ value for ADP was determined by varying the amount of ADP between 0 and 1.6 μmol , and maintaining PEP constant at 5.6 μmol and KCl at 100 μmol . The reaction was started by adding PEP to pyruvate kinase and the other assay components after equilibration for 3 min. Fru-1,6- P_2 was present at 5 μmol where

indicated. The progress of the reaction was followed by monitoring the loss of 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 of NADH/min under the conditions described above.

Determination of protein concentration

The concentration of the pure yeast pyruvate kinase was determined spectrophotometrically using an A_{280} of 0.51 for a 1 mg/ml solution [20]. Protein concentration during purification was measured by the dye-binding method of Bradford [21] with BSA as standard.

Growth and harvesting of yeast overexpressing pyruvate kinase

On day 1, 10 ml samples of appropriate selection media (one for each litre of yeast culture required) were inoculated with a single colony of transformed yeast and grown for 24 h (wild-type) or 48 h (S384P mutant) at 30°C in a shaking incubator (200 rev./min). The starter cultures were then added to a 1-litre volume of the same medium and grown until A_{600} reached 1.0–1.5. There is strong evidence that yeast pyruvate kinase is cold-labile, and so all purification steps were performed at room temperature [8]. The yeast cells were harvested by centrifugation (2400 g for 10 min; r_{av} 8.6 cm). The cells were pooled, washed once by resuspension in lysis buffer, and then centrifuged as before. Lysis buffer comprised 50 mM Tris/HCl, pH 7.5, 3 mM MgCl_2 , 1 mM dithiothreitol (DTT) and 20% (v/v) glycerol. The harvested cells were used immediately.

Yeast cell lysis

The harvested cells were resuspended in a 250 ml-capacity centrifuge pot with 10 ml lysis buffer. To this cell suspension were added protease inhibitors to the following concentration: benzamidine hydrochloride (1 mM), PMSF (0.1 mM), DCIC (0.1 mM), E64 (0.1 mM) and 1,10-phenanthroline (0.1 mM). Sufficient 0.2 mm-diameter glass beads were added to the suspension to form a thick slurry. The slurry was stirred from overhead, with a Teflon pestle mounted in a variable-speed power drill, for 6 min at 600 rev./min. This procedure was performed twice with a 5 min incubation on ice between treatments. After lysis, the glass beads were washed with 200 ml of lysis buffer and centrifuged (2400 g for 20 min; r_{av} 8.6 cm) to remove cell debris and extraneous glass beads. The supernatant from this step constituted the cell-free extract (CE in Table 1). A sample of the pellet from this step was examined under an optical microscope at 400× magnification to assess the extent of cell lysis. If insufficient lysis had occurred, the pellet was resuspended as before and lysed again according to the procedure outlined above. Usually only one lysis step was necessary.

$(\text{NH}_4)_2\text{SO}_4$ fractionation

To the cell-free extract was added sufficient finely divided solid $(\text{NH}_4)_2\text{SO}_4$ to bring the solution to 40% (w/v) satn. The solution was stirred at room temperature for 2 h before being centrifuged (14000 g for 30 min; r_{av} 7.0 cm). The resulting pellet (40P) was discarded. Further solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant (40S) to bring it to 70% (w/v) satn. The solution was stirred and centrifuged exactly as before. The resulting pellet (70P) contained approx. 90% of the pyruvate kinase activity. The supernatant (70S) was discarded.

Gel filtration

The pellet from the second $(\text{NH}_4)_2\text{SO}_4$ precipitation (70P) was resuspended in 5 ml of lysis buffer and applied to a Sephacryl S200 gel-filtration column (90 cm $\times$ 1.6 cm) equilibrated in lysis buffer, to which had been added NaN_3 to 0.04 % (w/v). The column was eluted with the same buffer, and the flow rate was kept constant at 20 ml/h with a Gilson Micropuls 2 peristaltic pump. The pyruvate kinase activity was eluted just after the void volume of the column. Fractions containing pyruvate kinase activity were pooled (S200) and the enzyme was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 75 % (w/v) satn.

