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PURIFICATION AND MOLECULAR PROPERTIES OF BOVINE HEART PYRUVATE KINASE

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Summary

A rapid method is presented for the purification of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from bovine heart. The enzyme obtained is homogeneous by criteria of sodium dodecyl sulphate polyacrylamide electrophoresis and ultracentrifugation and has a specific activity of 260 units/mg.

It is a tetramer of 220 000 daltons and $S^0_{20,w}$ = 10.0 S and possesses no free amino-terminal residue. The amino acid composition is similar to that of the M_1 isozyme of rabbit and bovine skeletal muscle. The enzyme is subject to polymerisation to a hexamer of the basic tetramer. The polymeric species has a molecular weight of 1320 000, is promoted at low ionic strength and is undetectable at ionic strength greater than 0.2 by either sedimentation equilibrium or sedimentation velocity measurements. The polymerisation is independent of temperature in the range 5—20°C implying that charge interactions rather than apolar interactions are responsible for the process.

Introduction

Pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) has been previously purified from several animal sources [1—8] and occurs in at least three isozymic forms. These have been designated types M_1, M_2 and L by Imamura and Tanaka [9] and correspond to the forms M, K and L respectively noted by Cardenas et al. [10]. Some naturally occurring hybrids have also been proposed [9,11]. In the rat heart the principle forms encountered are M_1 and M_2 but minor differences may exist between rat and bovine forms, particularly with respect to the effects of regulatory molecules [5,10]. The pyruvate kinase of bovine heart has not been previously purified and although the pig heart enzyme has been purified [2] no detailed account of its molecular properties

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has been given. A form of the enzyme from turtle heart has been purified [12] but the best molecular characterisation of mammalian enzymes has been made for the M_1 and L isozymes from skeletal muscle and liver respectively [6,7].

The present report describes a rapid method for the purification of beef heart pyruvate kinase. The subunit structure and molecular weight of the enzyme have been investigated and it is shown to be a tetramer similar to the M_1 and M_2 isozymes. The tetramer undergoes polymerisation reactions at low ionic strength and a computer analysis of sedimentation equilibrium data reveals that a hexamer of the basic tetramer is the main polymer formed.

Materials and Methods

Reagents. Analytical grade reagents were purchased from British Drug Houses (Poole, England). ADP, phospho*enol*pyruvate, NADH and lactic dehydrogenase (EC 1.1.1.27) were purchased from C.F. Boehringer & Soehne (Mannheim, Germany). Phosphocellulose was a product of Whatman (Maidstone, England). Sephadex G 200 was a product of Pharmacia (Uppsala, Sweden). Marker proteins for SDS electrophoresis came from Sigma Chemical Co. (London).

Protein concentration. During the purification the protein concentration was determined by the method of Warburg and Christian [13]. The concentration of the purified enzyme was obtained from ultraviolet absorbance measurements at 280 nm. Spectra were measured on a Perkin-Elmer 356 spectrophotometer and the molar absorbance was determined by the interferometric method of Babul and Stellwagen [14]. $E^{1\%,1cm}_{280nm}$ was 5.55.

Enzyme assays. Pyruvate kinase was routinely assayed by the procedure described by Bücher and Pfleiderer [15] involving coupling to lactic dehydrogenase. The concentrations of reagents made up in 0.1 M Tris · HCl, pH 7.5, were 0.15 mM NADH, 2.00 mM ADP, 1.15 mM phospho*enol*pyruvate, 10 mM $MgSO_4$, 80 mM KCl and 7.2 units of lactic dehydrogenase in a volume of 1 ml. The assay was started by the addition of pyruvate kinase. An enzyme unit is defined as the quantity of enzyme catalysing the phosphorylation of 1 μmol ADP per minute at 30° C and corresponds to an absorbance change at 340 nm of 6.21 units per minute in the above assay.

