THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 238, No. 10, October 1963
Printed in U.S.A.

Cleavage of Sedoheptulose 1,7-Diphosphate by a Purified Rat Liver Diphosphatase*

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(Received for publication, January 14, 1963)

The existence of a specific D-fructose 1,6-diphosphatase in mammalian liver was first shown by Gomori in 1943 (1). The enzyme has subsequently been purified by Pogell, Mokrasch, and McGilvery and some of its properties, such as specificity, optimal pH, ion requirement, and activation by proteolytic enzymes, have been reported (2, 3).

We have recently shown (4) the presence of a sedoheptulose 1,7-diphosphatase activity in the soluble fraction of rat and rabbit liver. This activity is also present in purified preparations of fructose diphosphatase obtained according to Pogell and McGilvery (5). Data were also presented for the chromatographic and enzymatic characterization of the reaction product as sedoheptulose 7-phosphate.

A simple purification procedure of the diphosphatase activity from rat liver is described in the present paper together with evidence for the specificity, kinetics, and stoichiometry of the cleavage reaction. The results obtained provide several lines of evidence that a single enzyme catalyzes the cleavage of both D-fructose 1,6-diphosphate (FDP)[1] and sedoheptulose 1,7-diphosphate (SDP) according to the equations

$$SDP + H_2O \rightarrow \text{sedoheptulose 7-P} + P_i \quad (1)$$
$$FDP + H_2O \rightarrow \text{D-fructose 6-P} + P_i \quad (2)$$

EXPERIMENTAL PROCEDURE

Materials

D-Glucose 6-phosphate, D-ribose 5-phosphate, D-fructose 1,6-diphosphate, D-gluconate 6-phosphate (sodium salts), D-glucose 1-phosphate (potassium salt), TPN, DPN, and DPNH were obtained from the Sigma Chemical Company. D-Fructose 6-phosphate (calcium salt), D-fructose 1-phosphate (barium salt), hexosephosphate isomerase, aldolase, D-glucose 6-phosphate dehydrogenase, D-glyceraldehyde 3-phosphate dehydrogenase, and glycerophosphate dehydrogenase containing triosephosphate isomerase were purchased from Boehringer und Soehne. Sedoheptulose 7-phosphate and sedoheptulose 1-phosphate were prepared by the procedure of Horecker, Smyrniotis, and Klenow (6). Sedoheptulose 1,7-diphosphate was prepared according to Smyrniotis and Horecker (7). D-Xylulose 5-phosphate was prepared as described by Srere *et al.* (8). D-Ribulose 5-phosphate was obtained from the enzymatic decarboxylation of D-gluconate 6-phosphate as reported elsewhere (9). D-Ribulose 1,5-diphosphate was kindly supplied by Dr. B. L. Horecker of New York University. Sedoheptulosan employed as standard for the colorimetric procedures was a gift of Dr. N. K. Richtmyer of the National Institutes of Health. Transketolase, D-gluconate 6-phosphate dehydrogenase, D-xylulose 5-phosphate 3-epimerase, and D-ribose 5-phosphate isomerase were prepared as described elsewhere (10).

Methods

Assay of Enzyme—The routine assay measures the release of inorganic phosphorus with the procedure of Fiske and SubbaRow (11). The incubation mixture (0.5 ml) contained: FDP, 1.5×10^{-3} M, and $MnCl_2$, $\times 10^{-4}$ M, or SDP, 1.5×10^{-3} M, and $MnCl_2$, 7×10^{-4} M, in 5×10^{-2} M triethanolamine buffer, pH 9.0, and enzyme in the range of 0.5 to 1.5 units. After incubation for 10 minutes at 37° the reaction was stopped by the addition of 0.1 ml of 30% trichloroacetic acid. After centrifugation for 5 minutes at 5000 r.p.m. an aliquot (0.4 ml) of the supernatant fluid was assayed for P_i. One unit of enzyme was defined as that amount which releases 1 μmole per hour of P_i under the assay conditions. Specific activity is expressed as units per mg of protein. The amount of P_i released is, under our conditions, proportional to the amount of enzyme added.

