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Yeast Pyruvate Kinase. Native and Subunit Molecular Weight*

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ABSTRACT: Yeast pyruvate kinase was shown to have a molecular weight of 162,000 to 168,000 as calculated with the Svedberg equation from data obtained under two sets of solvent conditions given below, and by the high-speed equilibrium method at low protein concentration. The enzyme sediments as a single symmetrical peak with an $s^0_{20,w}$ of 8.85 S and a $D^0_{20,w}$ of 4.84×10^{-7} cm^2 sec^{-1} in 0.1 M tetramethylammonium cacodylate buffer, pH 6.2, containing 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, and 10^{-3} M fructose 1,6-diphosphate. In 0.1 Tris·HCl, pH 7.5, the values obtained are 8.34 S for $s^0_{20,w}$, and 4.52×10^{-7} cm^2 sec^{-1} for $D^0_{20,w}$.

The enzyme was shown to be a tetramer, each polypeptide chain having a molecular weight of 42,000 to 45,000. Complete dissociation was obtained in 6 M guanidine hydrochloride–0.15 M 2-mercaptoethanol. Utilizing sedimentation equilibrium under these solvent conditions, similar values for $M_w{}^0$ and $M_z{}^0$ were obtained indicating the subunits had approximately equal molecular weights.

Dissociation was also obtained by extensive treatment of the enzyme with maleic anhydride, resulting in a symmetrically sedimenting peak with $M_w{}^0(s/D)$ of 42,200 excluding bound maleyl groups. Some physical characteristics of yeast, rabbit muscle, and rat liver pyruvate kinases are compared.

Investigations in this laboratory have been directed toward a clearer understanding of the conformational changes of yeast and muscle pyruvate kinase associated with the binding of substrates and effectors. Fundamental for an analysis of such changes is a characterization of the molecular weight of the enzymes and determination of the size and number of subunits associated therewith. We report the results of an investigation of the native and subunit molecular weights of yeast pyruvate kinase. These results are compared with those obtained with other preparations.

Methods

Preparation of Enzyme. Pyruvate kinase was isolated from fresh "Budweiser" Baker's yeast (Anheuser-Busch, Inc.) according to the procedure of Hunsley and Suelter (1969a) and stock enzyme was stored as a suspension in 90% saturated (3.6 M) $(NH_4)_2SO_4$. Protein concentrations were estimated from the absorbance at 280 nm ($E^{0.1\%}_{1\ cm}$ 0.653) (Hunsley and Suelter, 1969a). Kinetic assays were performed under the conditions and with reagents described by Hunsley and Suelter (1969b). All enzyme preparations had a minimum specific activity of 210 μmoles/min per mg at 30°. Stock enzyme was equilibrated with appropriate buffers by chromatography on Sephadex G-25 (coarse). Aliquots of the protein were tested for complete removal of $(NH_4)_2SO_4$ with saturated $BaCl_2$. Unless otherwise noted, the enzyme, after passage over Sephadex, was allowed to stand at room temperature for at least 3 hr before initiation of ultracentrifugal studies (Kuczenski and Suelter, 1970).

Ultracentrifugal Analysis. A Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and an RTIC unit was used for all sedimentation experiments. Sedimentation velocity experiments were run at 59,780 rpm near 20°. Diffusion coefficient experiments were performed in double-sector synthetic boundary cells at 4908 rpm at 20° and the coefficients were calculated using height-to-area analysis (Schachman, 1957).

A molecular weight for native enzyme was also determined using the meniscus depletion technique of Yphantis (1964). Runs were performed at 20° with a rotor speed of 15,200 rpm using Rayleigh interference optics and the six-channel Kel-F centerpiece designed by Yphantis (1964). Rayleigh patterns were recorded on Kodak 11-G photographic plates.

The short-column sedimentation equilibrium technique of Van Holde and Baldwin (1958) was used for molecular weight determinations in 6 M guanidine hydrochloride. These experiments were run for 30 hr (results remained constant from 30 to 40 hr) near 20° with a solution column depth of 1.7 mm. Enzyme for these experiments was prepared by extensive dialysis (48 hr) against the appropriate guanidine

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TABLE I: Sedimentation and Diffusion Coefficients of Yeast Pyruvate Kinase.