Analytical ultracentrifugation. Ultracentrifugation was performed in a Spinco Model E machine equipped with monochromator and photoelectric scanner. Sedimentation velocity measurements were made at 60 000 rev./min and a temperature of 10° C unless otherwise stated. Equilibrium centrifugation was performed according to the low-speed method of Van Holde and Baldwin using 3-mm solution columns [16]. Alternatively the high-speed method of Yphantis was used [17]. Meniscal protein concentration was determined in low-speed experiments as described previously [18]. All data from the photoelectric scanner were plotted as $\log_{10}$ (absorbance) vs. r^2. The baseline for absorbance measurements was determined by over-speeding the rotor and depleting the meniscus of protein at the end of the run. Initial protein concentrations used in conjunction with sedimentation equilibrium data obtained from the Schlieren optical system were determined in synthetic boundary experiments using double sector synthetic boundary cells and meniscal protein

concentration was determined by application of the mass conservation condition to the integrated Schlieren pattern [18]. Initial concentrations used in conjunction with sedimentation velocity measurements were determined by Schlieren integration and extrapolation to zero time. Peak areas were determined by measurement of width at half height [19].

A partial specific volume of 0.74 ml/gm was used in all calculations. Densities and viscosities of solutions were computed from data in International Critical Tables.

Amino acid analysis. 500 μg of enzyme were hydrolysed at 106° C in 3 ml of 6 M HCl containing 10 mM phenol. Hydrolyses were performed for 20, 36 and 72 h respectively in sealed, evacuated tubes. The resulting hydrolysates were analysed on an Eel 136 amino acid analyser. Correction was made for loss of serine and threonine and for increases in the amounts of valine, leucine and isoleucine with time. Cysteine was estimated by reaction with N-ethyl maleimide in the presence of 6 M guanidinium chloride. On hydrolysis the production of *S*-(1,2-dicarboxyethyl)cysteine gave a measure of the cysteine residues present [20].

N-terminal analysis. This was performed according to the method of Grey [21]. 500 μg of protein were performate-oxidised and dissolved in 1% SDS and *N*-ethyl morpholine. The enzyme was treated with dansyl chloride, hydrolysed in 6 M HCl and subjected to two dimensional polyamide layer chromatography. The solvent systems used were:

1st direction: H_2O/90% formic acid (200 : 3 v/v)

2nd direction: Benzene/glacial acetic acid (9 : 1 v/v), 1 M ammonia/ethanol (1 : 1 v/v), 0.05 M trisodium phosphate/ethanol (3 : 1 v/v).

Maleylation of pyruvate kinase. 5 mg of enzyme in 0.2 M K_2HPO_4 was maintained at pH 8.0 in a Radiometer pH-stat by addition of 0.1 M KOH. Maleylation was achieved by addition of 4 mg of solid maleic anhydride at 30° C and the reaction was allowed to proceed until alkali addition had ceased. The enzyme was then dialysed against 0.1 ionic strength phosphate, pH 7.0.

Electrophoresis. Electrophoresis on polyacrylamide and on SDS polyacrylamide gels have been described previously [22]. The molecular weight standards used on SDS polyacrylamide gels were: lysozyme (14 300), chymotrypsinogen (25 700), yeast alcohol dehydrogenase (37 000), ovalbumin (46 000), human serum albumin (66 000) and phosphorylase (100 000).

Purification of pyruvate kinase. The buffers used were:

Buffer 1: 0.05 ionic strength (0.033 M) phosphate, pH 6.5, containing 3.33 g KH_2PO_4 1.49 g K_2HPO_4 and 1.5 g disodium EDTA per l.

Buffer 2: 0.2 ionic strength (0.09 M) phosphate, pH 7.0, containing 4.84 g KH_2PO_4, 9.55 g K_2HPO_4 and 1.5 g disodium EDTA per l.

The purification was carried out at 4° C and all buffers were prechilled.