At different stages of purification the colorimetric assay was checked by enzymatic evaluation of the products formed. For fructose diphosphatase activity this was accomplished by following the rate of synthesis of fructose 6-P in the same assay system, which also contained hexosephosphate isomerase, D-glucose 6-phosphate dehydrogenase, and TPN. The formation of TPNH was followed at 340 mμ. For sedoheptulose diphosphatase activity, aliquots of the incubation mixture were analyzed with the cysteine-sulfuric acid reaction (12) for total ketoheptulose and by the enzymatic assay for the residual SDP. This was performed in the presence of aldolase, glycerophosphate dehydrogenase containing triosephosphate isomerase, and DPNH according to the procedure previously reported (13). Comparison of the results obtained in the different assay conditions revealed no appreciable differences.

Other Methods—Protein was determined by the method of Lowry *et al.* (14) and by the turbidimetric procedure of Bücher (15). SDP was analyzed for FDP content in the presence of aldolase, glyceraldehyde 3-phosphate dehydrogenase, and DPN. *The total content of FDP in our SDP* preparation was found to be 0.3%.

Purification of Enzyme

All the following operations were carried out at 2° except when otherwise indicated.

* Aided by Research Grant A-5228 from the National Institutes of Health and by a grant from the Consiglio Nazionale delle Ricerche.

[1] The abbreviations used are: FDP, fructose 1,6-diphosphate; SDP, sedoheptulose 1,7-diphosphate.

TABLE I

Summary of enzyme purification

Fraction	Step	Sedoheptulose diphosphatase (*a*)			Fructose diphosphatase (*b*)			*a/b*
		Total units	Recovery	Specific activity	Total units	Recovery	Specific activity	
I	Soluble fraction	16,400	100	2.4	15,000	100	2.2	1.09
II	Acid fractionation	13,260	81	11.9	12,100	80	10.8	1.10
III	First ammonium sulfate fractionation	10,400	63	64.0	9,050	60	55.6	1.15
IV	Heat treatment	9,140	56	330	8,100	54	294	1.13
V	Second ammonium sulfate fractionation	6,100	37	1,640	5,300	35	1,420	1.15

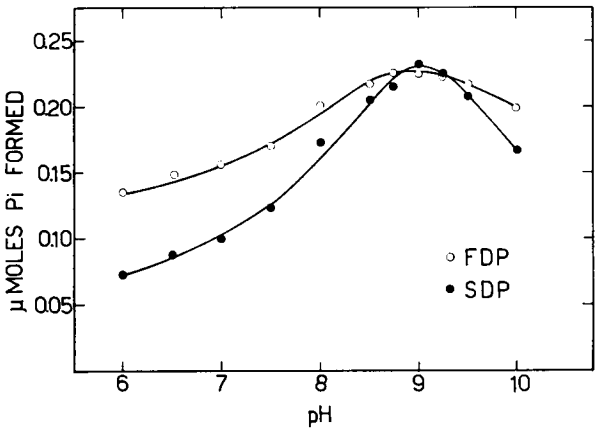

FIG. 1. pH optimum for FDP and SDP cleavage. Activity was determined by the routine assay, except that each reaction mixture was adjusted to the proper pH with NaOH.

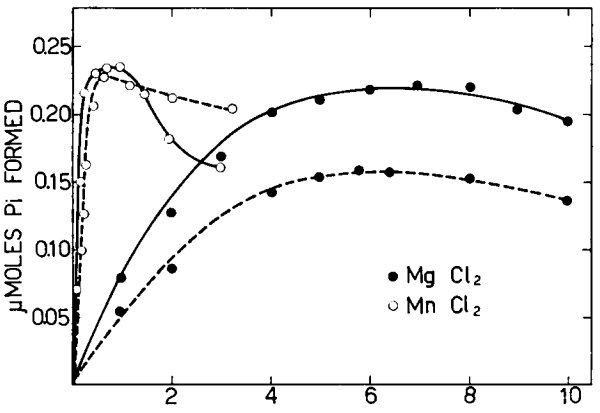

FIG. 2. Effect of Mg^{++} and Mn^{++} concentration on the rate of FDP or SDP cleavage. Activity of Fraction V was measured in the routine assay. The concentration of cations is expressed on the *abscissa* as molarity $\times 10^6$ for FDP (*full line*) and as molarity $\times 10^4$ for SDP (*dotted line*).

