Characterization of Two Fructose Bisphosphatase Isoenzymes from the Hydrogen Bacterium *Nocardia opaca* 1b

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Two distinct forms of fructose 1,6-bisphosphatase, designated form A and form B, have been partially purified from autotrophically grown cells of the hydrogen bacterium *Nocardia opaca* 1b. Form A was present when the organism was grown under autotrophic conditions but was not synthesized during heterotrophic growth. Form B was detected in both autotrophically and heterotrophically grown organisms, suggesting that it is a constitutive enzyme. The molecular weights were estimated to be 140000 for form A and 190000 for form B. Both purified enzymes showed fructose bisphosphatase as well as sedoheptulose bisphosphatase activity, with pH optima of 8.2 and 8.8 to 9.0 for forms A and B, respectively. The ratio of fructose bisphosphatase to sedoheptulose bisphosphatase activity was 0.6 for form A and about 4.5 for form B. The enzymes required Mg^{2+} for activity; Mn^{2+} could only partially replace Mg^{2+} . Form A was inhibited by ATP and ribulose biphosphate. AMP exerted a strong allosteric inhibition on form B which was also inhibited by NAD^+ and NADH. Phosphoenolpyruvate activated form B. On the basis of these results, different physiological roles for the two forms of fructose bisphosphatase are suggested.

INTRODUCTION

In the gluconeogenic pathway of heterotrophic organisms a number of enzyme reactions are involved which also occur in the Calvin cycle for the reductive assimilation of carbon dioxide in autotrophs. In both metabolic pathways, fructose bisphosphatase (EC 3.1.3.11), catalysing the hydrolytic cleavage of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate, is a principal regulatory enzyme (Bassham, 1971; Horecker *et al.*, 1975). The enzymes isolated from animal (Taketa & Pogell, 1965), plant (Racker & Schroeder, 1958) and bacterial sources (Fraenkel *et al.*, 1966; Springate & Stachow, 1972a) resemble each other in major characteristics but exhibit distinct regulatory properties which seem to suit their different physiological functions in heterotrophic and autotrophic metabolism. While fructose bisphosphatases from mammalian tissues and the majority of heterotrophic bacteria are strongly inhibited by AMP (Taketa & Pogell, 1965; Opheim & Bernlohr, 1975), fructose bisphosphatase from plant chloroplasts (Buchanan *et al.*, 1971) as well as photo- and chemolithoautotrophic bacteria (Joint *et al.*, 1972; Abdelal & Schlegel, 1974) are insensitive to AMP. Furthermore, the chloroplast fructose bisphosphatase and a related enzyme activity, sedoheptulose bisphosphatase, which is also part of the Calvin cycle, are reductively activated in the light (Buchanan *et al.*, 1976; Schürmann & Wolosiuk, 1978).

Very little information is available on whether facultatively autotrophic bacteria synthesize fructose bisphosphatases with different regulatory properties during autotrophic and

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heterotrophic growth. The purpose of this study was to examine the nature of the fructose bisphosphatases from autotrophically and heterotrophically grown cells of the facultatively chemolithoautotrophic hydrogen bacterium *Nocardia opaca* strain 1b. We report here the separation, partial purification and characterization of two bisphosphatase isoenzymes, both of which also exhibited a sedoheptulose bisphosphatase activity. Their differential synthesis and catalytic properties suggest a specific physiological role for each of the isoenzymes.

METHODS

Organism. *Nocardia opaca* strain 1b, DSM 427 (Aggag & Schlegel, 1973) was obtained from the culture collection of this institute.

Chemicals. Most enzymes, bovine serum albumin, most sugar phosphates and other biochemicals were from Boehringer. Ammediol (2-amino-2-ethyl-1,3-propanediol), phenylmethanesulphonyl fluoride, ribulose 1,5-bisphosphate, ribulose 5-phosphate and sedoheptulose 1,7-bisphosphate were from Sigma; urease (EC 3.5.1.5) was from Miles-Seravac, Maidenhead, Berks. All other chemicals were obtained from Merck.

Culture conditions and preparation of crude extracts. The organism was grown at 30 °C in a 10 l fermenter (Braun, Melsungen, West Germany) using the mineral salts medium of Schlegel *et al.* (1961). Autotrophic cultures were supplied with a gas mixture of H₂/O₂/CO₂ (8:1:1, by vol.) at 500 ml min⁻¹. For heterotrophic growth under air (aeration at 2 l min⁻¹), the medium was supplemented with 0.5 % (w/v) organic substrate. The maximum specific growth rate ($\mu_{\max}$) was 0.092 h⁻¹ for autotrophic growth, and 0.103 h⁻¹ and 0.277 h⁻¹ for heterotrophic growth with pyruvate and fructose, respectively. Organisms were harvested during mid-exponential phase by centrifugation and washed with 0.05 M-Tris/HCl buffer (pH 7.6) containing 2.5 mM-MgCl₂, 0.1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride and 1 mM-dithioerythritol (Tris/MEPD buffer).

