YEAST PHOSPHOFRUCTOKINASE: PHYSICAL PARAMETERS, MOLECULAR WEIGHT AND SUBUNIT STRUCTURE

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1. Introduction

According to Lindell and Stellwagen [1], Liebe et al. [2], and Freyer et al. [3] highly purified yeast phosphofructokinase has a molecular weight of about 570,000.

Regarding its subunit structure considerable uncertainty originates from the fact that the enzyme protein may be partially degraded by proteolytic attack during purification and storage as well as in media containing sodium dodecylsulphate (SDS) [4]. Lindell et al. [1] found a polypeptide chain molecular weight of 44,000; according to Liebe et al. [2] the enzyme may contain subunits of 60,000 Daltons. While these studies were in progress Wilgus et al. [4] presented evidence for the existence of a polypeptide chain molecular weight of 1.0×10^5 and considered the products of splitting just described as proteolytically degraded fragments.

In this contribution the values of physical molecular parameters of yeast phosphofructokinase are presented since they are necessary for a precise calculation of its molecular weight. Furthermore, the subunit structure of yeast phosphofructokinase is reexamined in more detail. Finally, evidence is presented that the true molecular weight of undegraded yeast phosphofructokinase is apparently higher than hitherto assumed.

2. Materials and methods

The enzyme (specific activity 110–140) was prepared according to [5] omitting heat treatment, in absence or presence of 1 mM phenylmethylsulfonylfluoride (PMSF) (Serva, Heidelberg) or the more soluble *p*-aminoethyl-phenylsulfonylfluoride* as protease inhibitors. In the analytical ultracentrifuge (Phywe U 60 L; equipped with Schlieren-, interference- and UV-optics) the enzyme is homogeneous. High speed sedimentation equilibrium studies were performed at 10° or 20° according to Yphantis [6], using a multichannel-cell. Diffusion measurements were accomplished with the Schlieren optics in the Phywe U 60 L at low speed (10,000 rpm) according to Kawahara [7]. The partial specific volume was determined pycnometrically [8]. We cordially thank Dr. P. Bohley (Physiologisch-chemisches Institut, Halle/S.) for the precision measurements of protein concentration [9].

Enzyme dissociation in 6 M guanidine-HCl (Gd-HCl) was accomplished by storing the enzyme for 5–20 days at 4° in this solution containing 0.02 M Tris-HCl, 0.01 M NaCl, 1 mM EDTA, 0.1 M 2-mercaptoethanol and 1 mM PMSF. SDS-electrophoresis was performed according to Weber and Osborn [10], routinely in the presence of 5 mM PMSF. Sucrose density gradient centrifugation was carried out following Freyer et al. [3] with the exception that

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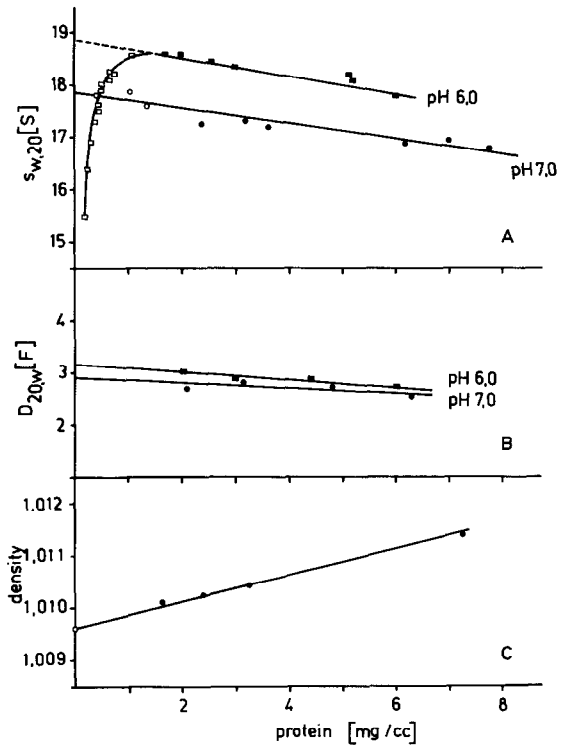

Fig. 1. Sedimentation (A) and diffusion (B) coefficients of yeast phosphofructokinase and medium density (C) as a function of protein concentration. Buffer: 0.1 M phosphate, 1 mM EDTA, 1 mM fructose 6-phosphate, 3 mM 2-mercaptoethanol, with or without 10 mM $(NH_4)_2SO_4$ pH 6.0 and 7.0. Full symbols: Schlieren optics; open symbols: UV-optics. Experiments in fig. 1A and 1B were performed at 20°; sedimentation velocity runs at 50,000 rpm, diffusion runs at 10,000 rpm. Measurements of density (fig. 1C) were carried out at 25° at pH 7.0 without $(NH_4)_2SO_4$. Accuracy of the microbalance was 1×10^{-6} g.