Conditions[a]	$s^0_{20,w}$ (S)	$[\Delta s_{20,w}/mg]^b$ (S)	$D^0_{20,w}$ ($\times 10^{-7}$ cm^2 sec^{-1})	$[\Delta D_{20,w}/mg]^b$ ($\times 10^{-7}$ cm^2 sec^{-1})	Concentration Range (mg/ml)
			Native Enzyme		
A	8.85 ± 0.02	−0.13			2–11
			4.84 ± 0.19	−0.010	2–13.5
B	8.35 ± 0.04	−0.081			1.3–12.5
			4.52 ± 0.03	−0.009	
			Maleylated Subunits		
C	1.97 ± 0.02	−0.105			2.1–7.1
			3.75 ± 0.06	+0.067	3.0–7.1

[a] All experiments were performed at 20° in (A) 0.1 M tetramethylammonium cacodylate buffer, pH 6.2, containing 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, and 10^{-3} M fructose 1,6-diphosphate, (B) 0.1 M Tris·HCl, pH 7.5, or (C) 0.1 M $K_2B_2O_7$ buffer, pH 9.0, containing 0.1 M KCl. Extrapolations to zero protein concentration were least-squares plots, ± standard deviations. [b] The Δ values represent the slope of the least-squares line derived from a plot of the indicated parameter *vs.* protein concentration.

hydrochloride solution at 4°. Densities were determined by pyncnometry. Viscosities of Gd·HCl[1] solutions were interpolated from data of Kawahara and Tanford (1966). All other viscosities were based on data from Bates and Baxter (1929), or from Svedberg and Pederson (1940). Calculations, including statistical analyses of the data, were performed on a Control Data Corporation 3600 digital computer using tested programs [sedimentation velocity, diffusion coefficients, and low-speed equilibrium, W. C. Deal, Jr., in preparation; high-speed equilibrium,[2] Small and Resnick (1965)].

Maleylation of Protein. A modification of the procedure of Freedman *et al.* (1968) was used to prepare maleylated yeast PK.[2] Stock enzyme (20 mg) in 1.0 ml was dialyzed extensively against 0.05 M sodium borate buffer, pH 9.0, at 0°. Maleic anhydride (100 μl; Aldrich) in acetone (0.5 g/ml) was added to this solution in 10-μl aliquots over a 30-min period. pH 9 was maintained with 5 N NaOH utilizing a Radiometer TTT-1–SBR2-SBU1–TTA31 automatic recording titrator. The protein was then dialyzed for 2 days against several changes of 0.1 M KCl–0.1 M potassium borate, pH 9.0.

Reagents. Gd·HCl was either Mann Ultra Pure and used directly, or was obtained from Eastman as the carbonate and converted into the hydrochloride according to the procedure of Kawahara *et al.* (1965). Mercaptoethanol (Sigma) was redistilled before use. All other reagents were used without further purification.

Results

Native Enzyme. Initial attempts to determine the molecular weight of the native enzyme were performed in 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, 10^{-3} M tetracyclohexylammonium, $FruP_2$, and 0.1 M tetramethylammonium cacodylate buffer, pH 6.2. The sedimentation constant in this solvent found by extrapolation to zero protein concentration ($s^0_{20,w}$) was 8.85 S (Table I). In a similar manner, the diffusion coefficients extrapolated to zero protein gave a value of $D^0_{20,w} = 4.84 \times 10^{-7}$ cm^2/sec (Table I). These values, together with a partial specific volume of 0.734 cc/g as calculated from the amino acid content (Hunsley and Suelter, 1969a) using the procedures of McMeekin and Marshall (1952), yielded a weight-average molecular weight ($M_w{}^0(s/D)$) of 166,500.

During the above (s/D) experiments, a slow precipitation of protein, particularly at higher protein concentrations, was observed. This problem was eliminated in 0.1 M Tris, pH 7.5, and thus the studies were repeated in this solvent. Values of $s^0_{20,w} = 8.34$ S (Table I), $D^0_{20,w} = 4.52 \times 10^{-7}$ cm^2/sec (Table I), and $M_w{}^0(s/D) = 168{,}100$ were obtained.

The molecular weights of the native enzyme as determined by the meniscus depletion technique are plotted in Figure 1 as number-average molecular weight *vs.* concentration in fringes for enzyme in 0.1 M Tris·HCl, pH 7.5, containing 0.23 M KCl, 0.025 M $MgCl_2$, 2×10^{-3} M $FruP_2$, and 10^{-2} M phospho(enol)pyruvate. The enzyme retained 92% of its initial activity when allowed to stand for 24 hr at 20° at 0.2 mg/ml in this solvent. The enzyme was not sufficiently stable for a 24-hr period at 0.2 mg/ml to determine molecular weight in the absence of $FruP_2$.