Stage 1. Homogenisation of bovine heart. 1 kg of heart was chopped and homogenised for 5 min in a Waring blendor in 2 l of a twofold dilution of Buffer 1. The homogenate was then centrifuged at 20 000 × *g* for 20 min. All pyruvate kinase activity was found in the supernatant.

Stage 2. Chromatography on phosphocellulose at pH 6.5. KCl was added to the filtered supernatant from Stage 1 to a concentration of 10 g/l and applied to a column of phosphocellulose (12 × 3 cm) equilibrated with Buffer 1.

The column was washed with 1 l of Buffer 1 containing 10 g KCl and this eluted much of the contaminating protein. The column was further washed with 500 ml Buffer 1 containing 6.25 KCl. This procedure eluted further protein, but the volume of eluant at the higher salt concentration was restricted in order to avoid leakage of pyruvate kinase from the column. Finally the enzyme was eluted by 500 ml of Buffer 1 containing 10 g KCl. This salt concentration was in excess of that needed to elute the enzyme but ensured a small volume of eluate. The first 150 ml of effluent were discarded and the remainder was dialysed, concentrated against Buffer 1 using an Amicon fibre concentrator and applied to a second column of phosphocellulose (5 × 15 cm). A 600 ml linear salt gradient (0—0.4 M KCl) was used to elute the enzyme. The flow rate was 30 ml/h and 5-ml fractions were collected. The enzyme eluted in fractions 70—85.

Stage 3. Gel filtration on Sephadex G 200 at pH 7. The enzyme from Stage 2 was concentrated to 4 ml by vacuum dialysis against Buffer 2 and applied to a Sephadex G 200 column (2.5 × 100 cm) by upward flow. The flow rate was 12.5 ml/h. The column void volume was 170 ml and the enzyme eluted at a volume of 270 ml. 4-ml fractions were collected.

Results

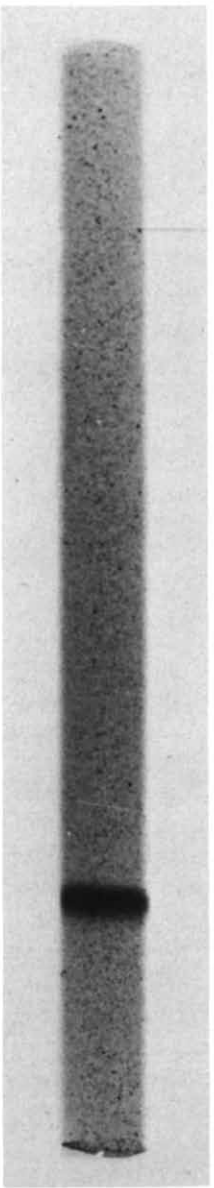
Purification and general properties. The purification procedure described takes less than three days to perform and its course is shown in Table I. The enzyme was homogeneous by criteria of SDS polyacrylamide electrophoresis and ultracentrifugation (Figs. 1 and 2) and had a specific activity of 260 units/mg. On conventional acrylamide electrophoresis it had very low mobility in the pH range 7.5—8.0 and its p*I* is therefore probably in this range. It behaves similarly to the M_1 and M_2 forms found in rat heart in this respect but only a single form of the enzyme was seen on electrophoresis. The amino acid composition of the protein is shown in Table II. No N-terminal amino acid was detectable and it is probable that the α amino group is acylated. The absorbance of a 1% solution of the protein at 280 nm was 5.55 as measured by the method of Babul and Stellwagen [14] and is similar to that determined for the rabbit muscle enzyme [23]. The corresponding molar absorption coefficient is $1.22 \cdot 10^6\ M^{-1} \cdot cm^{-1}$. The partial specific volume of the protein measured at

TABLE I

PURIFICATION OF HEART PYRUVATE KINASE

1 kg of bovine heart tissue was used.

