THE MOLECULAR WEIGHT OF A YEAST PYRUVATE KINASE AND ITS SUBUNITS

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Received 11 March 1971
Revised version received 7 May 1971

1. Introduction

Recent attempts to elucidate the structure of yeast pyruvate kinase (EC 2.7.1.40) have shown some discrepancies in the values quoted for the molecular weights of both the enzyme and its subunits. Kuczenski and Suelter [1] reported a value of 166,000–168,000 for the molecular weight of the undissociated enzyme and that dissociation in 6 M guanidinium hydrochloride (GdHCl) containing 0.1 M β-mercaptoethanol yielded subunits for which values of M_w and M_z of 41,400 and 45,900, respectively, were obtained. Bischofberger et al. [2] found that in 8 M urea and in 6 M GdHCl (with 10^{-2} mg/ml dithioerythritol) the enzyme dissociated into subunits of M_w 62,000 and that their native enzyme had a molecular weight in the range 185,000–200,000. It should be noted that in both of these studies [1, 2], the partial specific volume $\bar{v}$ (ml/g) in dilute buffers was accorded a value (0.734 ml/g) calculated from the amino acid composition; and for GdHCl as solvent, the approximate adjustment of Ullman et al. [3] was applied to $\bar{v}$ by Bischofberger et al. [2]. In the following, a re-examination of this problem is described in which, in particular, conditions for stability of the intact enzyme have been ascertained, and it is concluded that pyruvate kinase from *Saccharomyces cerevisiae* contains 8 subunits each of molecular weight 20,000.

* Contribution from the Oxford Enzyme Group.

2. Methods

Pyruvate kinase was prepared from fresh brewer's yeast (*S. cerevisiae*) obtained locally. Cytolysis and ammonium sulphate fractionation was carried out according to the modified procedure of Hess and co-workers [2, 4] and further purification was achieved by chromatography on CM- and DEAE-cellulose columns. The pyruvate kinase had a specific activity of 150–200 IU/mg determined by their assay method [4]. Only one protein band was observed on polyacrylamide gel electrophoresis at pHs between 6.5 and 9, or by micro-isoelectric focusing on polyacrylamide gels.

Sedimentation studies of the native enzyme were carried out at 8–10° in a Beckman Spinco Model E ultracentrifuge with 0.08 M KCl, 0.01 M tris-HCl (pH 7.0) plus 100 mM fructose-1,6-diphosphate ($FruP_2$) as the solvent. The viscosity of this solvent was determined in a capillary viscometer, and its density and all other densities (to determine, *inter alia*, $\bar{v}$ of the enzyme in the various solvents) were measured in a Digital Densimeter (Anton Paar, Graz, Austria). Sedimentation velocity experiments were conducted at 56,100 rpm and diffusion coefficients were obtained from runs in a synthetic boundary cell at 10,589 rpm using Schlieren optics. Sedimentation equilibrium at 4,609 rpm was performed with the omission of fluorcarbon oil as it seemed to cause aggregation of the native enzyme. The initial concentrations of pyruvate kinase were determined by differential refractometry at 8°. The white-light fringe method of Richards and Schachman [5] was used for the determination of the hinge points and measurements

of the photographs were made by means of a micro-comparator [6]. A molecular weight for the native enzyme was also determined at 16,200 rpm by the meniscus-depletion technique of Yphantis [7].

Recent attempts [1, 2] to dissociate pyruvate kinase have shown that a high concentration of reducing agent is necessary to prevent random aggregation of the subunits, so the following procedure was used. The enzyme was dialysed against 0.1 M phosphate buffer at pH 7.0 for 2–3 hr then treated for 2 hr at room temperature with an amount of *N*-ethyl maleimide (NEM) calculated to be 40 times that required to react with the –SH groups, on the basis of two cysteine per minimum mole weight [2]. Dissociation was completed by dialysis for 48 hr at room temperature against approximately 2 M or 6 M GdHCl. Any precipitate which formed in the phosphate buffer was filtered off; that which occasionally appeared during the NEM reaction redissolved in the guanidine solvent.

Sedimentation equilibrium of the resulting subunits was performed in the 2 M or 6 M GdHCl at 20° at speeds between 16,200 and 24,630 rpm which depended on the protein concentrations. The latter were determined by fringe measurements in synthetic boundary runs. Osmotic pressure measurements of the subunits in 6 M GdHCl were made at 25° by means of a Mechrolab 503 osmometer by procedures similar to those described by Paglini [8].

Dissociation of the enzyme was also effected by dialysis against 1% sodium dodecyl sulphate (SDS) and 1% β-mercaptoethanol in 0.1 M phosphate (pH 7.2) at 37° for two hours. Electrophoretic separation on 7% and 10% polyacrylamide gels containing SDS (0.1%) at pH 7.0 was performed by a modification of the method of Shapiro et al. [9].

3. Results and discussion

The purified enzyme sedimented at 20° and 56,100 rpm as a single symmetrical Schlieren peak of coefficient 7 S (at ca. 4–5 mg/ml) in 0.1 M KCl, 0.01 M tris (pH 7.0). However, repeated velocity runs under these conditions over 40 hr showed a slow loss of peak area with formation of 2 S material and some precipitate and this process was greatly accelerated by storage of the centrifuge cell at 4° between runs. This is in agreement with the observations of cold lability made by Kuczenski and Suelter [10]. When the buffer included 10 mM $FruP_2$, dissociation was not observed after cold storage for 12 hr, while an increase in $FruP_2$ concentration to 100 mM was effective in preventing dissociation for up to 5 days in the cold, as was demonstrated by the constancy over 5–6 days of the area of its Schlieren peak (ca. 7–8 S) and of the activity assayed in the presence of 50% glycerol. All subsequent ultracentrifuge studies on the native enzyme were therefore conducted in a solvent containing 100 mM $FruP_2$, 0.08 M KCl and 0.01 M tris-HCl (pH 7.0), and the temperature was kept below 12° since otherwise precipitation was observed. The extrapolated values of $s^0_{20,w}$ and $D^0_{20,w}$ in this solvent were 8.5 S and 4.7×10^{-7} cm^2 sec^{-1}, respectively, which together with the calculated $\bar{v}$ of 0.734 ml/g [1, 4], gave $M_{s/D}$ = 165,000.