The temperature was maintained at 0 to 4 °C throughout the preparation of crude extracts and the purification procedure. Organisms (2 to 100 g wet wt) were suspended at a concentration of 0.33 g ml⁻¹ in Tris/MEPD buffer and ruptured by passing twice through a French pressure cell at 140 MPa. The homogenate was centrifuged at 20000 g for 20 min to remove unbroken cells and debris and the supernatant was recentrifuged at 140000 g for 60 min. The resulting supernatant fraction contained all the fructose/sedoheptulose bisphosphatase activity and was used as crude extract. Protein was determined by Lowry's method.

Separation of the fructose bisphosphatase isoenzymes. The protein concentration of the crude extract from autotrophically grown organisms was adjusted to 20 mg ml⁻¹ by dilution with Tris/MEPD buffer and the extract was taken to 45 % saturation by adding saturated (NH₄)₂SO₄ solution (pH 7.6). The precipitated fraction contained virtually all the activity of the form A isoenzyme and was dissolved in a minimal volume of Tris/MEPD buffer. A second protein fraction, precipitating between 45 and 50 % saturation with (NH₄)₂SO₄, showed no fructose/sedoheptulose bisphosphatase activity and was therefore discarded. Protein precipitating between 50 and 80 % (NH₄)₂SO₄ saturation contained the form B isoenzyme; it was collected by centrifugation and dissolved in a minimal volume of Tris/MEPD buffer. Both enzyme solutions were dialysed against the same buffer. On (NH₄)₂SO₄ fractionation of crude extracts from heterotrophically grown organisms, fructose bisphosphatase activity was exclusively found in the fraction precipitating between 50 and 80 % salt saturation. The precipitate was treated as described before.

Partial purification of form A and form B fructose bisphosphatase.

(i) *DEAE-Sephadex chromatography.* The dialysed enzyme solutions from the (NH₄)₂SO₄ fraction, supplemented with 2 mM-fructose bisphosphate, were separately applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with Tris/MEPD buffer containing 2 mM-fructose bisphosphate. The column was first washed with 250 ml of the equilibrating buffer and the protein was then eluted with 1.5 l of a linear gradient of KCl (0 to 0.5 M) in the same buffer at a flow rate of 50 ml h⁻¹. Form A fructose bisphosphatase was eluted between 0.22 and 0.29 M-KCl, while the form B isoenzyme was eluted between 0.32 and 0.42 M-KCl. Fructose bisphosphatase from heterotrophically grown organisms was eluted at exactly the same ionic strength as the form B isoenzyme. The enzyme fractions were concentrated by (NH₄)₂SO₄ precipitation (80 % saturation), dialysed against Tris/MEPD buffer and re-chromatographed on the same column. Elution was with 1.5 l linear gradients of KCl in Tris/MEPD buffer containing 2 mM-fructose bisphosphate. The concentration ranges of the gradients were from 0.15 to 0.35 M-KCl for form A and from 0.2 to 0.4 M-KCl for form B or the enzyme from heterotrophically grown organisms. Again, form A was eluted between 0.22 and 0.29 M-KCl whereas form B or the enzyme from heterotrophically

grown organisms was eluted between 0.32 and 0.36 M-KCl. The protein of the enzyme solutions was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80 % saturation).

(ii) *Back-extraction with ammonium sulphate.* The enzyme preparations from the previous step were extracted stepwise with $(\text{NH}_4)_2\text{SO}_4$ solutions (prepared in Tris/MEPD buffer containing 2 mM-fructose biphosphate) in 2 to 5 % decrements ranging between 75 and 40 % saturation. Two successive extractions, each of 2 ml, were employed at each step. Form A dissolved between 45 and 42 % saturation, form B or the enzyme from heterotrophically grown organisms between 65 and 58 % saturation.

(iii) *Sephadex G-200 gel filtration.* The enzyme solutions from the $(\text{NH}_4)_2\text{SO}_4$ back-extraction were applied to a Sephadex G-200 column (2.5 × 95 cm; void vol. 190 ml) equilibrated with Tris/MEPD buffer containing 2 mM-fructose biphosphate and eluted with the same buffer at a flow rate of 10 ml h⁻¹ (2.2 ml fractions). The elution volume for form B or the enzyme from heterotrophically grown organisms was 261 ml and that for form A was 279 ml. To estimate the molecular weights of the enzymes (Reiland, 1971), the Sephadex G-200 column was calibrated with the following standards (2 mg of each protein): urease (mol. wt 483000), catalase (EC 1.11.1.6; 247000), aldolase (EC 4.1.2.13; 158000), bovine serum albumin (68000) and cytochrome *c* (12500). Fractions with similar specific activity were combined, concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80 % saturation), then dissolved in a minimal volume of Tris/MEPD buffer and finally dialysed against the same buffer. The enzyme solutions could be stored at -20 °C for several weeks without appreciable loss of activity.

