DETERMINATION OF THE CATALYTICALLY ACTIVE UNIT OF FRUCTOSE 1,6-DIPHOSPHATASE FROM RABBIT LIVER

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Received 20 March 1974

1. Introduction

'Neutral' fructose 1,6-diphosphatase (FDPase) (EC 3.1.3.11) from rabbit liver, a protein with a molecular weight of 140 000 possesses four identical subunits [1]. It is still unknown, which unit of the enzyme is catalytically active under conditions of the biological activity assay, that is extreme dilution and in the presence of substrate and magnesium or manganous ions, which the enzyme requires for catalytic activity [2–4].

The method of band centrifugation or 'Active Enzyme Centrifugation' (AEC) according to R. Cohen et al. [5–8] allows the determination of the catalytically active unit of an enzyme. In the present communication this procedure was applied to study purified fructose 1,6-diphosphatase from rabbit liver at a pH range from about 7–9 and in the presence of magnesium or manganese as activating ions.

2. Materials and methods

Fructose 1,6-diphosphate disodium salt, NADP, hexose phosphate isomerase (EC 5.3.1.9) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Boehringer, Mannheim. Phosphocellulose was obtained from Serva, Heidelberg. Fructose 1,6-diphosphatase activity was determined spectrophotometrically by a coupled assay with hexose phosphate isomerase and glucose 6-phosphate dehydrogenase as auxiliary enzymes according to the equation:

fructose 1,6-diphosphate + NADP + H_2O → 6-phosphogluconate + NADPH + P_i + H^+.

The formation of NADPH was followed at 340 nm in the Eppendorf photometer at 23°C. Unless otherwise stated the routine assay mixture contained:

40 mM triethanolamine buffer, pH 7.5, 0.1 mM NADP, 0.1 mM fructose 1,6-diphosphate, 2 mM $MgCl_2$, 1 μg/ml each of glucose 6-phosphatedehydrogenase and hexosephosphate isomerase; the protein concentration for the purified enzyme was calculated from the absorbance at 280 nm as described [1].

'Neutral' fructose 1,6-diphosphatase was isolated from rabbit liver following the procedure outlined by [1]. From 270 g fresh rabbit liver about 12 mg purified fructose 1,6-diphosphatase with a specific activity of about 12 U/mg protein was obtained.

'Active enzyme centrifugation' was performed as described by R. Cohen et al. [5–7]. Bandforming Vinograd-type double-sector cells (Beckman Nr. 331359) were used. About 300–400 μl assay solution was placed in the sector and 10 μl fructose 1,6-diphosphatase solution (30–100 μg) were added to the well. During centrifugation, the enzyme solution is layered over the substrate. The time of deposition was determined by the appearance of an infinity gradient observed with the Schlieren viewer. This time was noted in each experiment, since it is the actual beginning of the centrifugation, usually at about 1–2000 rpm. The change in the absorption of the substrate solution was directly monitored by the use of the photoelectric scanner. For higher precision of the scans a 10 inch recorder as used for

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the Beckman DB-photometer was adapted to the scanner. The apparent sedimentation coefficient was evaluated from the scanner tracings and after drawing of difference curves as described by Cohen [5,6] or as outlined by Schachman and Edelstein [9] for conventional sedimentation velocity runs using absorptions optics. Both methods of evaluation yielded identical results as also previously found by Taylor et al. [10]. The apparent sedimentation coefficient was corrected for standard conditions (20°C, water) as described [11]. The partial specific volume $\bar{V}$ was 0.73 ml/g according to Sia et al. [12]. The viscosity of the assay solution relative to distilled water was evaluated by a capillary viscosimeter as described [13]. The density was determined in a 5 ml picnometer calibrated with distilled water.

3. Results and discussion

The sedimentation of FDPase was followed during the centrifugation in the cell by the distribution of formed NADPH according to the bioassay (Materials and methods). Fig. 1 shows a scanner pattern at different time intervals after deposition of FDPase.

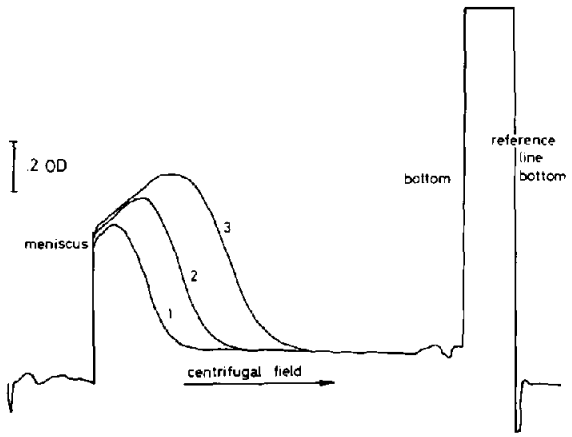

Fig. 1. Scanner pattern of an AEC experiment at different time intervals. The absorption of the assay solution was recorded at 340 nm. The rotor speed was 40 000 rpm, the temperature 22.1°C. At the time of deposition about 40 ng FDPase were added to 350 µl assay solution containing 100 mM TRAM buffer, pH 7.5, 2 mM $MgCl_2$. Substrate and auxiliary enzymes see Methods. Curve 1: 24 min, Curve 2: 40 min, Curve 3: 64 min after deposition of the enzyme.