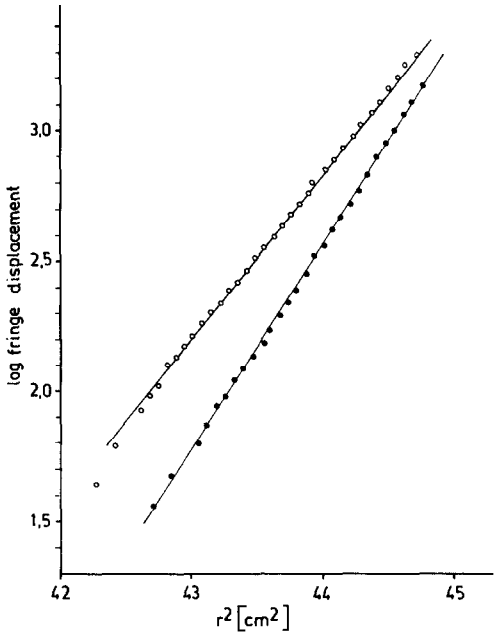

Fig. 2. Sedimentation equilibrium of yeast phosphofructokinase in 6 M guanidine hydrochloride. Open circles: Plot of log fringe displacement of the stored enzyme (1 mg/ml) against r^2 after 30 hr at 25,500 rpm and 20°. Incubation time in 6 M guanidine hydrochloride (see Materials and methods): 8 days. Full circles: Plot of fringe displacement of a freshly prepared enzyme (1 mg/ml) against r^2 under the same conditions; speed: 27,000 rpm.

the Beckman rotor Nr. SW 65 L Ti with a speed of 53,000 rpm (5 hr) was used. In calculations of physical constants the least square treatment was applied. The errors are expressed as standard deviations.

3. Results

Fig. 1 shows the dependence of sedimentation (A) and diffusion coefficiencts (B) as well as of medium densities (C) on enzyme concentration. A significantly higher sedimentation coefficient of the enzyme is observed at pH 6 than at pH 7. At pH 6 the enzyme dissociates below 1.5 mg protein per ml into smaller particles. At pH 7 a normal dependence of the sedimentation coefficients on protein concentration is obtained. From the slope of the straight line in fig. 1C, representing the buoyancy term $(1 - \bar{\nu}\rho)$ the partial specific volume $\bar{\nu}$ has been calculated to be 0.736 ± 0.008 cc per g. This value agrees well with that of rabbit muscle phosphofructokinase [11]. From these parameters the molecular weight of the enzyme was calculated using the Svedberg equation (table 1). The differences in the S-values of the enzyme at pH 6 and pH 7 evidently originate from different frictional ratios and do not arise from differences in the molecular weights. The molecular weights evaluated by sedimentation equilibrium and sucrose density centrifugation [2, 3] are also given. In the sedimentation equilibrium studies a straight

Table 1
Physical parameters of yeast phosphofructokinase at pH 6 and pH 7.

	Sedimentation constant (S)	Diffusion constant (F)	Molecular weight $M_{s,D} \times 10^{-3}$	Frictional ratio	Molecular weight sedimentation-equilibrium $M_W \times 10^{-3}$	Molecular weight density gradient-centrifugation $M \times 10^{-3}$ [2]
pH 6.0	$(s_{20,w})_c$ = 18.86(1−0.0083c)	$(D_{20,w})_c$ = 3.15(1−0.0241c)	567 ± 15	1.23	–	see lit. ref. [3]
pH 7.0	$(s_{20,w})_c$ = 17.85 (1−0.0082c)	$(D_{20,w})_c$ = 2.89(1−0.0187c)	586 ± 20	1.33	560 ± 20* (n = 4)	570 ± 28† (n = 12)

* Experimental conditions: The enzyme (0.8 mg/ml) was dialyzed about 40 hr against 0.1 M phosphate buffer pH 7.0, 2 mM fructose 6-phosphate, 3 mM 2-mercaptoethanol, speed: 10,600 rpm; temp. 10°, duration 24 hr.

