EVIDENCE FOR DIFFERENT OLIGOMERIC FORMS OF HUMAN ERYTHROCYTE PHOSPHOFRUCTOKINASE

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1. Introduction

Phosphofructokinases from mammalian tissues occur in different association states under a variety of experimental conditions [1–6]. These states depend on protein concentration and were found to be influenced by substrates and effectors as well as by changing pH values and ionic strengths. Layzer and coworkers [7, 8] described some properties of partially purified erythrocyte phosphofructokinase including a concentration-dependent sedimentation behaviour of the enzyme using density gradient centrifugation. The present paper reports the existence of a series of oligomeric forms of human erythrocyte phosphofructokinase in highly purified enzyme preparations. These are correlated with a product of splitting of the enzyme having a molecular weight of 104,000 which could be characterized by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis.

2. Materials and methods

For the experiments we used highly purified homogeneous phosphofructokinase from human red blood cells prepared according to a purification procedure which has been developed in our laboratory [9]. The final specific activity of the enzyme was 136 IU per mg protein.

Sucrose density gradient centrifugation was carried out with a SW-50 L rotor in a model L2-65 B ultracentrifuge at 40,000 rpm and 5° for 7 hr according to the procedure of Martin and Ames [10] using a linear gradient of sucrose between 5–20% (w/v) in 100 mM potassium phosphate buffer (pH 7.1) containing 0.5 mM EDTA, 3 mM mercaptoethanol and 2 mM F6P. The enzyme samples were dialyzed for 15 hr against sucrose-free buffer before applying them to the gradient. For calculation of the sedimentation coefficients, aldolase, catalase, muscle and yeast phosphofructokinases were taken as standard proteins.

Polyacrylamide gel electrophoresis was conducted in a linear gel concentration gradient (3–20% acrylamide) as described previously [11] (gel buffer: 0.25 M imidazole/HCl, pH 7.5; electrode buffer: 0.01 M imidazole diethylbarbituric acid, pH 7.0). With this procedure native proteins are separated predominantly in a manner dependent upon their molecular sizes. Therefore this method is suitable for molecular weight determinations of proteins. For visualization, the proteins were stained with Coomassie brilliant blue [12].

SDS-cleavage experiments were performed by incubation of the enzyme for 12 hr in a solution containing 1% SDS and 1% mercaptoethanol. SDS-polyacrylamide gel electrophoresis for estimation of subunit molecular weights was by the method of Shapiro et al. [13].

Analytical gel filtration experiments for determination of molecular weights of different oligomeric phosphofructokinase forms were done using a column of Sepharose 4 B (7.0 × 1.5 cm). The column was equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 2 mM F6P [14]. Standard proteins were used as indicated in fig. 3.

Enzyme activity was measured spectrophotometrically at 25°; the assay mixture contained (final volume 1.5 ml): 100 mM Tris-HCl (pH 8.0), 1.5 mM F6P, 0.25 mM ATP, 3 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1.3 mg