Soluble Liver Fraction—Livers of Sprague-Dawley rats (180 to 220 g) were used. The soluble fraction was prepared by centrifugation for 60 minutes at 32,000 $\times$ *g* of a 20% homogenate in 0.15 M KCl containing 2×10^{-4} M $NaHCO_3$ (Fraction I; 300 ml).

Acid Fractionation—The soluble fraction was adjusted to pH 3.7 with 0.5 N lactic acid. The heavy precipitate was immediately discarded by centrifugation for 7 minutes at 30,000 $\times$ *g* and the supernatant was brought to pH 7.7 with 1 N NaOH. A clear supernatant was obtained after removal of the precipitate as above (Fraction II; 310 ml).

First Ammonium Sulfate Fractionation—The proceeding fraction was treated with 75 g of ammonium sulfate and after 10 minutes the precipitate was centrifuged and discarded. To the supernatant solution were added 13.5 g of ammonium sulfate and the precipitate was collected and dissolved in water (Fraction III; 9.2 ml).

Heat Treatment—In order to reach the proper pH (4.7 and a suitable protein concentration (11 to 12 mg per ml), Fraction III was diluted with 9.3 ml of 0.2 M sodium acetate buffer, pH 4.5. The dilute fraction was heated in a water bath at 70° for 7 minutes and chilled to 0°, and the precipitate was centrifuged at 30,000 $\times$ *g* for 10 minutes and discarded (Fraction IV; 18 ml).

Second Ammonium Sulfate Fractionation—To Fraction IV an equal volume of a saturated ammonium sulfate solution (0°), pH 6.4, was added. The mixture was immediately centrifuged at 30,000 $\times$ *g* for 10 minutes and the precipitate was suspended in a 0.41 saturated ammonium sulfate solution (0°), pH 3.7 (Fraction V; 4.0 ml). The preparation was stored at $-10°$ with practically no loss of activity for 2 months.

In preliminary experiments the enzyme has been further purified to approximately 1500-fold by dialysis against increasing concentration of ammonium sulfate.

RESULTS

Purification of Enzyme—Table I summarizes the course of enzyme purification. The ratio of the two diphosphatases remains constant throughout the purification. The purified enzyme does not contain detectable amounts of aldolase, transketolase, or transaldolase.

pH Optimum—The maximal rate of cleavage for both SDP and FDP occurred at about pH 9.0 (Fig. 1). The shapes of the curves were similar for the two substrates. Identical results have been observed with different buffers such as triethanolamine, barbital, and Tris.

Effects of Cations—At all stages of purification Mg^{++} or Mn^{++} is required for activity with both substrates. The effect of cation concentration on the rate of hydrolysis is shown in Fig. 2. With FDP optimal concentrations were 1×10^{-4} M for $MnCl_2$ and 7×10^{-4} M for $MgCl_2$. With SDP optimal concentrations were 7×10^{-4} M and 7×10^{-3} M for $MnCl_2$ and $MgCl_2$, respectively. At high concentrations of both cations a decrease in the rate of hydrolysis has been constantly observed. These results are not dependent on the nature of the anion utilized. No requirement for cysteine was observed at any stage of purification.

Substrate Affinity—The K_m values were calculated by the method of Lineweaver and Burk (16). At pH 9.0 in triethanolamine buffer, with the optimal levels of $MnCl_2$ indicated in Fig. 2, the values for FDP and SDP were, respectively, 1.2×10^{-5} M and 3×10^{-4} M.

The two substrates behave as reciprocal competitive inhibitors. In Fig. 3 the effect of SDP on the rate of FDP hydrolysis is shown. The K_i for SDP calculated from the data reported in Fig. 3 was equal to 4×10^{-4} M, which corresponds within experimental error, to the K_m for this substrate. In the presence of 1×10^{-3} M FDP and an equal concentration of SDP, no detectable sedoheptulose 7-phosphate is formed and all of the P_i liberated closely corresponds to the fructose 6-phosphate formed.