Enzyme assays. Fructose bisphosphatase activity was measured either spectrophotometrically at 334 nm in a coupled assay by following the rate of reduction of NADP⁺, or colorimetrically based on the release of inorganic phosphate. Both assay procedures gave identical enzyme activities. Sedoheptulose bisphosphatase activity was determined only by the colorimetric assay. The stoichiometry of the sedoheptulose bisphosphatase reaction, i.e. the equimolar formation of sedoheptulose 7-phosphate and inorganic phosphate from sedoheptulose 1,7-bisphosphate, was established as described by Rosen (1966).

The standard reaction mixture for the spectrophotometric assay (final volume 0.60 ml) contained 100 mM-Tris/HCl buffer (pH 8.5), 2.5 mM-MgCl₂, 0.1 mM-EDTA, 0.2 mM-NADP⁺, 1.75 units phosphoglucose isomerase (EC 5.3.1.9), 1.4 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.1 mM-fructose biphosphate and an appropriate amount of enzyme. The reaction was started by the addition of the enzyme and the formation of NADPH was followed in a Zeiss PL4 spectrophotometer connected to a Servogor RE511 recorder.

The standard reaction mixture for the colorimetric assay (final volume 0.50 ml) contained 100 mM-Tris/HCl buffer (pH 8.5), 2.5 mM-MgCl₂, 0.1 mM-EDTA, 0.1 mM-fructose biphosphate or sedoheptulose biphosphate and enzyme. The reaction was stopped after 2.5 min by adding 0.1 ml of 2.5 M-H₂SO₄ and the precipitate, if any, was removed by centrifugation. The amount of inorganic phosphate in the supernatant was determined according to Ames (1966). Controls without enzyme or without substrate were included. All enzyme assays were performed at 30 °C. One unit of enzyme is defined as the amount catalysing the hydrolysis of 1 μmol fructose 1,6-bisphosphate or sedoheptulose 1,7-bisphosphate min⁻¹ at 30 °C.

RESULTS

Studies with crude extracts

Crude extracts of autotrophically grown organisms contained both fructose bisphosphatase and sedoheptulose bisphosphatase activity in the ratio 1.4:1 (Table 1). In organisms grown with pyruvate or fructose, sedoheptulose bisphosphatase was almost completely absent while fructose bisphosphatase was only partially depressed. Preliminary studies on the regulatory properties of the enzymes indicated that AMP exerted a much stronger inhibitory effect on the fructose bisphosphatase activity from heterotrophically grown organisms. These results suggested that different fructose bisphosphatases exist in *N. opaca* grown under autotrophic and heterotrophic conditions. Another question raised by these preliminary observations was whether the sedoheptulose bisphosphatase activity detected in autotrophically grown organisms was due to a separate specific enzyme or was the property of a single protein catalysing both the fructose bisphosphatase and sedoheptulose bisphosphatase reactions.

Table 1. *Specific activities of fructose biphosphatase and sedoheptulose biphosphatase in N. opaca under different growth conditions*

Activities were determined colorimetrically by the inorganic phosphate assay as described in Methods. Specific activities are expressed as units (mg protein)⁻¹.

Growth conditions (carbon source)	Fructose biphosphatase	Sedoheptulose biphosphatase
Autotrophic (carbon dioxide)	0.133	0.095
Heterotrophic (pyruvate)	0.080	< 0.001
Heterotrophic (fructose)	0.053	0.001

Separation and partial purification of two fructose biphosphatase isoenzymes

The two fructose biphosphatase isoenzymes, designated form A and form B, were effectively separated by a simple ammonium sulphate fractionation of crude extracts from autotrophically grown organisms at 45 % salt saturation. The form A isoenzyme, which catalysed the hydrolysis of the phosphate group on C-1 of sedoheptulose 1,7-bisphosphate in addition to that of fructose 1,6-bisphosphate, comprised about 60 % of the total fructose biphosphatase activity in autotrophically grown organisms. Crude extracts from organisms grown heterotrophically on pyruvate or fructose only contained fructose biphosphatase which precipitated between 50 and 80 % ammonium sulphate saturation. Further purification of the isoenzymes was achieved by DEAE-Sephadex A-50 chromatography, back-extraction with ammonium sulphate and gel filtration on Sephadex G-200 (Table 2). The purified enzymes were free of detectable amounts of aldolase. Both crude fractions and purified preparations of form A had fructose biphosphatase as well as sedoheptulose biphosphatase activity. The ratio of fructose biphosphatase to sedoheptulose biphosphatase activity remained constant at approximately 0.6 during the last purification steps. In contrast, form B showed no sedoheptulose biphosphatase activity in crude fractions but acquired this activity in the course of the purification procedure. However, the ratio of fructose biphosphatase to sedoheptulose biphosphatase activity was never less than about 4.5.