Stage	Enzyme (units)	Volume (ml)	Protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Homogenate	20 000	2000	(20 000)	(1)	1	100
Phosphocellulose eluate	14 500	80	242	60	60	72.5
Sephadex G 200 eluate	12 500	56	48	260	260	62.5



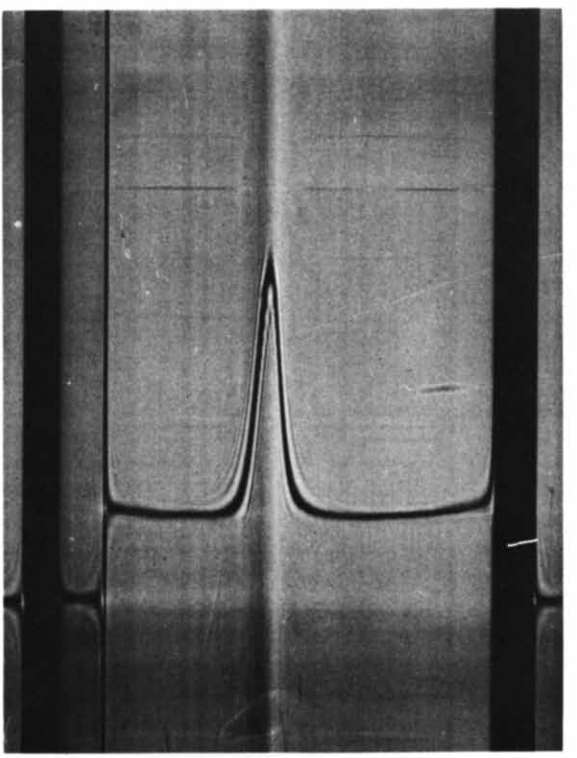


Fig. 1. Dodecyl sulphate polyacrylamide electrophoresis of heart pyruvate kinase. The origin is at the bottom of the figure.

Fig. 2. Sedimentation velocity of heart pyruvate kinase. The photograph was taken 47 min after reaching a rotor speed of 60 000 rev./min. The protein concentration was 7 mg/ml, rotor temperature was 10°C and the Schlieren analyser angle was 65°C. The buffer was 0.2 ionic strength (0.09 M) phosphate, pH 7.

0.5 mg/ml in 0.02 ionic strength (0.01 M) phosphate pH 7 by centrifugation in H_2O and ^2H_2O according to the method of Edelstein and Schachman [24] was 0.743. A similar value (0.740) was obtained from the amino acid composition shown in Table II [25].

TABLE II

AMINO ACID COMPOSITION OF HEART PYRUVATE KINASE

The composition was determined as described under Materials and Methods. A value of 55 000 was taken as the subunit molecular weight for the purpose of the calculation.

Amino acid	Moles per mole of subunit
Asparate	46
Threonine	26
Serine	28
Glutamate	48
Proline	25
Glycine	42
Alanine	60
Valine	38
Methionine	19
Isoleucine	33
Leucine	40
Tyrosine	10
Phenylalanine	16
Lysine	36
Histidine	11
Arginine	20
Cysteine	8

Subunit molecular weight. The polypeptide chain molecular weight of the enzyme was determined by SDS polyacrylamide electrophoresis and a value of 55 000 was obtained. The enzyme co-electrophoresed with a commercial sample of the rabbit muscle enzyme suggesting that it has a similar subunit size. Partial maleylation of the enzyme resulted in a species of $S_{20,w}$ = 3.05 S and molecular weight 122 000. Further maleylation in the presence of 5 mM 2-mercaptoethanol produced further dissociation. A molecular weight of 55 000 was obtained by the method of Yphantis [17] for the subunit but some aggregation was noted.