† Stored enzyme

line is obtained by plotting the logarithm of the fringe displacement against r^2. These three independent methods result in a molecular weight of yeast phosphofructokinase of 560,000–580,000. With SDS-electrophoresis a subunit molecular weight of 96,000 is obtained with the exception that the performic acid oxidation product was only of about 85,000 Daltons (table 2).

The subunit molecular weight of phosphofructokinase in 6 M Gd-HCl was determined by sedimentation equilibrium centrifugation. Prior to centrifugation the enzyme was incubated at 4° for 5–20 days in the buffer described in the methods section. The experiments were performed by varying the duration of centrifugation between 25–40 hr. Equilibrium was reached after about 25 hr at 25,000 rpm. By plotting the logarithm of the fringe displacement against r^2 a straight line was obtained (fig. 2). Hence no systematic variation of the apparent molecular weight dependent on the distance from the centrifugation axis occurs. Following Hade and Tanford [12] we used for computation of the classical buoyancy term $(1-\bar{\nu}\rho)$ in 6 M Gd-HCl solution an apparent partial specific volume ϕ' which was assumed to be 0.01 or 0.02 cc/g respectively lower than the partial specific volume of the native enzyme. Taking these values into consideration, the experimental data of the fragmentation products obtained in 6 M Gd-HCl give molecular weights of 56,900 ± 1,500 (n = 5) or 53,500 ± 1,100 (n = 5), respectively. Hence, the SDS cleavage product is apparently composed of 2 polypeptide chains. Upon removing the Gd-HCl by dialysis and carrying out SDS-electrophoresis (see table 2), a reassociation occurs with formation of the 96,000 Dalton species, this being therefore evidently a dimer.

The experiments described below give strong evidence that a significant proteolytic alteration of the enzyme takes place during purification and storage of the isolated homogeneous enzyme at 4°.

If the time necessary for the preparation of homogeneous phosphofructokinase is considerably reduced (to about 20 hr), always in the presence of PMSF, significantly higher sedimentation coefficients (19 S) of the undissociated enzyme are found than with material from a preparation lasting 4–5 days. Within a few days after termination of such an accelerated preparation a gradual decrease of the $s_{20,w}$-value is observed, reaching a plateau at about 17 S, which corresponds to the value described above (fig. 3). Moreover, the 19 S enzyme molecule is dissociated by SDS not only into one subunit species, as with the 17 S molecule, but into 2 different cleavage products of 96,000 Daltons (fraction 1) and 131,000 Daltons (fraction 2) (fig. 4A). Both products are present in about equal amounts in fresh enzyme preparations as judged by protein staining intensity.

Fraction 2 disappears within a few days (fig. 4B). This finding is consistent with the observation presented in fig. 3. If the 131,000 Dalton fraction is eluted from the sliced gels and electrophoresed in SDS

Table 2
Subunit molecular weights of yeast phosphofructokinase determined by SDS-electrophoresis [10].

Conditions of preincubation	Molecular weights $\times 10^{-3}$
1) 12 hr, 0.01 M sodium phosphate pH 7.0 1% SDS, 1% 2-mercaptoethanol.	96 ± 6 (n = 8)
2) 15 hr, in 6 M urea, redialysis against buffer 1).	96 ± 4 (n = 4)
3) 15 hr, 6 M guanidine hydrochloride, redialysis against buffer 1) at 50°.	97 ± 2 (n = 3)
4) performic acid oxidation according to [15], redialysis against buffer 1).	about 85

in the absence of PMSF a time dependent conversion of this species to the 96,000 Dalton one is observed. Simultaneously smaller peptide material appears in these gels.

In 6 M Gd-HCl the 19 S molecule dissociates into products of 62,800 ± 1500 (n = 3) or 59,000 ± 1000 (n = 3) Daltons (depending on the $\Delta\bar{\nu}$-value taken; see above) evaluated by means of sedimentation equilibrium centrifugation (fig. 2). These values are significantly higher than those obtained after splitting of the 17 S molecule. It is assumed that the expected heterogeneity resulting from the splitting of both the 131,000 Dalton and the 96,000 Dalton subunits into 2 halves is not detectable by this method.

In agreement with [13] all proteolytic activity seems to be inactivated in 6 M Gd-HCl. Upon removing this denaturant by dialysis and subsequent SDS-electrophoresis these 2 bands are obtained again, even after keeping the enzyme in this solvent for several weeks at 4°.