Substrate Specificity—The relative activities with FDP or SDP as substrates did not change significantly throughout the purification, as mentioned above (Table I). A number of monophosphate and diphosphate esters, such as D-glucose 6-phosphate, D-glucose 1-phosphate, D-fructose 1-phosphate, D-fructose 6-phosphate, D-ribose 5-phosphate, D-ribulose 5-phosphate, D-xylulose 5-phosphate, D-ribulose 1,5-diphosphate, sedoheptulose 1-phosphate, and sedoheptulose 7-phosphate, were tested as substrates for the purified enzyme. Under the conditions of the standard assay, with substrate concentrations equal to 2×10^{-3} M, none of these compounds was cleaved at a significant rate.

Heat Denaturation—The purified enzyme has been exposed to different temperatures as shown in Table II. The relative rates of hydrolysis of the two substrates did not change during the course of heat inactivation.

Products of Cleavage and Stoichiometry of Reactions—The cleavage products obtained from FDP and SDP with the purified enzyme preparation have been characterized as D-fructose 6-phosphate and sedoheptulose 7-phosphate, respectively. The identification was based on enzymatic and chromatographic analysis of the reaction products as described elsewhere (5).

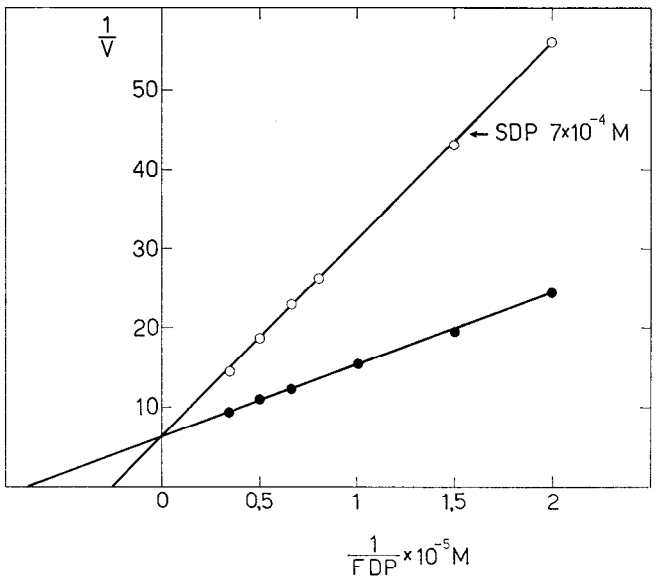

FIG. 3. Effect of FDP and SDP concentration on the rate of FDP cleavage. Activity in Fraction V was measured by the enzymatic method which evaluates the appearance of fructose-6-P. FDP was present at the concentrations indicated either alone or with SDP, 7×10^{-4} M. $MnCl_2$ concentration was 1×10^{-4} M; triethanolamine buffer was 4×10^{-2} M at pH 9.0; temperature, 37°.

TABLE II

Heat inactivation

Samples of purified enzyme (Fraction V) were heated at different temperatures as indicated. After cooling in ice, the samples were centrifuged and the supernatant solutions were tested for sedoheptulose diphosphatase and fructose diphosphatase activity with the routine assay.

Temperature	Heating period	Relative activity remaining	
		Fructose diphosphatase	Sedophetulose diphosphatase
	min		
		100	100
70°	10	96	94
75	7	92	93
75	15	88	86
80	7	52	50
80	15	32	30

TABLE III

Occurrence of diphosphatase in mammalian tissues

Tissues were homogenized in 4 volumes of isotonic KCl and centrifuged for 60 minutes at 30,000 × *g*. The supernatant solutions were tested for fructose diphosphatase (activity A) and for sedoheptulose diphosphatase (activity B) with routine assay. Activity is expressed as micromoles per hour of P_i released per g of wet tissue (total activity) and per mg of soluble protein (specific activity).

Tissue	Total activity		Specific activity	
	A	B	A	B
Rat liver	270	290	2.4	2.6
Rabbit liver	240	275	2.1	2.4
Guinea pig liver	170	185	1.5	1.7
Pig liver	390	410	3.2	3.4
Calf liver	210	175	1.9	1.6
Rat kidney	320	360	4.2	4.7
Guinea pig kidney	165	190	2.1	2.4

The stoichiometry of the over-all reaction was found to be in perfect agreement with Equations 1 and 2; the amounts of P_i liberated, of monophosphate ester formed, and of diphosphate ester which disappeared were equivalent.

