The Preparation and Properties of Pyruvate Kinase from Yeast

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A new method is described for the preparation of pyruvate kinase from yeast. This eliminates proteolysis during the preparation. The molecular weight of yeast pyruvate kinase is 215000, and it is composed of four subunits. Such properties of the enzyme as its extinction coefficient, cold-lability, thiol-group reactivity and binding of Mn^{2+} ions are compared with those previously reported for yeast pyruvate kinase prepared by different methods. The specific activity is significantly higher than previously observed, but otherwise the enzyme is similar, apart from its molecular weight and Mn^{2+} -binding characteristics, to preparations from *Saccharomyces cerevisiae* obtained in this laboratory (e.g. Fell *et al.*, 1972, and references therein) and that of C. H. Suelter (e.g. Kuczenski & Suelter, 1971, and references therein), and is different from the enzyme isolated from *Saccharomyces carlsbergensis* by B. Hess and his co-workers (e.g. Wieker & Hess, 1972, and references therein).

There have been discrepancies in the values quoted for the molecular weight of yeast pyruvate kinase and of its subunits: Bischofberger *et al.* (1971) found a mol. wt. of 190000 for the enzyme from *Saccharomyces carlsbergensis*, and of 45000–51000 for its subunits. Kuczenski & Suelter (1970b) reported a value of 162000–168000 for the enzyme isolated from *Saccharomyces cerevisiae*, and a value of 42000–45000 for the subunits; Ashton & Peacocke (1971) found a mol. wt. of 161000 for the pyruvate kinase of *S. cerevisiae* in this laboratory, but obtained eight monodisperse subunits, each of mol. wt. 20000, when the enzyme was maleylated and dissolved in 6M-guanidinium chloride. Other properties of the enzymes obtained in the three laboratories also differed. Thus the enzyme from *S. cerevisiae* is cold-labile (Kuczenski & Suelter, 1970a; Ashton, 1971), whereas that from *S. carlsbergensis* is not (B. Hess, personal communication), and the reports of Cottam *et al.* (1972) and Fell *et al.* (1972) on the binding of Mn^{2+} and other ligands to the pyruvate kinase of *S. cerevisiae* show some differences.

In an attempt to repeat the experiments of Ashton & Peacocke (1971), we found the molecular weight of

the subunits was about 42000 by sedimentation-equilibrium measurements in 6M-guanidinium chloride, but electrophoresis of the reduced protein on sodium dodecyl sulphate–polyacrylamide gels gave multiple bands, suggesting that the polypeptide chains had been degraded. An analogy can be drawn here with the studies on yeast hexokinase; in different laboratories and with different preparative methods, the properties and specific activity of this enzyme were not consistent. However, Lazarus *et al.* (1966) demonstrated that the discrepancies arose through the proteolytic degradation in the preparations, and that autolysis was not a suitable method for releasing soluble enzyme from yeast cells because it entailed exposure to the endogenous proteolytic enzymes. Since the preparative methods in use for the isolation of yeast pyruvate kinase all started with autolysis (Haeckel *et al.*, 1968; Hunsley & Suelter, 1969a; Ashton, 1971), we devised a new preparative method to decrease the possibility of proteolysis, and we then investigated the properties of the enzyme so prepared.

Materials and Methods

Materials

Fructose diphosphate (tetracyclohexylammonium or sodium salt), phosphoenolpyruvate (potassium salt), ADP (di- or tri-sodium salts), NADH (disodium salt) and rabbit muscle lactate dehydrogenase [as a

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precipitate in $(\text{NH}_4)_2\text{SO}_4$] were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.; Tris (reagent grade), sodium dodecyl sulphate and pyruvate were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; cacodylic acid was from Serva Feinbiochemica G.m.b.H., Heidelberg, Federal Republic of Germany; 5,5'-dithiobis-(2-nitrobenzoate) was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., and from BDH Chemicals Ltd., Poole, Dorset, U.K.; iodo-[1- ^{14}C]acetamide was from The Radiochemical Centre, Amersham, Bucks., U.K.; the spin-label *N*-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyliodoacetamide) was from Synvar, Palo Alto, Calif., U.S.A.; the liquid-scintillation fluid, Aquasol, was from New England Nuclear Corp., Boston, Mass., U.S.A.; DEAE-cellulose (DE-52) and cellulose phosphate (P-11) were from Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and Sephadex G-25 (coarse) was from Pharmacia, Uppsala, Sweden. All other chemicals were BDH AnalaR grade, except that Aristar $(\text{NH}_4)_2\text{SO}_4$ was used when the purified enzyme was precipitated, and 'Biochemical reagent'-grade guanidinium chloride was used for the sedimentation-equilibrium studies. Dialysis sacs were prepared from Visking tubing that had been boiled for 5 min in 1% NaHCO_3 -1% EDTA, washed several times in distilled water and stored in distilled water at 4°C.

Preparation of pyruvate kinase

S. cerevisiae (3.5 kg wet wt.) was obtained from Morrells' Brewery, Oxford, immediately after fermentation of the beer had ceased. The yeast was washed twice by suspension in 3 litres of 5 mM-EDTA-10 mM- MgCl_2 and 40 mM- NaHCO_3 , pH 7, at 4°C and centrifugation (1000g for 10 min), and the yeast paste was stored overnight at 4°C.