The molecular weight of the native enzyme. At ionic strengths of 0.2 or greater pyruvate kinase was found to be monodisperse by sedimentation equilibrium analysis. Its weight- and z-average molecular weights were measured by use of both absorption and Schlieren optics at a range of concentrations by both low and high speed sedimentation equilibrium methods. The molecular weight determined was 220 000 ± 3000 (mean and S.D. on 9 determinations). At lower ionic strengths the enzyme polymerised and exhibited heterogeneity in sedimentation equilibrium experiments. In the presence of polymerisation reactions of this sort the concentration distribution in the ultracentrifuge cell is given by a variant of the Rinde equation [26,27]:

$$C_r = \frac{\bar{C}_1 X \exp AM(r^2-a^2)}{(\exp X)-1} + \frac{\bar{C}_2 2X \exp 2AM(r^2-a^2)}{(\exp 2X)-1} + \ldots \frac{\bar{C}_n nX \exp nAM(r^2-a^2)}{(\exp nX)-1} \quad (1)$$

where C_r = total concentration at radial position r; $X = AM(b^2 - a^2)$; $A = \omega^2(1 - \bar{v}\rho)/2RT$; and a and b are the radial positions of the meniscus and solution bottom respectively. M is the molecular weight of the smallest macromolecular species present and $\bar{C}_1, \bar{C}_2, \bar{C}_3 \ldots \bar{C}_n$ are the mean concentrations of

monomer, dimer, trimer . . . n'mer of this species at sedimentation equilibrium, averaged over the total cell volume. They are equivalent to the coefficients g_i defined by Sophianopoulos and Van Holde [28] but do not correspond to the initial concentrations of species.

Eqn. 1 was fitted to the experimental data by use of Nottingham Algorithm E0 4GAF on an IBM 1900/CDC7600 system computer. The routine minimises the sums of squares of residuals of non-linear functions. Eqn. 1 was written in the polynomial form:

$$C_r = \bar{C}_1 Y_1 + \bar{C}_2 Y_2 + \bar{C}_3 Y_3 + \ldots \bar{C}_n Y_n \tag{2}$$

for introduction into the routine. Here

$$Y_i = \frac{iX \exp AM(r^2 - a^2)\, i}{(\exp iX) - 1}$$

Y_i values were computed and the program selected $\bar{C}_i$ values to give the best fit to the set of simultaneous equations obtained by applying data for different values of r.

The data of Fig. 3 are the result of a sedimentation equilibrium experiment in 0.05 ionic strength (0.024 M) phosphate pH 7 at 5°C. It can be seen that the polymerisation data are best fitted by Eqn. 1 when monomer (220 000 daltons) and hexamer (1320 000 daltons) are the principle species present in solution. The polydispersity is accounted for by the presence of approximately 87% monomer and 12% hexamer at sedimentation equilibrium. There was negligible difference in the proportion of monomer and hexamer present at 8, 15 and 20°C. However, the polymerisation was concentration dependent and at an enzyme concentration of 1.5 mg/ml no polymerisation was detectable.

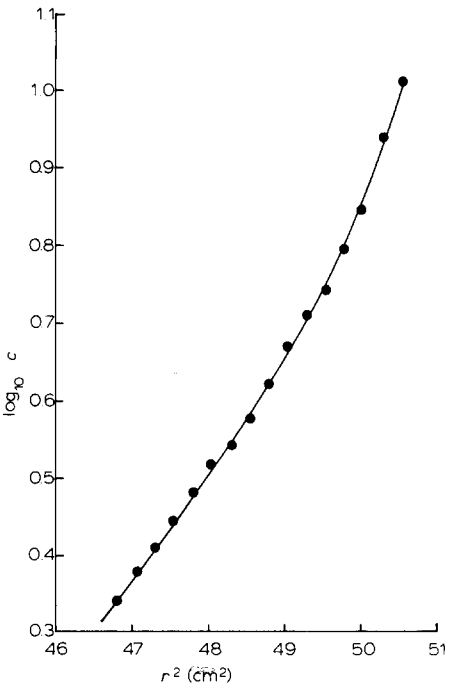
While Eqn. 1 may be applied to reversibly interacting protein systems the coefficients $\bar{C}_i$ do not represent the initial concentrations of species present prior to centrifugation [29]. It may, however, be readily shown by application of the mass conservation condition that they represent the mean concentrations of each species present in the centrifuge cell at sedimentation equilibrium [30]. Sophianopoulos and Van Holde [28] have shown that the perturbation of the chemical equilibrium caused by centrifugation may be estimated for a monomer-dimer equilibrium and a similar approach has also been adopted by Reinhardt and Squire [31]. In the more complicated situation of a monomer-polymer equilibrium the following relation holds:

