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PURIFICATION OF HUMAN LIVER FRUCTOSE-1,6-BISPHOSPHATASE

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Summary

Human liver fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) has been purified 1200-fold using a heat treatment step followed by absorption on phosphocellulose at pH 8 and specific elution with buffer containing the substrate (fructose 1,6-bisphosphate) and allosteric effector (AMP).

The enzyme is homogeneous in electrophoresis in polyacrylamide gel, in the presence and absence of denaturing agent. It has a molecular weight of 144 000 and is composed of four identical or nearly identical subunits. Fluorescence spectra indicate that the enzyme does not contain tryptophan residues. The pH optimum is 7.5 and the K_m is determined as 0.8 μM. The enzyme is inhibited by AMP in cooperative manner with a $K_{0.5}$ of 6 μM.

Introduction

Although fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) from the time of its discovery in 1943 [1] has been purified from a number of different sources, purification of this enzyme from primates was not reported (for review see Horecker et al. [2]). Its basic properties like pH optimum, allosteric properties, specific activity etc., are rather complex functions depending on bivalent and monovalent ion concentrations, temperature and presence of allosteric effector (AMP). This enzyme is extremely labile to the action of endocellular proteases which may dramatically alter fructose-1,6-bisphosphatase pH optimum and sensitivity to AMP inhibition [3]. The protection of this enzyme against proteolysis and elimination of contaminating proteins are the main problems in the isolation of liver fructose-1,6-bisphosphatase.

In this paper a simple and fast purification procedure is presented which yields homogeneous and nondegraded human liver fructose-1,6-bisphosphatase within 10 h. Some catalytic and molecular properties of this enzyme are described.

Methods

Fructose-1,6-bisphosphatase activity was measured spectrophotometrically by coupled enzymatic assay according to Traniello et al. [4]. 1 ml of the standard mixture contained: 50 mM Tris-HCl/5 mM $MgCl_2$/0.5 mM EDTA/150 mM KCl/0.2 mM fructose 1,6-bisphosphate/0.2 mM NADP/2 μg glucose-6-phosphate dehydrogenase/2 μg phosphoglucose isomerase, pH 7.5.

1 unit of activity was the amount of the enzyme that produced 1 μmol fructose 6-phosphate and P_i per min at 25° C under standard assay conditions.

For the determination of K_m values, purified fructose-1,6-bisphosphatase and auxiliary enzymes were dialyzed against 50 mM Tris-HCl/0.1 mM EDTA/5 mM $MgCl_2$, pH 7.5 at 5° C for 4 h. Tissue homogenate was dialyzed for 8 h against the same buffer.

The rate of fructose 1,6-bisphosphate hydrolysis was measured by two methods when K_m values were determined. 1. Monitoring of fructose 6-phosphate formation. The assay mixture contained the following components: 50 mM Tris-HCl/0.1 mM EDTA/5 mM $MgCl_2$/0.2 mM NADP/2 μg glucose-6-phosphate dehydrogenase/2 μg phosphoglucose isomerase and variable concentrations of fructose 1,6-bisphosphate, pH 7.5. The increase of absorbance at 340 nm was followed with a Beckman Acta M VI spectrophotometer using 0—0.02 absorbance scale. 2. A colorimetric determination of P_i release was performed according to Tashima and Yoshimura [5]. The assay mixture contained all the above components except the auxiliary enzymes and NADP.

The determination of molecular weight of fructose-1,6-bisphosphatase was performed according to Andrews [6]. The molecular weight of the subunits was determined on polyacrylamide gel in SDS according to Weber and Osborn [7].

Disc gel electrophoresis was performed at pH 8.9 according to Davis [8].

For column chromatography phosphocellulose Whatman P-11 was recycled with 0.1 M HCl and 0.1 M NaOH, filtered on a Buchner funnel and washed until the filtrate was nearly neutral.

Results and Discussion

Purification of the human liver fructose-1,6-bisphosphatase.

Extraction. Human liver obtained from autopsy was passed through a meat grinder, then 200 g of the ground liver were suspended in 900 ml of 0.25 M saccharose containing 0.5 mM EDTA and adjusted to pH 7.5. The suspension was stirred at 4—6° C for 0.5 h and centrifuged at 10 000 × *g* for 0.5 h at 4° C.

Heat treatment. The supernatant was divided into small portions of about 150 ml and heated with a gentle shaking in a boiling water bath until the temperature of the solution reached 68° C. This temperature was maintained for

3 min then it was cooled rapidly and centrifuged at 10 000 × *g* for 0.5 h at 4° C. The precipitate was discarded.