The yeast was added in small amounts to 5 litres of toluene at -20°C. The temperature of the mixture was kept below -10°C by frequent additions of solid CO_2 . After 6 h at -10°C, the toluene was decanted and the slurry transferred to a water bath at 4°C to melt. When the temperature of the yeast had risen above 0°C, 2.5 litres of 0.1 M-Tris-HCl (pH 8.0)-0.1 M-KCl-1 mM-EDTA-2 mM- MgCl_2 at 4°C were added. α -Toluenesulphonyl fluoride in propan-2-ol to a final concentration of 1 mM was added either at this point or at the start of the dialysis. After a further 12-24 h at 4°C, during which time the pH of the slurry was periodically checked and adjusted back to 7.5 with dilute NH_3 if necessary, the cell supernatant was separated by centrifugation at 6300g for 20 min. In this step and all subsequent stages of the preparation, the temperature was not allowed to rise above 5°C. Pyruvate kinase activity was precipitated from the supernatant by the addition of $(\text{NH}_4)_2\text{SO}_4$ (350 g/l of

solution) and stirring for 1 h. The precipitate was collected by centrifugation at 6300g, for 45 min, and the supernatant was assayed. If more than 80% of the enzyme activity had not precipitated, more $(\text{NH}_4)_2\text{SO}_4$ was added. The precipitated pyruvate kinase was dissolved in 600 ml of 0.1 M-Tris-HCl (pH 7.5)-2 mM- MgCl_2 -1 mM-EDTA containing $(\text{NH}_4)_2\text{SO}_4$ (114 g) by stirring for 1 h. Undissolved protein was removed by centrifugation at 35000g for 5 min, $(\text{NH}_4)_2\text{SO}_4$ (20 g/100 ml of supernatant) was added, the mixture stirred for 1 h, and the precipitate collected by centrifugation as before.

The precipitate was then suspended in a minimal volume (about 50 ml) of distilled water, and dialysed against several 1 litre changes of 10 mM-sodium phosphate (pH 7.5)-glycerol (1:1, v/v). The protein dissolved as the $(\text{NH}_4)_2\text{SO}_4$ dialysed out. The pyruvate kinase was then purified by chromatography on DEAE-cellulose and cellulose phosphate by the method of Hunsley & Suelter (1969a), and was collected as a precipitate by dialysis of the column eluate against a saturated solution of Aristar $(\text{NH}_4)_2\text{SO}_4$.

Enzyme assays

Pyruvate kinase was assayed in a system containing 0.1 M-potassium cacodylate (pH 6.2), 0.1 M-KCl, 25 mM- MgSO_4 , 0.5 mM-EDTA, 5 mM-ADP, 5 mM-phosphoenolpyruvate, 1 mM-fructose diphosphate and 0.15 mM-NADH. Lactate dehydrogenase [1 mg/ml in 50% (v/v) glycerol] (0.02 ml) was added to 1 ml of the assay medium, and the reaction started by the addition of 0.02-0.05 ml of a suitable dilution of pyruvate kinase in 50% (v/v) glycerol. Initial rates were measured at 25°C by the disappearance of absorbance at 340 nm in a Beckmann DU-Gilford spectrophotometer, and the activities calculated in International Units.

Electrophoresis

Electrophoresis of the enzyme was carried out on 7.5% (w/v) polyacrylamide gels containing 0.9 M-acetic acid and 6 M-deionized urea with 0.9 M-acetic acid as the tank buffer. Protein samples were dialysed against the gel buffer plus 1% 2-mercaptoethanol. The gels were fixed with 10% (w/v) trichloroacetic acid and stained with Amido Black.

Electrophoresis on 6% polyacrylamide gels containing 0.1% sodium dodecyl sulphate and 0.1 M-sodium phosphate buffer, pH 7.2, was carried out as described by Shapiro *et al.* (1967). The proteins were dissociated with 6 M-urea-1% 2-mercaptoethanol-1% sodium dodecyl sulphate before application to the gels. After electrophoresis overnight, the gels were fixed and washed with 7% trichloroacetic acid-25% propan-2-ol, and stained with Coomassie Blue. The

mobilities of the standard proteins relative to the Bromophenol Blue marker were plotted against $\log(\text{mol.wt.})$.

Ultracentrifugation

Sedimentation experiments were performed at, or close to, 20°C with a Beckmann–Spinco model E analytical ultracentrifuge equipped with both schlieren and Rayleigh interference optics. Sedimentation-velocity measurements were made at 56100 or 59780 rev./min, and the results corrected to the values at 20°C with water as the solvent in the conventional manner, by using viscosities taken from tables and measured values of densities.

Solution columns of 3 mm depth were used for the low-speed equilibrium runs to determine the molecular weight of the subunits, and the initial concentration of the protein was found from the number of interference fringes across a boundary formed between the solution and its diffusate in a synthetic boundary cell. The hinge point at equilibrium was determined by the white-light fringe method of Richards & Schachmann (1959) without making the initial adjustment of refractive index of the solvent. High-speed equilibrium runs were by the method of Chervenka (1970). The weight-average molecular weight, $\bar{M}_w$, was obtained from measurements of the interference photographs taken at equilibrium by plotting the log of the protein concentration, c , at a radial distance, r , against r^2 . The z -average molecular weight, $\bar{M}_z$, was calculated from measurements of the schlieren photographs taken at equilibrium by plotting

$$\log \left(\frac{1}{r} \cdot \frac{dc}{dr} \right)$$

against r^2 (Lamm, 1929). $\bar{M}_w$ was extrapolated to zero concentration by plotting $1/\bar{M}_w$ against the arithmetic mean, $\bar{c}$, of the concentrations at the two extremes of the measured region of the solute column. $\bar{M}_z$ was extrapolated by plotting $1/\bar{M}_z$ against $2\bar{c}$ (Van Holde & Cohen, 1964).