Distribution of Enzymatic Activity—Both fructose diphosphatase and sedoheptulose diphosphatase activities have been detected in the soluble fractions of rabbit, guinea pig, calf, and pig liver and in the supernatant fraction of rat and guinea pig kidney. Neither activity is present in cell extracts of rabbit bone marrow, Ehrlich ascites carcinoma, or Crocker ascites sarcoma 100 (see Table III).

DISCUSSION

A diphosphatase which converts SDP to sedoheptulose-7-P has been identified in the soluble liver fraction of different animals. The enzyme activity has been purified almost 700-fold from rat liver and shown also to hydrolyze FDP to fructose-6-P.

Several lines of evidence strongly suggest that the same enzyme is responsible for the cleavage of both SDP and FDP. The ratio of activities with the two substrates remains constant throughout the purification. Each substrate behaves as a

competitive inhibitor in the hydrolysis of the other substrate. K_m for SDP is equal to K_i for this compound when it is tested as an inhibitor of FDP hydrolysis. Finally, both activities show similar responses to Mg^{++} or Mn^{++}, pH changes, and heat treatment.

None of a variety of other monophosphate and diphosphate esters tested as substrates was cleaved by the purified enzyme at significant rates. These results suggest an absolute requirement for the presence of two phosphate groups and also for the configuration found in FDP and SDP. D-Ribulose 1,5-diphosphate differs in the configuration at C-3 and is not attacked.

With respect to the physiological role of the diphosphatase, although it has been generally recognized that fructose diphosphatase may represent a key enzyme in the synthesis of glycogen from 3 carbon fragments, no direct evidence for a role of sedoheptulose diphosphatase has yet been obtained.

Three possibilities may be considered with respect to the physiological significance of the latter activity. (*a*) It may be merely an expression of the lack of complete specificity of fructose diphosphatase; (*b*) it may operate as an apparent fructose diphosphatase in the presence of catalytic amounts of either sedoheptulose 7-phosphate, D-erythrose 4-phosphate, or SDP, according to the following equations.

$$\text{FDP} \xleftrightarrow{\text{aldolase}} 2 \text{ triose phosphate} \quad (1)$$

$$\text{Sedoheptulose-7-P} + \text{triose phosphate} \xleftrightarrow{\text{transaldolase}} \text{fructose-6-P} + \text{erythrose-4-P} \quad (2)$$

$$\text{Erythrose 4-phosphate} + \text{triose phosphate} \xleftrightarrow{\text{aldolase}} \text{SDP} \quad (3)$$

$$\text{SDP} \xrightarrow{\text{sedoheptulose diphosphatase}} \text{sedoheptulose-7-P} + P_i \quad (4)$$

$$\text{Sum: FDP} \rightarrow \text{fructose-6-P} + P_i$$

This series of reactions has been shown to occur in a reconstructed system with specific sedoheptulose diphosphatase purified from *Torula* yeast (17). (*c*) Sedoheptulose diphosphatase may operate to maintain optimal concentration levels of erythrose 4-phosphate. This compound has been shown (18) to be a powerful inhibitor of the hexosephosphate isomerase, and it is likely that the level of erythrose 4-phosphate will affect the balance between the oxidative shunt and other metabolic pathways utilizing fructose-6-P.

Further work is in progress to evaluate these hypotheses through a comparative study of their occurrence in cells which are metabolically oriented in the direction of rapid glucose synthesis.

SUMMARY

A simple procedure is described for the purification, from rat liver, of a diphosphatase activity which cleaves both fructose diphosphate and sedoheptulose diphosphate to fructose 6-phosphate and sedoheptulose 7-phosphate, respectively.

Evidence that a single enzyme is responsible for both reactions is derived from the results of the purification steps, from kinetic measurements, and from other properties. The enzyme has no activity with a variety of monophosphate esters and other diphosphate esters.

Both activities are present in the liver of all mammalian species studied.

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