Chromatography on phosphocellulose. The fractions obtained by heat treatment were treated with the moist phosphocellulose. 1 g of the moist phosphocellulose per 10 ml solution was used. During the addition of the ionic exchanger the pH was kept equal at 8 with 2 M NaOH or 2 M HCl and the temperature maintained at 5° C. The suspension was stirred for 1 h at the same pH and temperature. After 1 h the suspension was filtered and washed several times with 50 mM Tris-HCl/0.5 mM EDTA, pH 7.5 on the Buchner funnel until the reddish colour disappeared. Following the absorption, the phosphocellulose was packed into a Pharmacia K 16/40 column, and subsequently washed with the same buffer until the absorbance at 280 nm of the eluate had fallen below 0.03 and the pH had reached 7.5. The enzyme was then eluted with 1 mM fructose-1,6-bisphosphatase/1 mM AMP/50 mM Tris-HCl/0.5 mM EDTA, pH 7.5. Fractions were collected and analysed for fructose-1,6-bisphosphatase activity and those of the highest activity were pooled. The enzyme can be stored as a precipitate in 70% satd. $(NH_4)_2SO_4$. The results of the purification procedure are summarized in Table I. The method is highly reproducible. The recoveries ranges from 25 to 35% of the original activity and specific activity varied from 12 to 14.

The elution with fructose 1,6-bisphosphate and AMP from the phosphocellulose column at acidic pH has been previously used as a step in the purification of fructose-1,6-bisphosphatase from other sources [9]. The advantage of our procedure is the absorption of this enzyme at pH 8. At that pH fructose-1,6-bisphosphatase is absorbed on this ionic exchanger while at the same time the majority of other proteins remain in the solution. Thus in a single step approx. 560-fold purification can be achieved. The absorption on phosphocellulose at pH 8 is quite unique for human enzyme and cannot be used without modification for isolation of rabbit or pig liver enzymes.

Homogeneity. Disc gel electrophoresis of purified human liver fructose-1,6-bisphosphatase showed only one sharp protein band (Fig. 1a). Twice as much protein applied for SDS gel electrophoresis revealed one slightly diffuse protein

TABLE I

PURIFICATION OF THE HUMAN LIVER FRUCTOSE-1,6-BISPHOSPHATASE

Fraction	Volume (ml)	Enzyme activity at pH 7.5 (units/ml)	Protein concentration (mg/ml)	Enzyme total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
Extract	860	0.4	36 *	344	0.011	100	1
Heated fraction	775	0.32	14 *	248	0.023	72	2
Phosphocellulose eluate	7.5	10.7	— **	80	—	—	—
Sephadex G-25 eluate	12	7.5	0.58 ***	90	12.9	26	1172

* Protein concentration was determined spectrophotometrically at 280 nm assuming $E^{1\%}_{1cm} = 1$.

** Protein concentration could not be determined spectrophotometrically because of the presence of AMP.

*** For the purified fructose-1,6-bisphosphatase, $E^{1\%}_{1cm} = 0.73$ at 280 nm was used in analogy to rabbit liver enzyme [4].

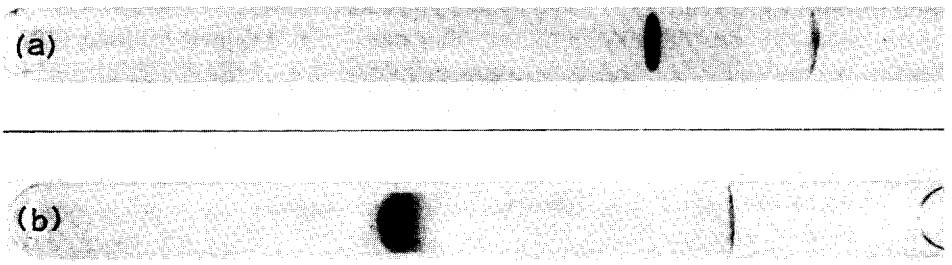


Fig. 1. Gel electrophoresis of human liver fructose-1,6-bisphosphatase. a, Disc gel electrophoresis, 20 μg of the protein were applied on the top of the gel (right-hand end on this figure). B, Gel electrophoresis in SDS, 40 μg of the protein were applied on the top (right-hand end) of the gel.

band, indicating rather minor heterogeneity of the enzyme subunits (Fig. 1b).

Molecular and catalytic properties. To investigate whether purified enzyme resembles the native protein K_m value, AMP inhibition profile, Hill coefficients, $K_{0.5}$ value and pH 9.3/pH 7.5 activity ratio were determined for the homogenate and purified fructose-1,6-bisphosphatase. All these parameters appear to be identical except the apparent K_m value which is higher for the homogenate than for the purified enzyme (Table II). This may be due to the presence of interfering substances in the initial extract, rather than reflecting the real changes in the enzyme molecules caused by the purification procedure (i.e. heat treatment). The K_m value of 0.8 μM, determined with two methods for the purified enzyme, is comparable with that found for rabbit (0.74 μM) and rat (1.2 μM) liver fructose-1,6-bisphosphatase [5,10].