The partial specific volumes used in the calculation of molecular weights were 0.734 ml · g⁻¹ for the native enzyme (Kuczenski & Suelter, 1970b; Bischofberger *et al.*, 1971; Ashton & Peacocke, 1971) and 0.726 ml · g⁻¹ for the subunits in 6M-guanidinium chloride. Densities of the solvents used in the sedimentation experiments were measured by pycnometry.

Native protein was prepared for sedimentation experiments by dissolving a sample of the (NH₄)₂SO₄ precipitate of the enzyme in a small volume of buffer, chromatographing the solution on a column (10 ml) of Sephadex G-25, and completing the equilibration with the buffer by dialysis for a further 4 h with rapid stirring. Subunits were prepared by dialysis of the

protein against two changes of 6M-guanidinium chloride for at least 24 h.

Diffusion measurements

Diffusion coefficients were determined from the autocorrelation function of intensity fluctuations of laser light scattered by the enzyme. The autocorrelation function was measured by a single-clipped technique (Foord *et al.*, 1970) with a Precision Devices and Systems 'Malvern' 24-channel autocorrelator. The light source was a 30 mW Scientific Cook He–Ne laser, and for the most dilute enzyme solutions studied (1 mg/ml) the scattered photon count rate at 90° was 3400/s. Sampling times of either 10 or 5 μs were selected and contributions from 10⁸ sample times were accumulated, ensuring a theoretical statistical uncertainty (Jakeman *et al.*, 1970) of less than 1% in the values of the autocorrelation function $g^2(\tau)$. The temperature was maintained at 19.6°C.

The diffusion coefficient, D , was calculated from the slope of the plot of $\ln[g^2(\tau)-1]$ against delay time (Foord *et al.*, 1970). Corrections for temperature, solvent viscosity and solvent refractive index were made in the determination of $D_{20,w}$.

Enzyme solutions were clarified by centrifugation for 60 min at 40000 rev./min in an MSE Superspeed centrifuge with a 3 × 5 ml swinging-bucket rotor. After centrifugation, 1 ml of solution from near the middle of the centrifuge tube was transferred to a scrupulously clean spectrofluorimeter cell by means of a Pasteur pipette attached by rubber tubing to a syringe. The pipette was mounted rigidly on a jack which allowed it to be lowered to the desired level in the tube without significant disturbance of the tube contents. Linearity of the logarithmic plots of $g^2(\tau)-1$ indicated that scattering from contaminating particles was negligible.

Carbohydrate content

The protein was tested for its content of neutral sugar by the phenol–H₂SO₄ method of Dubois *et al.* (1956), with glucose as standard. Some urea–acetic acid gels were stained for carbohydrate by the periodic acid–Schiff–base method of Fairbanks *et al.* (1971).

N-terminal amino acid

The dansyl chloride method of Gray (1972) was used in an attempt to identify the N-terminal amino acid.

Thiol-group reactivity

Pyruvate kinase was equilibrated with the buffers used for the reactions by chromatography on a

column of Sephadex G-25. The reaction with 5,5'-dithiobis-(2-nitrobenzoate) (Ellmann's reagent, Ellmann, 1959) was carried out with excess of reagent (0.8mM) in 0.1M-Tris-HCl (pH7.5)-0.1M-KCl, and was started by the addition of sufficient enzyme to give a final concentration of about 1 μ M in the solution in the cuvette (1ml). The time-course of the reaction was followed by the increase in absorbance at 412nm in a Beckmann DU-Gilford spectrophotometer at 25°C. After the reaction with the native enzyme was complete, a 10% (w/v) solution of sodium dodecyl sulphate was added to the cuvette to a final concentration of 0.5% sodium dodecyl sulphate, and the reaction of the remaining thiol groups was followed. After the reaction with the native enzyme was complete, the E_{412} was observed to decrease. The rate of this process could be measured at this stage in the reaction, and it was attributed to re-oxidation of reduced 5,5'-dithiobis-(2-nitrobenzoate) (NbS) by O₂ dissolved in the solutions, since it could be slowed by preliminary degassing with a water pump. The kinetics of the overall process could be treated as two consecutive first-order reactions [the first was assumed to be pseudo-first-order because of the excess of 5,5'-dithiobis-(2-nitrobenzoate) (NbS₂), and the second may be conveniently represented as such, since the fraction of this reaction which was observed was small]. The reactions can be written as:

[NbS] is the quantity measured experimentally, and it was found that k_1 was much greater than k_2 . Given this condition, the appropriate expression for [NbS] (Moore, 1963) was easily shown to simplify to:

$$a_1 \cdot e^{-k_2 \cdot t} - [\text{NbS}] = a_1 \cdot e^{-k_1 \cdot t}$$

where a_1 is the initial concentration of protein thiol groups reacting at rate k_1 , and a_1 is the total concentration of reacting groups, i.e. a_1 plus the concentration of groups that react at a rate much faster than k_1 (instantaneously relative to the time-scale of the experiment). a_1 was determined from the absorbance when the reaction of the protein was complete, when $a_1 \cdot e^{-k_1 \cdot t} \approx 0$, by using the above equation and the experimental value of k_2 . $\ln(a_1 \cdot e^{-k_2 \cdot t} - [\text{NbS}])$ was plotted against time (t) to give $\log a_1$ as the intercept on the ordinate and $-k_1$ as the slope. This treatment was not necessary for the reaction of the enzyme in the presence of sodium dodecyl sulphate as the amount of air oxidation in the short time taken for this more rapid reaction was insignificant.