Anion-exchange chromatography

The surface of the pellet from the gel-filtration step was rinsed gently with a small volume of anion-exchange buffer [20 mM Tris/HCl, pH 8.5, 3 mM MgCl_2 , 1 mM DTT, 20 % (v/v) glycerol] to lower the concentration of $(\text{NH}_4)_2\text{SO}_4$. The pellet was then resuspended in 5 ml of anion-exchange buffer and applied to a QA-52 anion-exchange column (10 cm $\times$ 2.7 cm) equilibrated with identical buffer. The column was eluted under gravity with the same buffer. The flow rate was constant at 30 ml/h. Contaminants were adsorbed to the matrix and the pyruvate kinase was eluted just after the void volume of the column. Fractions containing pyruvate kinase activity were pooled (QA-52) and the enzyme was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 75 % (w/v) satn. The enzyme from this step was over 95 % pure as determined by SDS/PAGE and densitometric analysis of the resultant gel.

Electrophoresis

Samples of protein from the various stages of purification were analysed by PAGE in the presence of SDS [22]. The gel contained 10 % (w/v) acrylamide monomer. The degree of cross-linking, expressed as a percentage (w/w) of methylenebisacrylamide, was 6.67 %.

CD spectroscopy

Far-UV CD spectra of the wild-type and mutant enzymes were obtained at the Scottish CD Facility, University of Stirling, Stirling, Scotland, U.K. with the assistance of Dr. N. C. Price and Dr. S. M. Kelly. A Jasco J600 spectropolarimeter was used. The data were collected and analysed by the associated software. Spectra were obtained in the region 190–260 nm at a scan speed of 10 nm/min. Measurements were made at 25 °C in a 1.2 ml-capacity quartz cuvette with an effective pathlength of 0.02 cm. The secondary-structure content was calculated using the program CONTIN [23] using a mean residue M_r of 109.3. The buffer contained 20 mM Tris/HCl, pH 8.5, 3 mM MgCl_2 , 1 mM DTT and 20 % (v/v) glycerol. Protein concentration was in the range 0.1–0.2 mg/ml.

RESULTS AND DISCUSSION

Expression of yeast pyruvate kinase

Plasmid pMA91pyk is a yeast/*Escherichia coli* shuttle vector bearing the yeast pyruvate kinase gene (*PYK1*) under the control of the yeast phosphoglycerate kinase promoter [24]. Yeast strain *PYK1::URA3* was transformed with plasmid pMA91pyk and the resultant transformants expressed yeast pyruvate kinase to very high levels (see Figure 2). The transformants also recovered the ability to grow on D-glucose as carbon source, demonstrating

Table 1 Purification of wild-type pyruvate kinase

Values presented are for a typical preparation from 1 litre of culture. Yeast cells were grown on selective media as described in the Materials and methods section. CE, cell-free extract; 40S, supernatant from 40 % (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; 70P, pellet obtained from 70 % (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; S200, pooled gel-filtration fractions; QA-52, pooled anion-exchange fractions.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity	Yield (%)	Fold
CE	192	1.8	346	51	17 650	100	1.0
40S	213	1.5	320	56	17 920	100	1.1
70P	7.5	10	75	94	7 050	40	1.8
S200	6.0	3.0	18	245	4 410	25	4.8
QA-52	5.5	1.5	8.3	325	2 670	15	6.4

that the highly expressed wild-type and S384P mutant pyruvate kinases were active *in vivo*.

Purification of yeast pyruvate kinase

The purification protocol was optimized for overexpressed wild-type pyruvate kinase. The mutant was subsequently purified under identical conditions. Table 1 presents a typical purification of wild-type yeast pyruvate kinase from 1 litre of cultured overexpressing yeast. A similar range of values was obtained for the S384P mutant-overexpressing cells.

Figure 2a shows the results of SDS/PAGE at each stage of the purification. On comparing lanes 1 and 2, the supernatant from the 40 % (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation (40S) does not have a significantly different profile from that of the cell-free extract. This suggests that few soluble proteins have been removed by this treatment. However, a large pellet was precipitated by this procedure (40P) and this suggests that insoluble matter, including cell-wall fragments, intracellular organelles and nucleic acids, have been removed. The 40P profile (Figure 2a, lane 3) shows high background staining but few specific bands and, most importantly, very little protein in the region corresponding to the position of pyruvate kinase. The further addition of $(\text{NH}_4)_2\text{SO}_4$ to the 40S fraction to bring it to 70 % (w/v) saturation results in the efficient precipitation of the majority of pyruvate kinase. A large proportion of the other proteins remain in the supernatant (70S, lane 4). In preliminary experiments it was found that significant pyruvate kinase activity remained in the supernatant from a 60 % (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation. The pellet from a 60 % (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation has been used by other workers (e.g. Murcott et al. [11]) as the material for further purification steps.