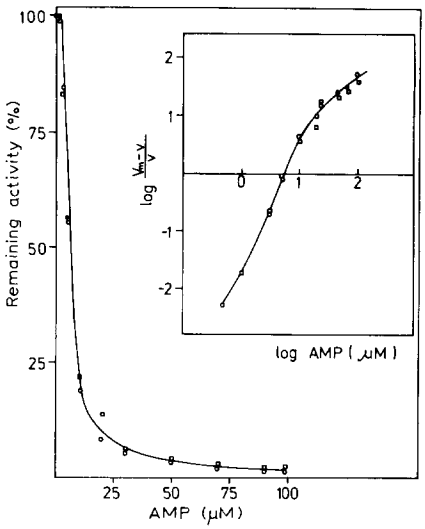
Human liver fructose-1,6-bisphosphatase interacts with AMP cooperatively (Fig. 2). High Hill coefficients found for the purified enzyme and homogenate indicate that no alteration in allosteric properties occurred during the enzyme purification [10]. According to the analysis of the Hill plot developed by Cornish-Bowden and Koshland [12], the results show positive cooperativity for the second AMP molecule binding, and negative cooperativity can be tentatively postulated for the fourth AMP molecule binding. The $K_{0.5}$ value of 6 μM found from the Hill plot is lower than the corresponding value for rat (21 μM) or ox (15.67 μM) liver fructose-1,6-bisphosphatase [10,13], indicating that

TABLE II

KINETIC PARAMETERS OF THE HUMAN LIVER FRUCTOSE-1,6-BISPHOSPHATASE

Hill coefficient was calculated from 'straight line' portion of a Hill plot for AMP concentration between 3 and 10 μM (Fig. 2). $K_{0.5}$ concentration of AMP giving 50% inhibition, calculated from a Hill plot (Fig. 2). The pH activity ratio was determined as described in Methods but instead of standard Tris-HCl buffer the solution contained 50 mM Tris/50 mM glycine/50 mM imidazol. This solution was adjusted to pH 7.5 or 9.3 with HCl and NaOH, respectively.

	K_m (μM)	Hill coefficient	$K_{0.5}$ (μM)	pH 9.3/pH 7.5 activity ratio
Purified enzyme	0.8	2.4	6	0.42
Homogenate	3.3	2.4	6	0.42



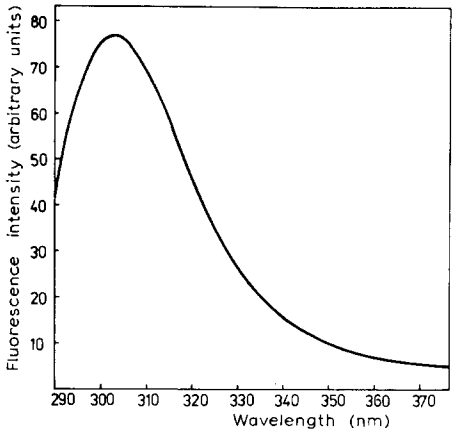


Fig. 2. Inhibition of human liver fructose-1,6-bisphosphatase by AMP. The reaction mixture (1 ml) contained: 50 mM Tris-HCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mM fructose 1,6-bisphosphate, 0.2 mM NADP, 2 μg glucose-6-phosphate dehydrogenase, 2 μg phosphoglucose isomerase, 0.005 units of fructose-1,6-bisphosphatase and variable concentrations of AMP pH 7.5. Each point represents the mean value of four measurements for the purified enzyme (○———○) and two measurements for the initial extract (□———□). The inset shows the Hill plot calculated from the original data.

Fig. 3. Fluorescence spectra of human liver fructose-1,6-bisphosphatase. Protein sample (0.1 mg/ml) in 50 mM Tris-HCl/0.5 mM EDTA/100 mM KCl, pH 7.5. Excitation wavelength 280 nm. Excitation and omission slits were 5 nm.

human liver enzyme is more sensitive to AMP inhibition than enzymes from ruminants or rodents.

It was found that, like all 'neutral' fructose-1,6-bisphosphatase isolated from higher organisms, human enzyme has molecular weight of 144 000. The subunits molecular weight was found to be 36 000.

Because of controversy concerned with the presence of a tryptophan residue in rabbit liver fructose-1,6-bisphosphatase [14—16], it is particularly important to note that human enzyme exhibits a typical tryptophan-free fluorescence spectrum (Fig. 3). Currently, it is believed that nondegraded forms of rabbit and rat liver enzymes do not contain tryptophan [10,17] whereas this amino acid presumably occurs in the ox liver fructose-1,6-bisphosphatase [18].

Although human liver fructose-1,6-bisphosphatase has basic molecular and catalytic properties typical for mammalian liver enzymes, this protein exhibits some distinct features, i.e. high affinity toward the phosphocellulose and different AMP inhibition profile. Further studies are required to elucidate the physiological meaning of these rather subtle differences.

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