The reaction of the protein with iodoacetamide was carried out in 0.05M-Tris-HCl (pH7.5)-0.05M-KCl, with 1mM-iodo[1-¹⁴C]acetamide of specific radioactivity 2.4mCi/mmol, at a protein concentration of about 2mg/ml. Samples (0.1ml) were withdrawn from the reaction at various times, and the protein was precipitated with 5ml of 10% trichloroacetic acid.

The precipitates were washed once with 5ml of the same solution, and the protein was dried overnight over silica gel at 37°C. The labelled protein was redissolved in 1M-KOH (1.5ml) and samples (1ml) were added to 'Aquasol' scintillation fluid (10ml) together with acetic acid (0.5ml) to suppress chemiluminescence. The samples were counted for radioactivity by using an LKB-Wallac scintillation counter, and the count rate was converted into mol of iodoacetamide/mol of protein by reference to an appropriate standard. The amount of free iodoacetamide passing through the precipitation and washing was insignificant.

Proton-relaxation enhancement and electron-spin-resonance measurements

These measurements were made on solutions of pyruvate kinase equilibrated with 0.08M-KCl-0.02M-Tris-HCl, pH7.2, by dialysis overnight. The experimental conditions and methods of analysis were as previously described by Fell *et al.* (1972). Electron-spin-resonance (e.s.r.) spectra were measured in X-band on either a Varian E4 spectrometer or a JEOL spectrometer.

Results and Discussion

Preparation and characterization

To prevent degradation during preparation of the enzyme the following precautions were taken. (1) The temperature was kept low throughout the preparation. This included the initial rupturing of the cells to release soluble enzyme; autolysis was clearly unsuitable and was replaced by a freeze-thawing method described by Lazarus *et al.* (1966). Although this method is slow and not completely efficient, it does allow the temperature to be controlled. (2) The pH of the preparation was kept above 6 at all times to prevent activation of inactive yeast proteases (Lenney, 1956). (3) α -Toluenesulphonyl fluoride was added before dialysis as the yeast proteases known to be active above pH6 are sensitive to inhibitors of serine proteases (Hata *et al.*, 1967; Doi *et al.*, 1967).

The preparation is summarized in Table 1. Pyruvate kinase prepared by this method has a specific activity when pure of 340-400 units/mg of protein. This is much higher than the values previously reported for this enzyme (see Table 2). The protein gave a single band on electrophoresis in sodium dodecyl sulphate-polyacrylamide gels, although slight contamination was visible on urea-acetic acid gels (Fig. 1). It sedimented as a single species in the ultracentrifuge, apart from a small amount of a faster-moving component which is probably aggregated material (cf. Kuczenski & Suelter, 1970b) (Fig. 2).

Table 1. *Summary of the preparation of yeast pyruvate kinase*

For further details see the text.

Fraction	Volume (ml)	Protein		Activity			Yield (%)
		(mg/ml)	Total (g)	(units/ml)	$10^{-4} \times$ Total (units)	(units/mg)	
1. Supernatant from freeze-thawed yeast	4080	24	98*	146	60	6.1	100
2. First $(\text{NH}_4)_2\text{SO}_4$ precipitate resuspended in 0.1 M-Tris-HCl buffer	670	24.5	16*	672	45	28	75
3. Supernatant from step 2 after centrifugation at 35000g for 5 min	670	19.5	13*	608	41	31	68
4. Second $(\text{NH}_4)_2\text{SO}_4$ precipitate after solution and dialysis for loading on to DEAE-cellulose column	85	123	10.5*	3100	26	25	43
5. Total eluate from DEAE-cellulose column	—	—	9.3†	—	19	—	32
6. Cut taken from step 5 for loading on cellulose phosphate column	510	—	0.830†	300	15	192	25
7. Total pyruvate kinase eluted from cellulose phosphate column	—	—	—	—	14	—	23
8. Pure pyruvate kinase recovered from cellulose phosphate column	—	—	0.270‡	—	10	340+	16

Protein assays were by: * micro-biuret, calibrated with bovine serum albumin (Itzaki & Gill, 1964); † assuming $E_{1\text{cm}}^{0.1\%}$ at 280nm = 1.0 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$; ‡ assuming $E_{1\text{cm}}^{0.1\%}$ at 280nm = 0.65 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$.