Figure 3(a) shows the elution profile of wild-type yeast pyruvate kinase from the Sephacryl S200 column. Enzyme activity is eluted just after the void volume of the column. The column matrix excludes proteins with M_r greater than 200 000 and so this value can be assigned as the minimum size of the enzyme. This is consistent with the enzyme being a homotetramer of subunit M_r 54 545 [10]. The S384P mutant enzyme was also eluted from the gel-filtration column just after the void volume. This indicates that, under the conditions of purification, this form of the enzyme exists as an oligomer, presumably also a tetramer. The presence or absence of the effector Fru-1,6- P_2 during the purification does not affect the elution position, and hence does not cause a change in the dimer/tetramer equilibrium. This is in contrast with the mammalian M2 isoenzyme, which associates to tetramers in the presence of Fru-1,6- P_2 [25].

Figure 2 SDS/PAGE showing the stages of purification of wild-type pyruvate kinase

(a) Lane 1, cell-free extract; lane 2, supernatant from 40% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 3, pellet from 40% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 4, supernatant from 70% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 5, pellet from 70% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 6, pooled gel-filtration fractions; lane 7, pooled anion-exchange fractions; lane 8, commercially available preparation of rabbit muscle pyruvate kinase. Leaders indicate size and position of M_r standards analysed under identical conditions: rabbit muscle myosin, M_r 205 000; *E. coli* β -galactosidase, M_r 116 000; rabbit muscle phosphorylase *b*, M_r 97 000; BSA, M_r 66 000; egg ovalbumin, M_r 45 000; bovine carbonic anhydrase, M_r 29 000. (b) Densitometric trace of lane 7; density is shown in arbitrary units.

Figure 3(b) presents the elution profile of wild-type pyruvate kinase from the anion-exchange column. The enzyme is eluted just after the break-through volume, which is surprising as its isoelectric point is below 8.5 [20]. The unusual binding properties of pyruvate kinase to ion-exchange columns is not an isolated phenomenon [26]. The calculated pI for yeast pyruvate kinase determined using the ISOELECTRIC POINT program [27] is 7.66. It is possible that several normally charged residues are obscured and are not available to undergo charge interaction with the ion-exchange matrix, leading to the anomalous binding behaviour observed. The S384P mutant enzyme displayed an identical elution profile from the anion-exchange column indicating that the size and charge distribution of this form of the protein is largely similar to that of the wild-type.

A densitometric scan of lane 7 of the SDS/polyacrylamide gel shown in Figure 2(a) revealed that the protein eluted from the anion-exchange column was more than 95% pure (Figure 2b). A similar degree of purity was observed in preparations of the S384P mutant enzyme. The reason for the slight lack of co-

Figure 3 Elution profile of wild-type pyruvate kinase from gel-filtration and anion-exchange columns

Wild-type enzyme was purified as described in the text. The mutant enzyme was purified and isolated in an identical manner. Pyruvate kinase was assayed as described in the Materials and methods section. (a) Elution of wild-type pyruvate kinase from Sephacryl S200 gel-filtration column. Conditions were as described in the Materials and methods section. (b) Elution of wild-type pyruvate kinase from Whatman QA-52 anion-exchange column. Conditions were as described in the Materials and methods section. $\square$, A_{280} ; $\blacklozenge$, percentage of maximum activity, expressed relative to fraction 7 which had an activity of 86 μmol of NADH consumed/min.

incidence between the protein and enzyme activity peaks in Figure 3(b) is not known, but may relate to injudicious pooling of the eluted material.