To get further information about the extent of proteolytic alteration during purification, the enzyme was subjected on each step of the procedure to sucrose density gradient centrifugation (table 3). During preparation the s-values are considerably higher than these of the purified enzyme, especially after keeping it several days at 4°. Assuming the relationship between sedimentation coefficient and molecular weight to be the same for degraded as well as undegraded yeast phosphofructokinase and the standard globular proteins the respective molecular weights of the former were estimated and also reported in table 3.

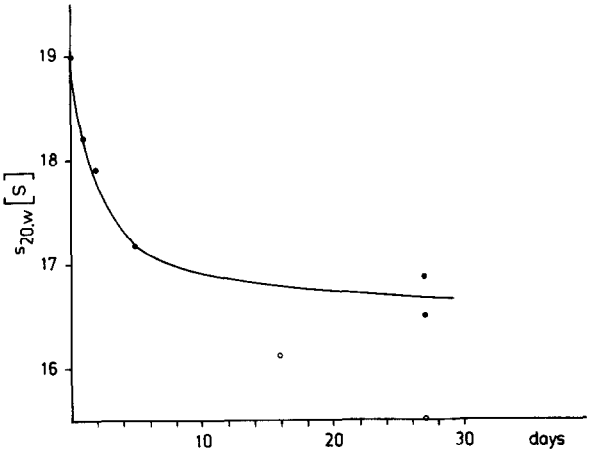

Fig. 3. Sedimentation coefficient of yeast phosphofructokinase as a function of the time of storage. Full circles: The enzyme (6 mg/ml) was stored in 0.1 M phosphate buffer pH 7.0, 1 mM fructose 6-phosphate, 1 mM EDTA, 3 mM 2-mercaptoethanol, 0.25 M $(NH_4)_2SO_4$, 1 mM PMSF. Prior to sedimentation velocity runs (20°; 50,000 rpm) the protein was dialyzed for 3 hr against the same buffer without $(NH_4)_2SO_4$. Open circles: The enzyme was kept in the buffer without $(NH_4)_2SO_4$.

4. Discussion

In this study evidence is presented that yeast phosphofructokinase (M.W. 580,000) may be composed of 6 subunits having molecular weights of 96,000. These subunits seem to contain 2 fragments each of about 50,000 Daltons. We are tempted to consider these species as the polypeptide chains of yeast phosphofructokinase. The 96,000 Dalton subunit has been identified by SDS-electrophoresis, whereas the polypeptide chain could be characterized by sedimentation equilibrium centrifugation in 6 M Gd-HCl. A crucial point for molecular weight calculation of this particle is the definition of the partial specific volume of the protein in the denatured state. Following the extensive studies of Hade and Tanford [12], for molecular weight calculation a small decrease of the apparent partial specific volume ($\Delta\bar{\nu}$ 0.01 or 0.02 cc/g, respectively) of the denatured protein in 6 M Gd-HCl solution as compared to the value in the native state has been taken into account. It may be objected to this interpretation that owing to uncertainties in the apparent partial specific volume of the protein in 6 M