Table 2. *Comparison of published reports of the properties of pyruvate kinases from yeasts*

Measurement technique	<i>S. cerevisiae</i> , the present paper	<i>S. cerevisiae</i> , prepared by the method of Hunsley & Suelter (1969a)*	<i>S. cerevisiae</i> , prepared by the method of Ashton (1971)†	<i>S. carlsbergensis</i> , prepared by the method of Haeckel <i>et al.</i> (1968), or of Röschlau & Hess (1972)‡
$\bar{M}_w$, native enzyme, sedimentation equilibrium	214000	—	161000	190000
$\bar{M}_z$, sedimentation equilibrium	213000	—	—	—
Molecular weight, calc. from Svedberg eqn.	217000	167000	162000	191000
$s_{20,w}^0$	9.5	8.3	8.0–8.3	8.7
$10^7 \times D_{20,w}^0$ (cm 2 $\cdot$ s $^{-1}$)	4.0	4.52	—	4.2
$\bar{M}_w$, subunits, sedimentation equilibrium	55000	42000	21000	51000
$\bar{M}_z$, sedimentation equilibrium	52000	45000	—	—
Molecular weight, subunits, sodium dodecyl sulphate–polyacrylamide gels	56000	—	18000–20000	55000–60000
$E_{1\text{cm}}^{0.1\%}$ at 280nm	0.66	0.65	0.65	0.76
Specific activity (units/mg)	340–400	220 (30°C)	180–200	250
Cold-labile	Yes	Yes	Yes	No

Additional references: * Kuczenski & Suelter (1970b); † Ashton & Peacocke (1971); ‡ Bischofberger *et al.* (1971).

The enzyme contained no carbohydrates as shown by staining urea–acetic acid gels with the carbohydrate-specific periodic acid–Schiff stain or by the phenol–H $_2$ SO $_4$ method for the estimation of neutral sugars. Since for many proteins the specific refractive increment (dn/dc) is approx. 0.184 ml/g (e.g. Halwer

et al., 1951), it was possible to determine the extinction coefficient from extinction measurements on dialysed protein solutions whose concentration had been estimated by refractive-index measurements made in a Brice–Phoenix differential refractometer. The value obtained of $E_{1\text{cm}}^{0.1\%} = 0.66$ litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$ at 280nm is

Fig. 1. Polyacrylamide-gel electrophoresis of yeast pyruvate kinase

Microdensitometer scans of (a) a 7.5% gel containing 6M-urea-0.9M-acetic acid, and (b) a 6% gel containing 0.1M-sodium phosphate buffer, pH 7.2, and 0.1% sodium dodecyl sulphate.

in good agreement with the value of 0.65 quoted for the pyruvate kinase from *S. cerevisiae* by Hunsley & Suelter (1969b) and by Ashton (1971). Bischofberger *et al.* (1970), however, reported a value of 0.76 for the enzyme from *S. carlsbergensis*.

No *N*-terminal amino acid was detected by chromatography of an acid hydrolysate of the protein that had been reacted with dansyl chloride. This is consistent with the report by Bornmann *et al.* (1972) that the *N*-terminal residue of pyruvate kinase from *S. carlsbergensis* is acetylated.

Cold-lability

Although the enzyme activity is stable at room temperature (60% of the activity is retained after 48h), it exhibits cold-lability. In 0.1M-Tris-HCl buffer, pH 7.5, at 0°C, and at an enzyme concentration of 1 mg/ml, only 20% of the original activity remains after 24h. The presence of 1 mM-fructose diphosphate greatly accelerates the loss of activity at 0°C. Phosphoenolpyruvate and ADP did not promote the inactivation. These observations demonstrate the similarity of the enzyme to the cold-labile pyruvate kinase isolated from *S. cerevisiae* by Kuczenski & Suleter (1970a), who used the method of Hunsley & Suelter

(1969a). Pyruvate kinase from *S. carlsbergensis* is not cold-labile, nor is it unstable in the presence of fructose diphosphate (B. Hess, personal communication).

Molecular-weight measurements

The molecular weight of the native enzyme was determined by high-speed equilibrium runs, with, as solvent, 0.1M-Tris-HCl (pH 7.5)-0.23M-KCl-0.01M-MgCl₂-1mM-fructose diphosphate-5mM-phosphoenolpyruvate, or 0.1M-potassium phosphate buffer (pH 7.5), or 0.1M-KCl-0.01M-potassium phosphate buffer (pH 7.5). The molecular weights obtained differed more than would be expected for this technique, presumably because of the slow aggregation which invariably occurs in solutions of pyruvate kinase, causing them to become turbid. Thus the high-speed Chervenka method (Chervenka, 1970) was used, as equilibrium is rapidly attained and the determination of absolute concentrations is simple, whereas in the more accurate low-speed method equilibrium is reached more slowly and the determination of absolute concentrations can be difficult if solute precipitates in the cell. From those runs judged as reliable from the linearity of the plots of $\log c$ and

$$\log \left(\frac{1}{r} \cdot \frac{dc}{dr} \right)$$

against r^2 , and from the agreement to within 5% of the corresponding molecular weights, the mean weight-average mol.wt. was 214000 (s.d. $\pm 7\%$) and the *z*-average mol.wt. was 213000 ($\pm 7\%$). There was no detectable dependence of the molecular weight on concentration in the range measured (0.1-2mg/ml).