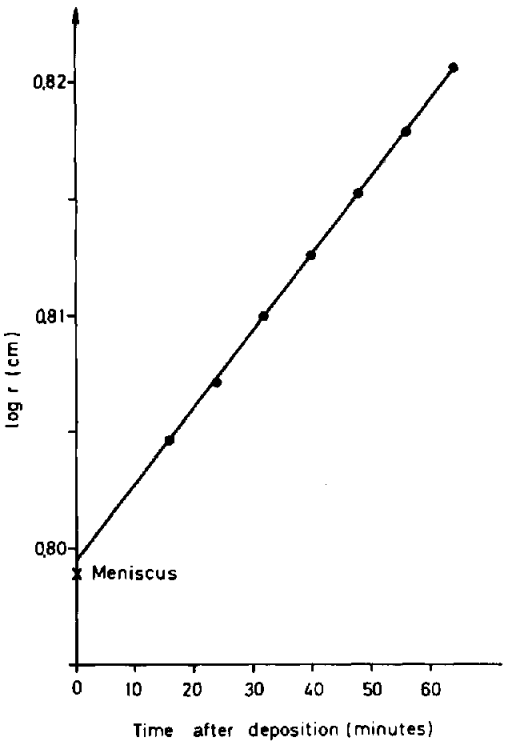

Fig. 2. Determination of the sedimentation coefficient of FDPase. The logarithm of the boundary from the center of rotation (log r) is plotted versus the time after deposition. The experimental conditions are identical to fig. 1.

The gradient, which represents the amount of formed NADPH proceeds towards the bottom of the cell.

In fig. 2, the logarithm of the distance of the gradient from the center of rotation versus the time after deposition of FDPase is plotted. The experimental points fit a straight line. On extrapolation to the time of deposition, which is the actual beginning of the centrifugation of FDPase, the position of the meniscus is obtained. This indicates, that the sedimentation of FDPase had started at the meniscus of the assay solution and proceeded in a constant rate towards the bottom of the cell. From the slope of the plot the apparent sedimentation coefficient was calculated and corrected for the increased viscosity and density of the assay solution to standard conditions (20°C, water).

The results of 16 experiments, carried out at different pH and in the presence of manganese or mag-

Table 1
Sedimentation coefficients of purified FDPase from rabbit liver using the 'active enzyme centrifugation' technique

Buffer	pH	Metal ion	n	$S_{20,w}$
0.1 M TRAM*	7.5	2 mM $MgCl_2$	6	7.20 ± 0.30
0.1 M TRAM	7.5	0.25 mM $MnCl_2$	4	7.39 ± 0.14
0.1 M TRAM	9.2	2.5 mM $MnCl_2$	2	7.39 ± 0.26
0.1 M GLYCINE	9.2	2 mM $MgCl_2$	2	7.31 ± 0.24
0.1 M GLYCINE	9.2	5 mM $MnCl_2$	2	7.53 ± 0.05
			16	7.33 ± 0.24

± Standard deviation.
* TRAM = triethanolamine.

nesium ions including the statistical variation are listed in the table 1. The mean sedimentation coefficient was 7.33 ± 0.24 S. As seen in the table 1, the presence of magnesium or mangenous ions for activation of FDPase or a shift in the pH from 7.5–9.2, did not affect the rate of sedimentation of FDPase significantly. The S-value was in each case between 7.2–7.5 S.

As reported previously, the sedimentation coefficient of purified 'alkaline' FDPase was 7.2 at 15°C by sucrose gradient centrifugation [14] and the same value was found by Sia et al. [12] at 20°C. Using the photoelectric scanner, a sedimentation coefficient of 7.4–7.5 S (20°C, water) and a molecular weight of 140 000 was obtained [15]. The protein concentration was between 200 and 500 μg/ml. In this work, the FDPase concentration was about 1000 fold more diluted and still an S-value of the same order of magnitude was found.

The data of this communication suggest, that the catalytically active unit of FDPase is the tetrameric form with a molecular weight around 140 000.

Acknowledgements

The author is indebted to Prof. Dr O. H. Wieland for his interest in this work and for reading of the manuscript and to Miss Jutta Drechsel for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

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