Subunits. Preliminary sedimentation equilibrium experiments with yeast PK in 6 M Gd·HCl indicated the need for a high concentration of reducing agent to eliminate what appeared to be a random aggregation of the protein. Figure 2 presents data, plotted according to Van Holde and Baldwin (1958), from a typical sedimentation equilibrium experiment with yeast PK in 6 M Gd·HCl containing 0.1 M 2-mercaptoethanol. Data for M_w and M_z of yeast PK at several concentrations are plotted and extrapolated to zero protein concentration in Figure 3. Values for $M_w{}^0$ and $M_z{}^0$ were calculated to be 41,400 and 45,900, respectively. Under the same solvent conditions the enzyme sedimented as a single symmetrical peak ($s^{0.8\%}_{20,w} = 1.09$ S).

To further characterize the subunits, efforts were made to dissociate the enzyme through the introduction of negative charges by maleylation. Under the conditions described in Methods, 230 moles of maleate/168,000 g of protein (11.9%

[1] Abbreviations used are: PK, pyruvate kinase; $FruP_2$, fructose 1,6-diphosphate; Gd·HCl, guanidine hydrochloride.

[2] The assistance of Mr. John Gerlt in adapting this program to the CDC 3600 is gratefully acknowledged.

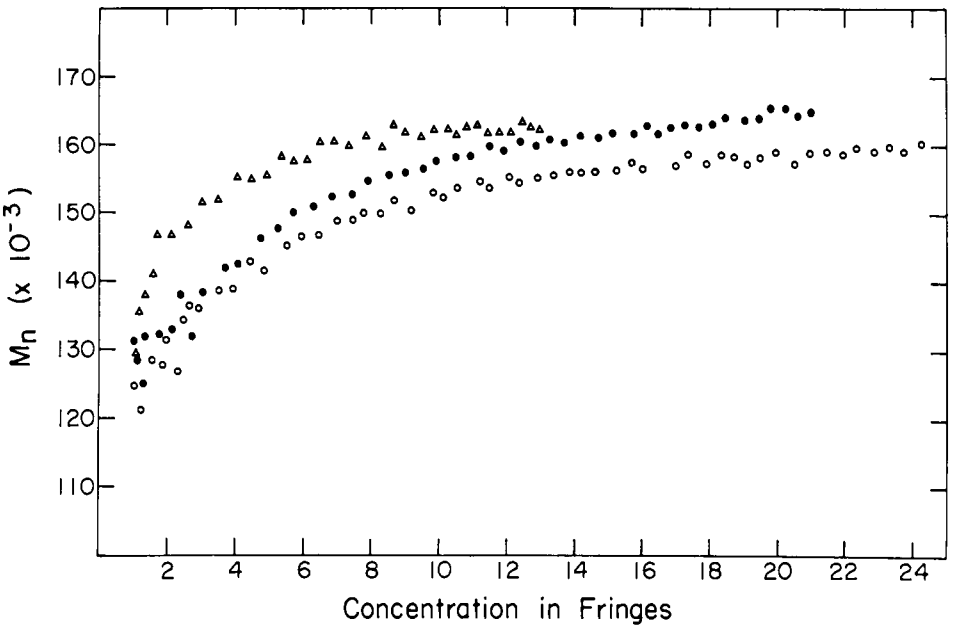

FIGURE 1: Number-average molecular weight as a function of fringe concentration for native yeast pyruvate kinase at 20° in 0.1 M Tris·HCl, pH 7.5 containing 0.23 M KCl, 0.025 M $MgCl_2$, 2×10^{-3} M $FruP_2$, and 10^{-2} M phospho(enol)pyruvate. Δ represents an initial concentration of 0.20 mg/ml; ●, 0.40 mg/ml and ○, 0.60 mg/ml.

of the resultant molecular weight) were incorporated as determined by the spectrophotometric assay of Freedman *et al.* (1968). Extensive dialysis and a G-25 Sephadex treatment of the maleylated enzyme did not alter the absorption coefficient of the enzyme at 250 nm, suggesting complete removal of unbound maleate.

Results for the sedimentation and diffusion coefficients of the maleylated enzyme determined as a function of protein concentration are shown in Table I. The values of $s^0_{20,w} = 1.97$ S and $D^0_{20,w} = 3.75 \times 10^{-7}$ cm² sec⁻¹ yielded a molecular weight of $M_w{}^0(s/D) = 47{,}900$. Subtracting 5700 for the contribution of the bound maleyl groups gave a net molecular weight of 42,200 for the subunits. Addition of 0.1 M 2-mercaptoethanol to the maleylated enzyme affected neither the sedimentation nor the diffusion coefficients.