The sedimentation coefficient of the native enzyme was measured in 0.1M-potassium phosphate buffer (pH 7.5) and in 0.1M-KCl with either 0.01M-Tris-HCl (pH 7.5) or 0.01M-potassium phosphate (pH 7.5) as the buffer. In 0.1M-potassium phosphate, the $s_{20,w}^0$ was 8.65S (s.e.m. $\pm 1\%$) (Fig. 3). This is in agreement with the value of 8.7S obtained in the same buffer at pH 7.0 by Bischofberger *et al.* (1971), and is significantly higher than the 8.3S found by Kuczenski & Suelter (1970b), with 0.1M-Tris-HCl, pH 7.5, as solvent. However, 0.1M-potassium phosphate buffer was not considered an ideal solvent for sedimentation-velocity measurements, because the curvature of the schlieren baseline indicated that some redistribution of the low-molecular-weight solutes was occurring, and also Svedberg & Pedersen (1940) have indicated the possibility of errors owing to 'secondary charge effects' when the masses of the components of the electrolyte are very different. These authors recommended KCl as a suitable electrolyte for use in sedimentation-velocity experiments, so measurements were made in two solutions

Fig. 2. Sedimentation of pyruvate kinase in the ultracentrifuge

The photograph was taken 32min after the rotor reached the required speed (59780rev./min). The temperature was 20.2°C, and the analyser angle 70°. In the upper trace, the enzyme concentration was 5.9mg of protein/ml and the solvent was 0.1M-KCl-0.01M-Tris-HCl, pH7.5, and for the lower trace, 5.2mg of protein/ml were dissolved in 0.1M-KCl-0.01M-potassium phosphate, pH7.5.

Fig. 3. Extrapolation of the sedimentation coefficient to zero concentration of enzyme

Symbols: ○, results obtained in 0.1M-potassium phosphate buffer, pH7.5, and ----, the least-squares line for these points; ■ and ▲, results obtained in 0.1M-KCl with 0.01M-Tris-HCl, pH7.5, or 0.01M-potassium phosphate buffer, pH7.5, respectively. The solid line is the regression line for these points.

each containing 0.1M-KCl, and either 0.01M-Tris-HCl, pH7.5, or 0.01M-potassium phosphate, pH7.5, as buffer. The results obtained in these two solutions appeared comparable and were together extrapolated to give a value of 9.50S (s.e.m. $\pm 2\%$) for $s_{20,w}^0$, which is significantly higher than the value of 8.7S obtained in 0.1M-potassium phosphate buffer. Because of the considerations above, 9.50S is taken as the correct value.

The diffusion coefficient of the enzyme was measured by a laser light-scattering technique in 0.1M-potassium phosphate buffer, pH7.5, at 1.0, 2.2 and 4.4mg of enzyme/ml. (There should be no anomalies arising from secondary charge effects in this measurement as the solution remains macroscopically homogeneous.) There was no detectable dependence on concentration, so $D_{20,w}^0$ was taken (from the average of nine measurements) as $3.99 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (s.e.m. $\pm 1.3\%$). The Svedberg relationship was used to calculate a mol.wt. of 217000 (s.e.m. $\pm 3\%$) for the native enzyme from the combination of the sedimentation and diffusion coefficients. This is in close

Fig. 4. Extrapolation of the subunit molecular weight to zero concentration

Symbols: ● and —, points and the regression line for the z-average molecular weight; ○ and ----, points and the regression line for the weight-average molecular weight. The mean concentrations of protein (J) (defined in the Materials and Methods section) are expressed in numbers of Rayleigh interference fringes.

agreement with the values obtained from sedimentation equilibrium, and the average value of 215000 was taken as the molecular weight of yeast pyruvate kinase. Comparison of these results with those of other investigators (Table 2) shows closest agreement with those of Bischofberger *et al.* (1971) with the pyruvate kinase of *S. carlsbergensis*, and this suggests that previous preparations from *S. cerevisiae* have been degraded by proteolytic action.

Measurements of the molecular weight of the subunits by sedimentation-equilibrium runs (with both the high-speed Chervenka method and the low-speed method) in 6M-guanidinium chloride with 1% 2-mercaptoethanol, suggested that there are four identical subunits/mol of enzyme. The weight-average molecular weight was extrapolated to 55000 (S.E.M. $\pm 5\%$) at zero protein concentration, and the z-average molecular weight was extrapolated to 52000 (S.E.M. $\pm 4\%$) (Fig. 4). The mobility of the reduced dissociated protein on sodium dodecyl sulphate-polyacrylamide gels corresponded to a mol.wt. of 56000 (S.E.M. $\pm 4\%$) (Fig. 5).

Reactivity of thiol groups

This was investigated to find suitable conditions for the incorporation of a spin-label reporter group into a specific site on the enzyme. In the absence of any denaturant, 3.33 (S.D. ± 0.1) thiol groups/subunit reacted with 5,5'-dithiobis-(2-nitrobenzoate) at pH 7.5. In the presence of 0.5% sodium dodecyl sulphate, 5.67 (S.D. ± 0.35) thiol groups/subunit reacted. Of the groups reacting in the native state, 0.65 (S.D. ± 0.2)/subunit reacted within the time taken for mixing, whereas the remainder reacted with a half-life of 2 min.

Fig. 5. Calibration of 6% sodium dodecyl sulphate-polyacrylamide gels

Tr (and Tr₂) = human transferrin, mol.wt. 76000 (and 152000); G = components of γ -globulin, mol.wt. of G1 = 23500, of G2 = 50000 and of G3 = 73500; R = rabbit muscle pyruvate kinase, mol.wt. 59000; A = aldolase, mol.wt. 37000; T = chicken muscle triose phosphate isomerase, mol.wt. 26000. The open circle is yeast pyruvate kinase. Mobilities are expressed relative to Bromophenol Blue.