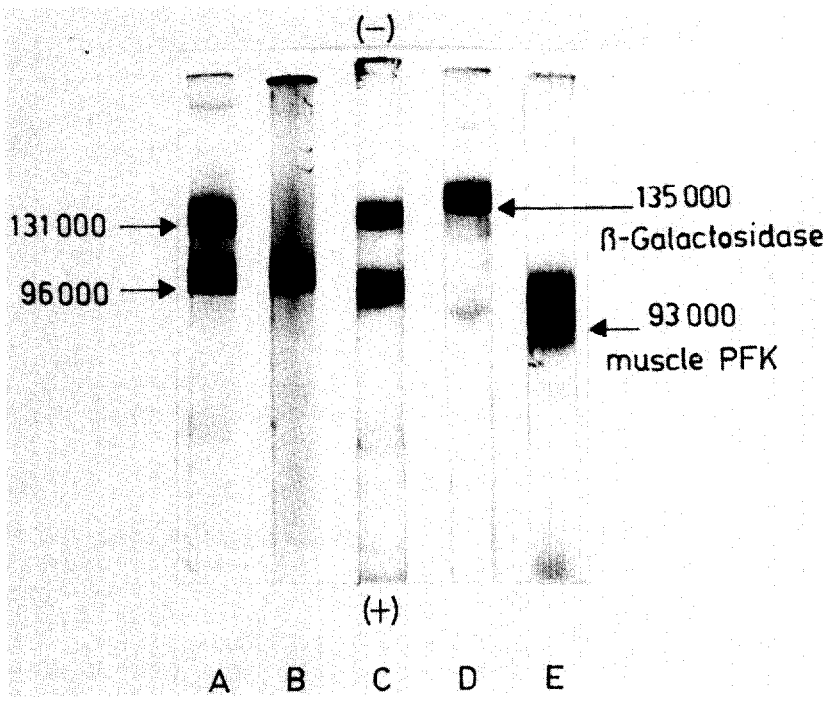

Fig. 4. SDS-electrophoresis of yeast phosphofructokinase. (A) Freshly prepared enzyme dissociated in 1% SDS and 1% 2-mercaptoethanol for 12 hr at 20°. (B) The same enzyme stored for 10 days at 4°, and then dissociated in 1% SDS and 1% 2-mercaptoethanol. (C) As gel B except that the freshly prepared enzyme was incubated for 10 days in 6 M guanidine hydrochloride and then dialyzed against 1% SDS at 50° prior to electrophoresis. Reference enzymes: (D) β-galactosidase (M.W. 135,000 [10]); (E) muscle phosphofructokinase (M.W. 93,000 [11]). In addition, the calibration curve usually contained aldolase (40,000), catalase (60,000) and bovine serum albumin (67,000).

Gd-HCl, the size of the resulting molecular species may not really be different in 6 M Gd-HCl and in SDS. For this to be true, one has to assume that the apparent partial specific volume of the denatured protein should not decrease but increase to a relatively large extent. However, from the classical work of Hade and Tanford [12] as well as Reisler and Eisenberg [17], this possibility seems to be highly improbable.

A critical assessment should be made of the question raised by Wilgus et al. [4] whether the enzyme is indeed composed of polypeptide chains of about 50,000 Daltons or whether these could be proteolytic degradation products of the 96,000 Dalton subunit. The fact that upon removing Gd-HCl the small molecules reassociate under reducing conditions to the 96,000 Dalton unit, which cannot be distinguished electrophoretically from that obtained by SDS-cleavage of the intact enzyme molecule may be taken as an argument that the smaller molecule, if it exists, cannot be a proteolytic artifact. Moreover, in 6 M Gd-HCl endogenous proteases are evidently inactivated. In SDS the polypeptide chain may not be randomly coiled as it is in 6 M Gd-HCl; therefore it cannot be precluded that in SDS the polypeptide chains may remain in an aggregated form [14, 18, 19]. The concept of a hexameric (or dodecameric) structure of the enzyme makes our preliminary trimeric model unlikely [2]. However, it corresponds to the results obtained by limited tryptic disgestion of this enzyme [3]. Evidently under those conditions different oligomeric states of the 96,000 Dalton unit occur.

The main question is related to the true molecular dimensions of the undegraded enzyme. In agreement with Wilgus et al. [4] 2 different SDS-cleavage products were obtained from freshly prepared enzyme. Significant differences in their molecular weights be-

Table 3
s-Values and estimated molecular weights of yeast phosphofructokinase during enzyme preparation.

Purification step	s-value (S) †	Molecular weights × 10^{-3}
Extract	20.4	710
Protamine sulphate precipitation	19.9	690
1. $(NH_4)_2SO_4$-precipitation	19.9	690
acetone precipitation	19.9	690
Sepharose chromatography	21.0	730
2. $(NH_4)_2SO_4$-precipitation	20.4	710
purified enzyme after storage for 1 week	17.7	585

† Standard proteins for calculation of s-values: aldolase (7.82 S), catalase (11.4 S), thyroglobulin (19 S). Estimation of molecular weights according to [16].

tween [4] and those presented in this study are evident. Assuming that the hexameric structure with subunits of about 131,000 Daltons is maintained during purification, the molecular weight of the undegraded enzyme could be calculated to be about 750,000 to 800,000. However, density gradient centrifugation performed immediately after ultrasonic disruption of the yeast cells with an inevitable time delay of about 6 hr after the moment of cell disruption gives a molecular weight of only about 700,000. Thus, despite the presence of protease inhibitor, the possibility of proteolytic attack occuring within this time cannot be excluded.

Acknowledgements

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