Sedimentation equilibrium experiments were carried out in the concentration range 4 to 27 fringes, and M_w was determined from the slopes of plots of ln J vs. r^2, which were linear (J = number of fringes at r cm from the centre of rotation). A plot of M_w^{-1} vs. J_0 (initial concentration in fringes) extrapolated to M_w = 161,000 at zero concentration (fig. 1). The meniscus-depletion study gave a plot of ln ΔY vs. r^2 (where ΔY is the fringe displacement from the meniscus value), which was slightly concave with respect to the r^2 axis. This is indicative of non-

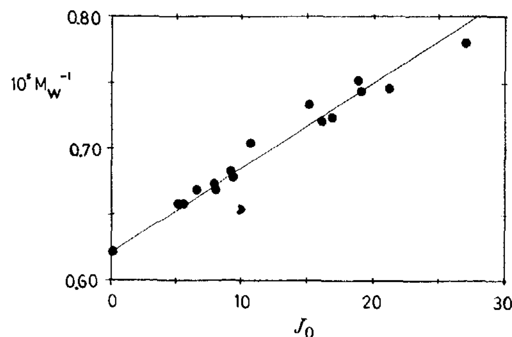

Fig. 1. Reciprocal of the weight-average molecular weight ($10^5 \times M_w{}^{-1}$) of yeast pyruvate kinase as a function of the initial concentration (J_0, units in fringes) in sedimentation equilibrium experiments. Solvent: 0.08 M KCl, 0.01 M tris buffer (pH 7.0), 100 mM $FruP_2$. Temperatures in the range 8–10°; 4,609 rpm. The intercept corresponds to M_w = 161,000.

ideality. The limiting slope yielded a value for M_w at J = 0.17 of 160,800.

Both 2 M and 6 M GdHCl appeared to be equally effective in causing the complete dissociation of the enzyme. The $\bar{v}$ of the enzyme in 2 M GdHCl was 0.724 ml/g by solution density measurements, and the same value was assumed for 6 M GdHCl. With these values, M_w in both 2 M and 6 M GdHCl was calculated from the linear four-fifths of the ln J vs. r^2 plots to be 21,000 ± 1,000 (S.D.) and to be independent of protein concentration. This substantial linearity indicated that the genuine subunits (having a molecular weight M_1) were of one size, but the departure from linearity near the bottom of the cell also indicated the presence of a species of larger molecular weight (M_2) which was estimated to be 80,000 by a subtraction method assuming a non-interacting mixture [11]. The analysis of Lloyd and Peacocke [6] was applied when curved Lamm plots were obtained and yielded values of M_2/M_1, which were fairly constant at 3.8 ± 0.30 at different r. Both analyses therefore indicated the presence mainly of subunits of molecular weight 19,500–22,000 and also of a much smaller proportion of a species of M_w of about 80,000–90,000.

Osmotic pressure (π) measurements, which were made on the same subunit preparations in 6 M GdHCl as were used in the ultracentrifuge studies, gave linear plots of π/c vs. c (where c is concentration in mg/ml) of zero slope, and showed that M_n was 19,620 ± 220 (S.D.). Electrophoresis on 10% polyacrylamide gels containing 0.1% SDS at pH 7.0 gave a single sharp band on each gel; the mobilities corresponded to molecular weights between 18,000 and 20,000 according to a calibration of these gels with 8 other proteins of known molecular weights in the range 17,000 to 147,000. On 7% gels an additional faint diffuse band in the 70,000–80,000 molecular weight region was obtained, and another faint band in the 160,000 region, which suggested that the enzyme was incompletely dissociated under these conditions. No other bands were observed on either gel.

4. Conclusion

Sedimentation studies have shown that the molecular weight of pyruvate kinase obtained from brewer's yeast is 161,000. This molecular weight agrees with that obtained by Kuczenski and Suelter [1] for pyruvate kinase from *S. cerevisiae* but not with that reported by Hess and coworkers [2, 4, 12] for their pyruvate kinase from *S. carlsbergensis*. Current studies [13] on the $FruP_2$ inactivation and cold lability of the pyruvate kinase preparations described in this note also confirm its similarity to those of Kuczenski and Suelter [10]. It appears that there may well be two distinct pyruvate kinases obtainable from yeasts. The existence of one size of subunit of molecular weight 20,300 ± 1000 in our pyruvate kinase was demonstrated by sedimentation equilibrium, osmotic pressure and gel electrophoresis studies, which is consistent with the minimum mole weight of 20,000 calculable from the amino acid analysis of Kuczenski and Suelter's enzyme [1] (the same value as calculated by Hess et al. for their enzyme [2]). This implies that our yeast pyruvate kinase contains 8 subunits. A small proportion of intermediates in the dissociation process, corresponding to 4 subunits, were observed during electrophoresis on acrylamide gels.

Acknowledgements

The authors gratefully acknowledge the support of the Medical Research Council by a Training Award (to K.A.) and in grants for equipment and running expenses (to A.R.P.). This work would not have been possible without the cooperation of Dr. D.G. Dalgleish, Mr. J.H. Diggle, Mr. D.A. Fell and Mr. J.D. McVittie to whom, with Mr. J.R.P. O'Brien, Director of this Department, we are much indebted.

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