Detailed analysis of the kinetics of labelling by iodoacetamide was not possible as denaturation and precipitation of the enzyme occurred before the reaction had reached completion. Qualitatively, it is apparent from the reaction curves that 0.3–0.5 groups/subunit were reacting very rapidly, and that the remaining groups were reacting much more slowly (Fig. 6). Iodoacetamide was shown to react first with the rapidly reacting thiol groups, which react with 5,5'-dithiobis-(2-nitrobenzoate), by withdrawing samples at various times from a solution containing 1 mM-iodoacetamide and 2.3 mg of pyruvate kinase/ml and reacting them with a limiting amount of the reagent. Under these conditions the rapidly reacting thiol groups still reacted within the time taken for mixing, but the remaining groups reacted more slowly, so the reaction curve itself could be extrapolated to zero time, giving the number of rapidly reacting groups. The reaction with iodoacetamide was making these groups, estimated as 0.6/subunit, unavailable to 5,5'-dithiobis-(2-nitrobenzoate) (Fig. 6). The maximal specific activity of the enzyme remained constant when these groups were reacting with iodoacetamide (cf. Wieker & Hess, 1972).

These results indicate that the stoichiometry of the reaction of the thiol groups is 0.5 groups/subunit that

Fig. 6. Reaction of the thiol groups of pyruvate kinase with iodoacetamide

○, The reaction was followed by the incorporation of ^{14}C ; enzyme concentration 1.6 mg of protein/ml, 1 mM-iodoacetamide, 0.1 M-KCl, 0.1 M-Tris-HCl buffer, pH 7.5, temperature 25°C. ●, The reaction was followed by loss of groups reacting rapidly with 5,5'-dithiobis-(2-nitrobenzoate); enzyme concentration 2.3 mg of protein/ml and other conditions as above.

Scheme 1. Rapid disulphide interchange in pyruvate kinase

react rapidly with both 5,5'-dithiobis-(2-nitrobenzoate) and iodoacetamide; 3.5 groups that are accessible to 5,5'-dithiobis-(2-nitrobenzoate) without addition of a denaturant, and a further 2.5 groups that react in the presence of a denaturant, giving a total of six groups. Bondar & Suelter (1971) reported that 1.5 groups/subunit of pyruvate kinase from *S. cerevisiae* reacted with 5,5'-dithiobis-(2-nitrobenzoate) in 10 s, and of a total of six groups, 3.5 reacted in the 'native' state of the protein. Wieker & Hess (1972) found that one group/subunit reacted rapidly with 5,5'-dithiobis-(2-nitrobenzoate), and three groups of a total of six reacted without the addition of denaturant. The most significant difference between these results is in the number of rapidly reacting thiol groups. (The degree of agreement is unexpected, since different

molecular weights were used to derive molar concentrations of enzyme in the three cases.) This may reflect genuine differences in the primary structure of the enzymes from different strains of yeast, which either affect the reactivity of the thiol groups directly or which affect the possibility of a rapid disulphide interchange of the type shown in Scheme 1, which affects the apparent stoichiometry of the reaction of the rabbit muscle pyruvate kinase with 5,5'-dithiobis-(2-nitrobenzoate) (Flashner *et al.*, 1972). The latter possibility is suggested since Wieker & Hess (1972) found that if their yeast enzyme was isolated after reaction of one thiol group/subunit, the protein did not contain 5-thio-2-nitrobenzoate (NbS).

The four subunits of yeast pyruvate kinase are believed to be composed of four identical polypeptide chains, since they are not resolved by electrophoresis in dissociating conditions, and Bornmann *et al.* (1972) have shown that degradation by CNBr or trypsin gives the number of peptides expected for identical chains. Therefore for it to be possible to observe less than four thiol groups reacting/mol of enzyme, either the four subunits are not equivalent in the molecule, or, less probably, there are four equivalent thiol groups at a site where steric hindrance prevents the entry of more than two iodoacetamide or thionitrobenzoate moieties. A heterologous tetramer has also been proposed by Kuczenski & Suelter (1971) to explain the results of their studies on the inactivation of the enzyme catalysed by fructose diphosphate. Similar instances have been documented by Levitzki *et al.* (1971) in connexion with 'half-the-sites' reactivity.

The presence of either of the substrates ADP or phosphoenolpyruvate did not significantly affect the reaction of the enzyme with either 5,5'-dithiobis-(2-nitrobenzoate) or iodoacetamide. However, the allosteric effector fructose diphosphate increased the rate of reaction with iodoacetamide and the extent of the reaction with 5,5'-dithiobis-(2-nitrobenzoate). In the latter case, although more than 3.5 groups reacted/subunit, the enzyme began to precipitate, so that the reaction could not be accurately quantitated.

Although it was possible to demonstrate the covalent bonding of a free-radical derivative of iodoacetamide [*N*-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl)iodoacetamide] to the rapidly reacting thiol groups by the reaction of the enzyme with 5,5'-dithiobis-(2-nitrobenzoate), the e.s.r. spectrum of the bound label was almost insensitive to the presence of phosphoenolpyruvate, ADP, pyruvate, ATP or fructose diphosphate.