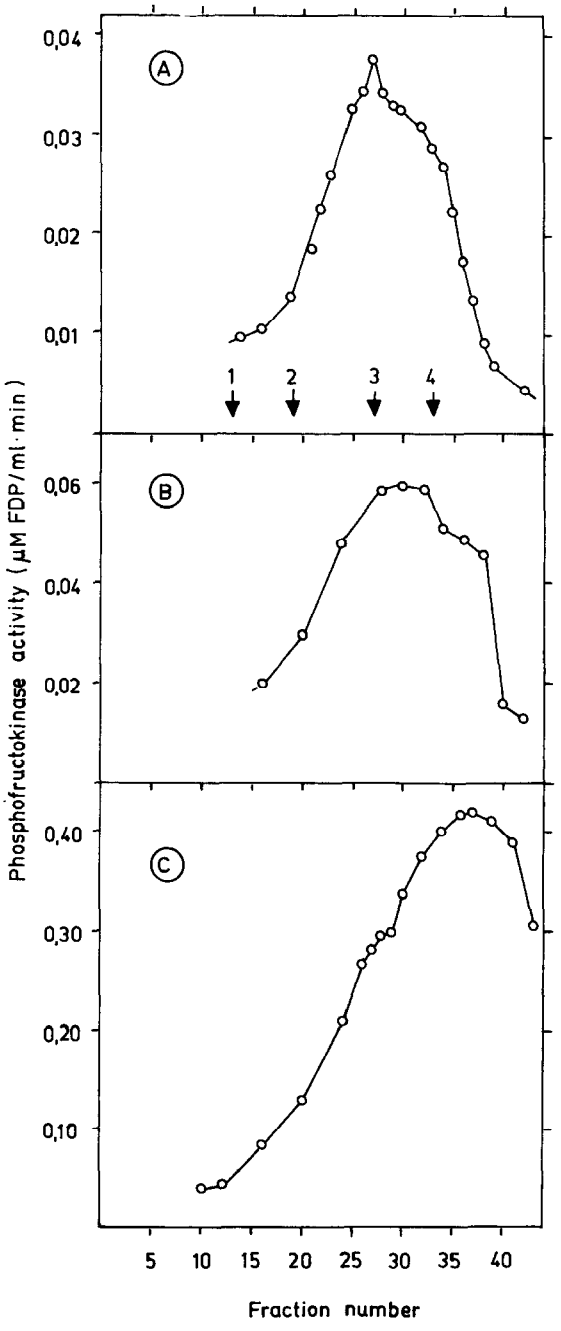

Fig. 1. Distribution of erythrocyte phosphofructokinase on the sucrose density gradient. On gradient A) 0.2, on gradient B) 0.28 and on gradient C) 1.7 units have been applied. Reference proteins (indicated in A): 1. aldolase ($s_{20,w}$ = 7.35), 2. catalase ($s_{20,w}$ = 11.1), 3. muscle phosphofructokinase ($s_{20,w}$ = 13.2), 4. yeast phosphofructokinase ($s_{20,w}$ = 17.8).

human serum albumin, 0.15 mM NADH, 5 µg aldolase, 5 µg triosephosphate isomerase, 5 µg glycerol-1-phosphate dehydrogenase.

Protein concentrations were determined by either the method of Janatova et al. [15] with human serum albumin as standard or spectrophotometrically at 280 nm according to Warburg and Christian [16].

3. Results

The distribution of highly purified erythrocyte phosphofructokinase activity after sucrose density gradient centrifugation and its dependence on enzyme concentration is shown in fig. 1. The enzyme activity is distributed over a broad range. Applying a low protein concentration (0.2 IU) the main fraction of the enzyme sediments at about 15 S (A). The curve shows a clear asymmetry towards the larger particles. At higher enzyme concentrations (1.7 IU) the sedimentation coefficient increases to 20.7 S (C), indicating an association of the enzyme. This is prevented neither by dialysis of the enzyme against 200 mM mercaptoethanol for 48 hr nor by adding 200 mM mercapto-

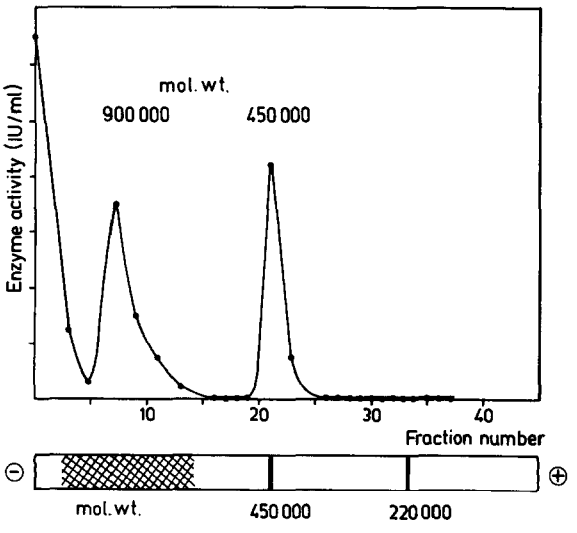

Fig. 2. Graded polyacrylamide gel electrophoresis of erythrocyte phosphofructokinase. Top: distribution of the enzyme activity determined by slicing an unstained gel into discs having 2 mm thickness, and measuring after homogenizing by adding the enzyme containing solution to the assay mixture. Bottom: protein distribution on the gel after staining with Coomassie brilliant blue.

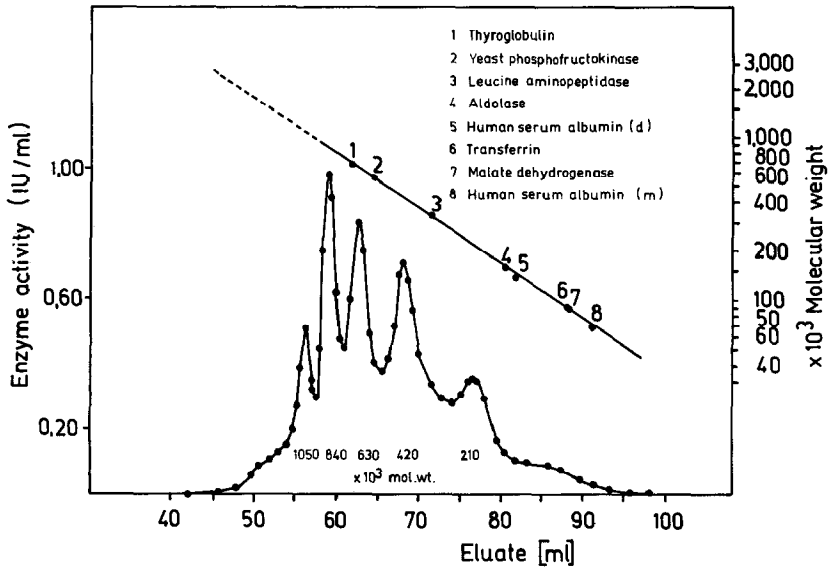

Fig. 3. Fractionation of erythrocyte phosphofructokinase by analytical gel filtration. Conditions, see text.

ethanol to the gradient before centrifugation.