Discussion

From sedimentation and diffusion data extrapolated to zero protein concentration under two different solvent

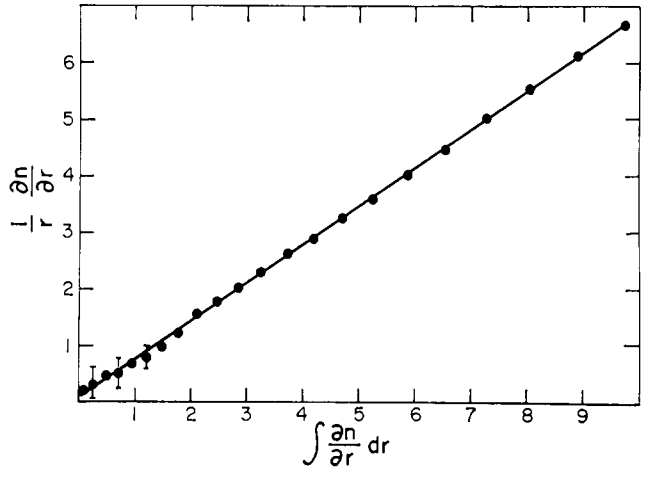

FIGURE 2: Molecular weight of yeast PK at 1.75 mg/ml in 6 M Gd·HCl containing 0.15 M 2-mercaptoethanol, as determined by the short-column sedimentation equilibrium technique after 30 hr. The rotor speed was 21,708 rpm and the temperature was 20°. $\partial n/\partial r$ is the refractive index gradient in arbitrary units and r is the radial distance (cm). The abscissa represents the integrated area under the refractive index gradient curve, beginning at the inner meniscus. The line is a least-squares plot.

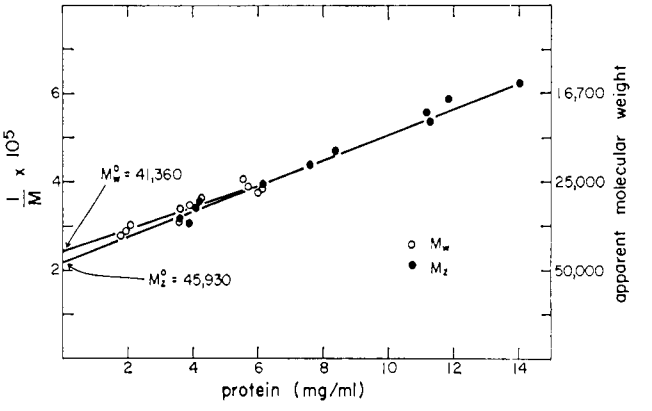

FIGURE 3: Extrapolation of the apparent weight-average ($M_w{}^{app}$) and apparent z-average ($M_z{}^{app}$) molecular weights of yeast PK to zero protein concentration. The solvent system contained 6 M Gd·HCl and 0.15 M 2-mercaptoethanol. The temperature was 20°. Centrifugation was carried out at 21,708 rpm for 30 hr. Concentrations were evaluated as $(c_m + c_b)/2$ for $M_w{}^{app}$ and $(c_m + c_b)$ for $M_z{}^{app}$, where c_m and c_b are the concentrations at the meniscus and bottom of the cell, respectively. The lines are least-squares plots.

TABLE II: Comparison of Physical Properties of Pyruvate Kinase Native Enzymes.

Parameter	Muscle	Yeast
$s^0_{20,w}$ (S)	10.04[a]	8.34
$D^0_{20,w}$ (cm^2 sec^{-1})	3.96×10^{-7} [a]	4.52×10^{-7}
f/f_0 (cc/g)	1.31[b]	1.29
$\bar{v}$	0.740[a,b]	0.734
$M_w{}^0$ (s/D)	237,000[a]	167,000
	Subunits	
$M_w{}^0$	57,100[c]	42,000
$M_z{}^0$	57,100[c]	45,000

[a] Warner (1958). [b] Steinmetz (1966). [c] Steinmetz and Deal (1966).