Proton-relaxation-rate enhancement

The binding of Mn^{2+} ion to the enzyme was re-investigated, by using a combination of the measurement of the proton-relaxation-rate enhancement of

Fig. 7. Binding of Mn^{2+} to yeast pyruvate kinase

Conditions were: 25°C; 0.08M-KCl-0.02M-Tris-HCl, pH 7.2; enzyme concentrations in the range 10–30 μM . ●, Values determined by e.s.r. spectrometry; ○, values obtained by proton-relaxation enhancement, calculated with a very approximate value of E_b obtained from some combined e.s.r. and proton-relaxation enhancement determinations. ----, Results obtained by Fell *et al.* (1972). -.-.-, Binding curve calculated from data of Mildvan *et al.* (1971).

water and e.s.r. measurements of free Mn^{2+} concentrations. In contrast with the previous results of Mildvan *et al.* (1971) and of Fell *et al.* (1972), it was found that the binding of Mn^{2+} to the enzyme was so weak that it was not possible to determine the number of binding sites or the dissociation constant from measurements in the experimentally accessible range of concentrations (Fig. 7). Further, as the e.s.r. measurements showed that the fraction of the total Mn^{2+} bound to the enzyme was small, it was not possible to determine accurately the enhancement characteristic of the binary enzyme- Mn^{2+} complex E_b . To calculate E_b it would have been necessary to assume the number of binding sites for Mn^{2+} and to have taken into account any heterogeneity (in terms of the dissociation constants and values of E_b) amongst the sites. However, these uncertainties would make little difference to the titrations with the substrate phosphoenolpyruvate. A possible explanation for the comparatively strong binding of Mn^{2+} reported previously is that this occurred at sites created by proteolysis of the enzyme. Mildvan *et al.* (1971) used enzyme prepared by the method of Hunsley & Suelter (1969a), and observed many bands in electrophoresis carried out under conditions where the enzyme had dissociated, and Fell *et al.* (1972) used enzyme prepared by the method of Ashton (1971). Both sets of workers claimed that only one enhancement value was needed to describe the binding curve,

but this is not necessarily inconsistent with heterogeneity of the sites (i.e. those reported in this study plus those which may have been a result of proteolysis). If the lifetime of the Mn^{2+} at each site was sufficiently short that, in the same time-scale of the experimental observations, a single Mn^{2+} ion could 'visit' each site, a single average enhancement would be observed. The limited binding we observed in the present study could have been either weak binding at the active site, or else binding to non-specific sites, or both. The present results would be consistent with the proposal, made by MacFarlane & Ainsworth (1972) on the basis of an analysis of the kinetics of yeast pyruvate kinase, that the essential bivalent metal ion did not bind at the active site until after both phosphoenolpyruvate and ADP had bound. In this respect the yeast enzyme differs from the rabbit muscle enzyme, for Ainsworth & MacFarlane (1973) showed that the latter exhibited random-order binding of phosphoenolpyruvate, ADP and Mg^{2+} , and this may be a sufficient explanation of the difference between the binding of Mn^{2+} to the yeast enzyme and to the rabbit muscle enzyme (e.g., Kayne & Price, 1972). In the present experiments, the enhancement was sensitive to the binding of phosphoenolpyruvate to the enzyme, although the half-point of the titration was at a higher substrate concentration than we had previously found (600 μM compared with about 150 μM).

Conclusions

This comparison of the properties of the yeast pyruvate kinase prepared by different methods underlines the problem of proteolysis during the isolation of enzymes from yeast, particularly if autolysis is used as the initial stage. Other examples of this are known, in addition to the work of Lazarus *et al.* (1966) on yeast hexokinase. The preparations from *S. cerevisiae* were more vulnerable in this respect, whereas the preparation from *S. carlsbergensis* gave a product of similar size to the one described here. However, the cold-lability and the different extinction coefficient previously observed for the pyruvate kinase from *S. cerevisiae* were not the result of partial proteolysis, as the same properties were found in these studies for the undegraded enzyme. Therefore these differences must have arisen during the divergence of these two strains of yeast.

That the allosteric effector, fructose diphosphate, affects the thiol-group reactivity and the cold-lability of the enzyme, whereas the substrate that exhibits co-operative interactions, phosphoenolpyruvate, does not influence these properties, suggests that any model of allosteric enzymes which assumes only two conformational states of the subunits will not be adequate to describe fully the allosteric behaviour of the pyruvate kinase of *S. cerevisiae*.

On the basis of their studies of the cold-lability of yeast pyruvate kinase, Kuczenski & Suelter (1970a) suggested that the enzyme was not a homologous tetramer, but a dimer of dimers. This conclusion was reinforced by their later demonstration of the existence of a dimer intermediate in the dissociation promoted by fructose diphosphate at 23°C (Kuczenski & Suelter, 1971). The observation of fractional reactivity of the thiol groups reported here would be consistent with their proposal. Thus there is a contrast between the quaternary structures of the yeast enzyme and of mammalian pyruvate kinase, which does not exhibit co-operative kinetics with phosphoenolpyruvate and fructose diphosphate, for the X-ray crystallographic studies of Muirhead & Stammers (1974) have shown that the four subunits of the enzyme from cat muscle are disposed in a symmetrical, tetrahedral arrangement.

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