Assuming the relationship between sedimentation coefficient and molecular weight to be the same for erythrocyte phosphofructokinase as for an average globular protein, for the peak fractions in fig. 1A and C, the approximate molecular weights of 420,000 (at low enzyme concentrations) and of 680,000 (at high enzyme concentration) have been deduced.

The enzyme is stable on the gradient between pH 6.5–9.0 in the presence of 2 mM F6P for at least 12 hr; without F6P the enzyme becomes rapidly inactivated. 2 mM ATP also stabilizes the enzyme at pH 7.1.

The enzyme is separated electrophoretically, using linear graded polyacrylamide gels, into two distinct bands and into a broad region of higher molecular weight apparently aggregated forms (fig. 2). The molecular weights relating to these bands were determined according to [11]. They were found to be about 220,000, 450,000 and for the broad region between about 600,000 and 900,000. In parallel runs the distribution of enzyme activity after slicing the gel was measured. The forms having a molecular mass of 450,000 dalton and those occurring in the broad region showed enzymatic activity. However, under these conditions the fraction with a molecular weight of about 220,000 appeared to be enzymatically inactive (fig. 2).

By means of analytical gel filtration on Sepharose 4 B, B enzymatic active peaks can be detected (fig. 3). 2 mM F6P was added to stabilize the enzymatic activity. From the linear relation between the eluting volume and the logarithm of the molecular weights of the reference proteins the molecular weights of the peak fractions were calculated. The 5 peaks were found to represent various enzymatic species having molecular weights of 210,000 ± 8000; 420,000 ± 15,000; 630,000 ± 30,000; 840,000 ± 35,000 and 1,050,000 ± 50,000 (n=15). In comparison, under the same conditions yeast phosphofructokinase is eluted as one single peak with a position as indicated in fig. 3. For testing stability of one distinct form, the peak fraction containing molecules with a mass of 840,000 dalton was rechromatographed on Sepharose 4 B. After this procedure a redistribution could be observed and the reappearance of 5 enzymatically active forms with the particle sizes shown above. In addition, the 840,000 molecules isolated by Sepharose 4 B chromatography rearrange during electrophoresis in the graded gel giving a pattern like that in fig. 2.

In SDS-polyacrylamide gel electrophoresis, the highly purified enzyme is homogeneous and yields a single distinct band (fig. 4). Fig. 5 shows the molecular weight determination of the SDS-fragmentation product in SDS-polyacrylamide electrophoresis. Trypsin,

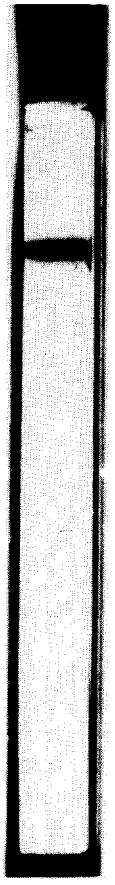

Fig. 4. SDS-polyacrylamide gel electrophoresis of erythrocyte phosphofructokinase.

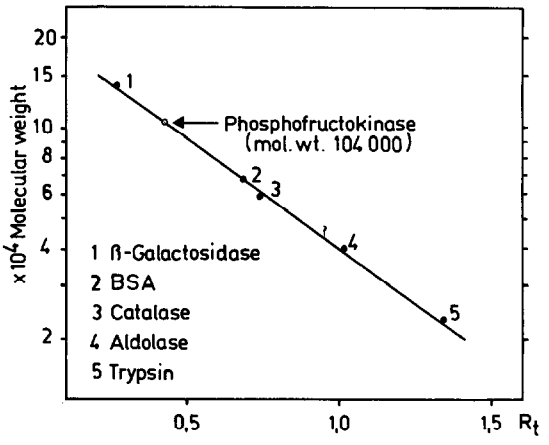

Fig. 5. Molecular weight determination of the SDS subunit by polyacrylamide gel electrophoresis.

aldolase, catalase, bovine serum albumin and β-galactosidase (see footnote *) were used as reference proteins. Using this method the molecular weight of the phosphofructokinase subunit was found to be 104,000 ± 3000 (n=5).

4. Discussion

Comparing the results from electrophoresis, gel filtration and density gradient centrifugation, it can be concluded that erythrocyte phosphofructokinase occurs in various associated forms.

The tendency towards aggregation with increasing protein concentrations seems to be a common property of mammalian phosphofructokinases [3, 5–7]. This is not shown by yeast phosphofructokinase [17].

The oligomeric forms of erythrocyte phosphofructokinase having molecular weights of 210,000, 420,000, 630,000, 840,000 and 1,050,000 can easily be correlated to the mass of the subunit obtained after SDS electrophoresis having a molecular weight of 104,000. From that, the conclusion may be drawn that with Sepharose 4 B, the dimer, tetramer, hexamer, octamer and decamer of the 104,000 unit can be separated. The results obtained with density gradient centrifugation and polyacrylamide gel electrophoresis accord with this model.

It should be emphasized that at present it can not definitely be said that SDS treatment yields indeed single polypeptide chains. Further experiments applying chemical modification procedures and end-group analysis are in progress in order to get a more thorough insight into the subunit structure of this enzyme. In addition, the polymerization process and its relation to the kinetic and allosteric behaviour of the enzyme are under study.

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