conditions, a weight-average molecular weight near 167,000 for native yeast pyruvate kinase was calculated with the Svedberg equation. A molecular weight near 165,000 was also determined under a third solvent condition using the high-speed equilibrium technique developed by Yphantis (1964). Since the molecular weight of the subunits is near 42,000 as determined for the dissociated enzyme in 6 M Gd·HCl containing 0.15 M 2-mercaptoethanol, and after maleylation, it is concluded that yeast pyruvate kinase, like muscle pyruvate kinase (Steinmetz and Deal, 1966), is composed of four subunits. That these subunits are very similar in size is consistent with the following observations: (1) maleylated enzyme sediments as a single nearly symmetrical peak; (2) the $M_w{}^0$ of 42,200 from sedimentation and diffusion data for the maleylated enzyme is in close agreement with $M_w{}^0$ of 41,400 obtained from sedimentation equilibrium data in Gd·HCl (Figure 3); (3) the homogeneous molecular weight distribution for the dissociated protein throughout the centrifuge cell (Figure 2); and (4) a single band of molecular weight near 42,000 obtained by Rose (1969) after electrophoresis of the enzyme in sodium dodecyl sulfate on sodium dodecyl sulfate–polyacrylamide gels (Shapiro *et al.*, 1967). No evidence is available regarding the chemical identity of the subunits in this preparation.

The plots of the molecular weight (M_n) *vs.* protein concentration in fringes (Figure 1) shows the presence of lower molecular weight components at low protein concentrations indicative of a protein concentration dependent dissociation. This is consistent with a previous demonstration (Kuczenski and Suelter, 1970) that yeast pyruvate kinase exhibits an instability inversely proportional to protein concentration which involves a protein dissociation to lower molecular weight species. The pattern of M_n as a function of protein concentration (Figure 1) obtained by the meniscus depletion technique (Yphantis, 1964) provides according to Harris *et al.* (1969) an adequate method for distinguishing between a protein participating in a rapid chemical equilibrium, and a mixture of nonequilibrating or slowly equilibrating protein species. A protein in chemical equilibrium should have molecular weight moments which are a function only of concentration throughout the cell. Hence, graphs of molecular weight *vs.* concentration should superimpose. On the other hand, a heterogeneous system not in chemical equilibrium, or one participating in a very slow equilibrium should have a distribution of mass which depends only on distance from the center of rotation (Harris *et al.*, 1969). The data presented in Figure 1 are consistent with the latter situation. Yet the enzyme at the higher concentration appears essentially homogeneous with a molecular weight approaching 165,000 in good agreement with the molecular weights calculated from the Svedberg equation.

Haeckel *et al.* (1968) have reported a molecular weight near 200,000 for yeast PK determined by gel filtration techniques. Data obtained in this laboratory (Hunsley, 1970) using the same technique yielded a nonhomogeneous system with molecular weights ranging as high as 300,000. Since the enzyme has been shown to aggregate to a higher molecular weight species in concentrated $(NH_4)_2SO_4$ (Kuczenski and Suelter, 1970), since preliminary sedimentation data in aqueous glycerol indicate the presence of higher molecular weight species, and since there is no evidence that yeast pyruvate kinase is a glycoprotein, the data are consistent with an aggregation being favored in polyhydroxylic solvents such as glycerol or when passed through Sephadex materials rather than a more expanded protein structure for many glycoproteins as observed by gel filtration techniques (Andrews, 1965).

Comparison of the physical properties of yeast and rabbit muscle PK given in Table II points out a marked difference in the molecular weight of the subunits of these two preparations. Similar differences have been reported for rabbit muscle and yeast enolase (Klotz and Darnall, 1969; Brewer and Weber, 1968). However, yeast and rabbit muscle aldolase have similar subunit molecular weights (Klotz and Darnall, 1969) precluding a clear pattern of molecular weight differences between glycolytic enzymes from yeast and rabbit muscle. In addition to the large difference in the molecular weights of the yeast and muscle PK, the yeast enzyme, but not the enzyme from muscle, exhibits cooperative saturation kinetics (Reynard *et al.*, 1961; Haeckel *et al.*, 1968; Hunsley and Suelter, 1969b). Still, each contains four subunits, requires both monovalent and divalent cations for catalytic activity, and catalyzes the same reaction. Although the rat liver enzyme resembles the yeast enzyme in kinetic properties (Taylor and Bailey, 1967), its molecular weight has been estimated at 208,000 (Tanaka *et al.*, 1967). The apparent similarities and dissimilarities among these enzymes suggest that an examination of their more subtle properties could lead to an increased understanding of the mechanisms of enzyme catalysis.

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