This revised purification protocol is an improvement on previous methods. The speed of the new method allowed pure protein to be purified in under 36 h after cell lysis. The yield of pure enzyme obtained from 1 litre of cultured cells was in the range 10–15 mg. The revised protocol was highly reproducible and has consistently produced high-quality enzyme preparations. The convenience of the new method lies in the small number of purification steps and different buffers which ensured speed and stability of the preparation. The enzyme prepared in the manner described remained active for at least 1 month when stored as a 75% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ suspension at room temperature, and remained active for up to 14 days at room temperature when stored in a sealed sterile glass test tube in anion-exchange buffer.

Figure 4 Kinetic data from wild-type and mutant pyruvate kinases

Enzyme assays were carried out as described in the text. The data were fitted to the Hill equation, and the resulting fitted curves are shown. Results are means $\pm$ S.E.M. for two determinations. Values of $S_{0.5}$ and h were derived from these curves and are given in Table 2. (a) Rate of reaction as a function of PEP concentration; (b) rate of reaction as a function of ADP concentration. $\circ$, Wild-type — Fru-1,6- P_2 ; $\triangle$, wild type + Fru-1,6- P_2 ; $\bullet$, mutant S384P + Fru-1,6- P_2 .

Kinetic and spectroscopic properties

It was discovered during the purification process that the mutant enzyme was essentially inactive under standard assay conditions in the absence of the allosteric activator Fru-1,6- P_2 . The mutant enzyme was only fully active in the presence of high concentrations (5 mM) of effector. The serine-to-proline mutation appears to have stabilized the T quaternary structure (or destabilized the R structure). The binding of the effector can overcome this effect although there may be some slight rearrangement of active-site residues as the k_{cat} is somewhat different. As the mutation was intended to disrupt communication across intersubunit contacts, the pronounced disturbance in the mode of action of the effector is, perhaps, not surprising. It has been reported that the pyruvate kinase from the yeast *Saccharomyces carlsbergensis* requires Fru-1,6- P_2 in order to achieve optimum V_{max} values [28]. Unfortunately, the amino acid sequence of the *S. carlsbergensis* enzyme is not known, and so comments on its ability to bind the effector and the mechanism by which it adopts an active conformation in the presence of the effector are limited.

The kinetic data obtained for both the wild-type and S384P mutant enzymes are shown in Figure 4. The parameters derived from these data are summarized in Table 2.

The kinetic data for the wild-type enzyme with PEP as substrate (Figure 4a) suggest that in the absence of the effector a strong positive homotropic interaction between PEP-binding sites occurs ($h = 2.93 \pm 0.07$). This confirms data obtained by other workers (e.g. Murcott et al. [11]). The kinetic data also indicate that the binding of the effector to the wild-type enzyme decreases the $S_{0.5}$ of the enzyme for PEP by a factor of 12 [$S_{0.5}$ (PEP) = 0.22 ± 0.004 mM], and reduces the co-operativity between PEP-binding sites. In the absence of effector the S384P mutant enzyme was inactive and so kinetic data were not obtainable by traditional enzyme assay. In the presence of 5 mM Fru-1,6- P_2 the mutant enzyme displayed a threefold higher $S_{0.5}$ for PEP than the wild-type under the same assay conditions [$S_{0.5}$ (PEP) = 0.67 ± 0.03 mM]. The effector caused a marked reduction in the co-operativity between PEP-binding sites in the wild-type enzyme ($h = 1.72 \pm 0.05$), and a comparable value was obtained for the mutant enzyme ($h = 1.95 \pm 0.04$). The addition of the effector

Table 2 Kinetic parameters of wild-type and mutant pyruvate kinases

—, Values not obtained because of inactivity of mutant enzyme in the absence of the effector. Results are means $\pm$ S.E.M. for two determinations.

Parameter	Wild-type	Mutant
$S_{0.5}$ PEP (— Fru-1,6- P_2) (mM)	2.76 ± 0.08	—
h	2.93 ± 0.07	—
$S_{0.5}$ PEP (+ Fru-1,6- P_2) (mM)	0.22 ± 0.004	0.67 ± 0.03
h	1.72 ± 0.05	1.95 ± 0.04
k_{cat} (— Fru-1,6- P_2) (s^{-1})	188 ± 5	—
k_{cat} (+ Fru-1,6- P_2) (s^{-1})	195 ± 2	314 ± 5
$S_{0.5}$ ADP (— Fru-1,6- P_2) (mM)	0.25 ± 0.006	—
h	1.53 ± 0.02	—
$S_{0.5}$ ADP (+ Fru-1,6- P_2) (mM)	0.15 ± 0.01	0.43 ± 0.03
h	1.01 ± 0.07	1.65 ± 0.07
pH optimum	6.5	6.5