$$\frac{K}{Q} = \frac{X^{n-1}(e^{nx} - 1)}{n(e^x - 1)^n} \tag{3}$$

where K is the dissociation constant of the polymer and Q is the apparent dissociation constant at sedimentation equilibrium, i.e.

$$Q = \frac{(\bar{C}_1)^n}{(\bar{C}_n)} \tag{4}$$

It should be pointed out that chemical equilibrium is maintained during centrifugation in each microscopic phase bounded by radii r and $r + dr$. It is the mean concentration of each reactant averaged over the whole cell which is dis-



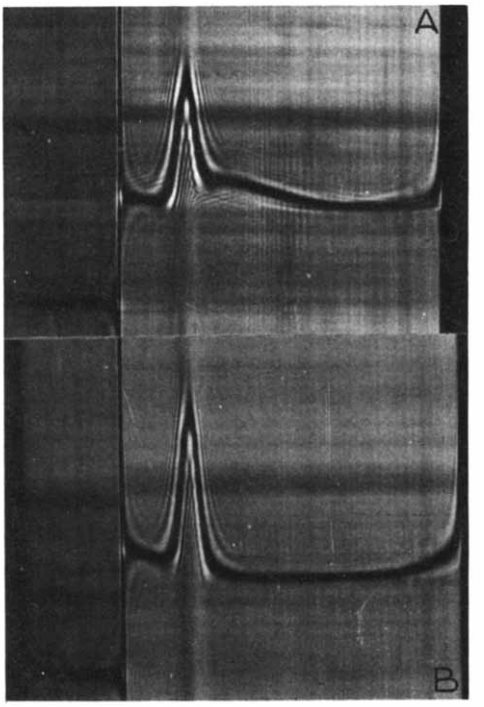


Fig. 3. Sedimentation equilibrium of heart pyruvate kinase at low ionic strength. The buffer was 0.05 ionic strength (0.024 M) phosphate, pH 7. Protein concentration was 4.75 mg/ml and temperature 5°C. Rotor speed was 4800 rev./min. Experimental points are shown together with a theoretical curve computed from Eqn. 1 with the following $\bar{C}_i$ values: $\bar{C}_1$ = 4.14 mg/ml; $\bar{C}_2, \bar{C}_3, \bar{C}_4$ = 0 mg/ml; $\bar{C}_5$ = 0.04 mg/ml; $\bar{C}_6$ = 0.57 mg/ml; $\bar{C}_7, \bar{C}_8, \bar{C}_9 \ldots$ = 0 mg/ml. This corresponds to the following initial distribution of components (see text): monomer = 4.49 mg/ml, pentamer = 0.03 mg/ml; hexamer = 0.23 mg/ml.

Fig. 4. Sedimentation velocity of heart pyruvate kinase at low ionic strength. A, pyruvate kinase in 0.05 ionic strength (0.024 M) phosphate, pH 7. B, sample from (A) after dialysis against 0.2 ionic strength (0.09 M) phosphate, pH 7. Photographs were taken 18 min after reaching a rotor speed of 60 000 rev./min. Schlieren analyser angle was 60° and temperature was 10°C. The protein concentration was 2.4 mg/ml.

placed from the initial concentration established by chemical equilibrium. Correction of the data of Fig. 3 by use of equations 3 and 4 gives for the initial distribution of species: C_1 = 4.49 mg/ml; C_5 = 0.03 mg/ml; C_6 = 0.23 mg/ml. Thus approximately 4.9% hexamer is initially present and this becomes exaggerated to 12% at sedimentation equilibrium. The contribution of pentamer is small and may not be significant.