destabilizes the T state and stabilizes the R state. The mutant has a higher $S_{0.5}$ for substrates even in the presence of effector, again suggesting small changes in the active-site pocket. It is perhaps surprising that the mutant enzyme displays a higher k_{cat} value than the wild-type enzyme under the same conditions. It may be that pyruvate kinase has evolved to optimize its regulatory properties at the slight expense of efficiency. The increased k_{cat} of the mutant may indicate that product release is favoured compared with the wild-type enzyme.

The saturation curve of the wild-type enzyme for the substrate ADP in the absence of Fru-1,6- P_2 was sigmoidal with $h = 1.53 \pm 0.02$. In the presence of effector, the plot became hyperbolic and the k_{cat} increased markedly (Figure 4b). Even so, this k_{cat} was less than that achieved by the conditions in Figure 4(a). The assay conditions in Figure 4(b) involved preincubation with ADP, and the reaction was started by the addition of PEP. The reduced k_{cat} value could be a consequence of the preincubation conditions. In the presence of 5 mM Fru-1,6- P_2 the data showed that $S_{0.5}$ for ADP was reduced to 0.15 ± 0.01 mM and h to

Figure 5 Far-UV CD spectra for wild-type and mutant pyruvate kinases

The far-UV CD spectra were collected as described in the text. Protein concentration was 0.137 mg/ml for wild-type and 0.2 mg/ml for the mutant. The spectra were corrected for protein concentration after collection. The mean residue ellipticity $[\theta]$ is shown as a function of wavelength for each enzyme. The spectrum of the wild-type enzyme is given as a continuous line, and that of the mutant as an interrupted line.

1.01 ± 0.07 . Thus the small degree of co-operativity between ADP-binding sites is abolished in the presence of the effector and further supports the notion of an effector-induced conformational change occurring in the wild-type enzyme. The kinetic parameters for the mutant enzyme with ADP as substrate were also determined. As the enzyme was inactive in the absence of effector, no kinetic data could be obtained. The data obtained in the presence of 5 mM effector gave an $S_{0.5}$ for ADP of 0.43 ± 0.03 mM and h of 1.65 ± 0.07 . Thus the point mutation has caused a threefold increase in $S_{0.5}$ for ADP and has increased slightly the co-operativity between ADP-binding sites.

The far-UV CD spectra in Figure 5 indicate that the wild-type and mutant enzyme generally adopt the same secondary structure under the same conditions. The program CONTIN [23] was used to determine the content of α -helix, β -strand and random-coil elements for each enzyme. The small decrease in α -helix content observed (from $29 \pm 0.51\%$ for the wild-type to $20 \pm 1.0\%$ for the mutant) is compatible with the nature of the mutation introduced. Thus any difference in polypeptide conformation between the wild-type and the mutant enzymes sufficient to cause the observed differences in kinetic parameters is likely to be at the interdomain and intersubunit levels. The fact that both forms of the enzyme were eluted from the Sephacryl S200 column near the void volume indicates that both are still tetrameric, and thus the mutation in the subunit contact region has not provoked a dissociation into dimers.

The overexpressed wild-type enzyme displays kinetic parameters typical of constitutively produced enzyme prepared by a number of other workers (e.g. Hunsley and Suelter [29] and Murcott et al. [11]). The mutant enzyme showed no measurable activity in the absence of Fru-1,6- P_2 . Thus the main effect of the

mutation appears to be to stabilize the low-affinity T state, and the allosteric effector is required to bring about the transition to the R state. The observed increase in k_{cat} could indicate changes in active-site architecture and hence rate-limiting step. This interpretation is compatible with independent findings that point mutations in subunit interface regions are capable of disrupting catalysis, intersubunit communication and protein conformation in other enzymes. For example, the dimeric glutathione reductase from *E. coli* has been reported as having k_{cat} reduced by 50%, an increased co-operativity between substrate-binding sites, and an altered thermal stability when the subunit interface residue Gly-418 was mutated to tryptophan [30].

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