Sedimentation behaviour of the native enzyme. The concentration dependence of the sedimentation coefficient of the enzyme was studied in 0.2 ionic strength (0.09 M) phosphate buffer, pH 7 and found to fit the following relationship:

$$S_{20,w} = S^{\circ}_{20,w}(1 - kc)$$

where $S^{0}_{20,w}$ has a value of 10.0 S and k has a value of $9.6 \cdot 10^{-3}$ ml/mg. The enzyme sedimented as a single, symmetrical boundary (Fig. 4).

At low ionic strength the enzyme was subject to polymerisation reactions

similar to those seen in sedimentation equilibrium experiments and boundaries faster than the 10.0-S boundary were seen (Fig. 4). The proportion of polymer decreased with increasing ionic strength, but owing to the complexity of interpretation of Schlieren patterns for polymerising protein systems [32,33] no attempt was made to estimate the proportion of monomer and polymer present. Forward 'trailing' of the boundary pattern was noted and was consistent with the presence of a monomer-hexamer equilibrium [33]. However, partial separation of a monomer boundary may imply that equilibration of monomer and polymer is not sufficiently rapid to be unperturbed by the effects of sedimentation. No polymerisation was detectable at ionic strengths greater than 0.1.

Discussion

The method described for the purification of heart pyruvate kinase is simple, rapid and yields homogeneous enzyme of comparable specific activity to previously described preparations of mammalian isozymes [1—8]. The advantage of the present method is that it is quick and does not entail procedures such as heat treatment or fractionation with organic solvents which might be disruptive of the native enzyme conformation. Most procedures for the purification of the enzyme from other tissues have employed at least one of these approaches.

During the present study only a single form of the enzyme was encountered in bovine heart and this had physical properties similar to the M_1 and M_2 forms seen in extracts of rat hearts [9]. However, its kinetic properties are slightly different from those of both the M_1 and M_2 forms (Parkinson, J. and Easterby, J.S., unpublished). It is not possible to say whether it is equivalent to the M_1 enzyme or a conformational variant of the M_2(K) enzyme as seen by Ibsen and Krueger [34]. These authors found it impossible to distinguish between the two species. The enzyme has a similar amino acid composition to the rabbit muscle enzyme [23] and lacks a free terminal amino group. It has recently been demonstrated that both the amino- and carboxy-termini of the rabbit muscle enzyme are blocked [35]. In the former case the terminal residue is *N*-acetylserine and the heart enzyme therefore probably also possesses an acylated N-terminus.

Subunit molecular weight studies demonstrate that the enzyme is a tetramer of 220 000 daltons and $S^0_{20,w}$ of 10.0 S. Maleylation experiments demonstrate the presence of two dissimilar planes of dissociation in the tetramer. Thus partial maleylation of the protein generates a dimeric species of 122 000 molecular weight and 3.05 S. The low sedimentation coefficient suggests either that the half molecule is partially unfolded or that it is subject to a large charge effect on acylation. The latter possibility seems very likely [18]. A similar conclusion regarding the assymmetry of dimers within the tetramer has been reached with regard to the rabbit muscle enzyme by Morawiecki et al. [36] and Steinmetz and Deal [37] studying dissociation of the enzyme by urea and by Davies and Kaplan investigating chemical crosslinking of subunits [38].

At low ionic strengths the native enzyme has been shown to undergo polymerisation reaction principally to a hexamer of the basic enzyme tetramer. Although this type of polymerisation has not been previously reported for

pyruvate kinase from other tissues it might partly account for the slight molecular weight discrepancies reported for pyruvate kinase from different laboratories [4—8,23,36—37]. The polymerisation was temperature-independent and it therefore seems unlikely that hydrophobic interactions are involved. The dependence of the process on ionic strength implies the involvement of charge interactions.

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