The fructose biphosphatase from pyruvate-grown organisms was purified by the same procedure as that described for form B. Furthermore, if purified form B was mixed with crude extracts from pyruvate-grown organisms only one enzyme activity was subsequently recovered. This suggested that form B from autotrophically grown organisms was identical with fructose biphosphatase from heterotrophically grown organisms. These two enzymes were identical with respect to all properties tested, lending further support to this suggestion.

Properties of the partially purified isoenzymes

Molecular weight. The molecular weights of the isoenzymes were estimated by gel filtration on Sephadex G-200 using urease, catalase, aldolase, bovine serum albumin and cytochrome *c* as standards. Molecular weights of 140000 for form A and 190000 for form B were obtained from the standard calibration curve (results not shown). The molecular weights of the enzymes did not change during purification.

Effect of pH and EDTA. In Tris/HCl or Ammediol/HCl buffers, the fructose biphosphatase as well as the sedoheptulose biphosphatase activity of form A was optimal at pH 8.2, whereas form B showed maximal activity between pH 8.8 and 9.0. The activity of both isoenzymes was increased about threefold by EDTA at concentrations higher than 10 μ M and 3 μ M for form A and form B, respectively, but the pH optima did not change. EDTA concentrations above 150 μ M were inhibitory.

Substrate and cofactor specificity. To differentiate the two isoenzymes from non-specific phosphatases, a number of sugar phosphates were tested for their ability to serve as

Table 2. Separation and purification of two fructose biphosphatase/sedoheptulose biphosphatase isoenzymes (form A and form B) from autotrophically grown *N. opaca*

Enzymes were extracted from 100 g (wet wt) organisms. Activities were determined colorimetrically by the inorganic phosphate assay as described in Methods; specific activities are expressed as units (mg protein)⁻¹.

Fraction	Total protein (mg)	Fructose biphosphatase (FBP)		Sedoheptulose biphosphatase (SBP)		Ratio of activities (FBP:SBP)
		Total activity	Specific activity	Total activity	Specific activity	
Crude extract	8100	1077	0.133	770	0.095	1.4
Form A						
(NH ₄) ₂ SO ₄ pellet (0-45 % satn)	3840	507	0.132	611	0.159	0.83
First DEAE-Sephadex A-50 eluate	958	374	0.39	567	0.592	0.66
Second DEAE-Sephadex A-50 eluate	130	328	2.52	568	4.37	0.58
(NH ₄) ₂ SO ₄ back-extraction (45-42 % satn)	45	249	5.53	403	8.96	0.62
Sephadex G-200 eluate	11	130	11.82	225	20.45	0.58
Form B						
(NH ₄) ₂ SO ₄ pellet (50-80 % satn)	1990	412	0.207	< 1	< 0.001	—
First DEAE-Sephadex A-50 eluate	712	368	0.517	59	0.083	6.24
Second DEAE-Sephadex A-50 eluate	127	221	1.74	40	0.315	5.53
(NH ₄) ₂ SO ₄ back-extraction (65-58 % satn)	81	172	2.12	32	0.395	5.38
Sephadex G-200 eluate	28	95	3.39	21	0.75	4.52

Fig. 1. Influence of sedoheptulose biphosphate on the fructose biphosphatase activities of form A (a) and form B (b) isoenzymes. Double-reciprocal plots of reaction velocity versus fructose biphosphate concentration are shown for fixed sedoheptulose biphosphate concentrations of 0 ($\circ$), 0.05 mm ($\bullet$) and 0.10 mm ($\square$). The insets show replots of the slopes versus sedoheptulose biphosphate (SBP) concentration. Activities were determined at pH 8.5 using the spectrophotometric assay.

Table 3. *Differential inhibition of the two fructose biphosphatase isoenzymes from N. opaca*

Fructose biphosphatase activities were determined colorimetrically by the inorganic phosphate assay at pH 8.5. Excess MgCl_2 in equivalent amounts was added to the reaction mixture when ATP was present.

Effector	Concn (mm)	Inhibition (%)	
		Form A	Form B
ATP	0.25	31	9
	1.0	70	49
AMP	0.025	0	87
	0.10	9	100
NAD^+	1.0	0	67
NADH	1.0	20	83
Ribulose 1,5-bisphosphate	0.25	24	0
	1.0	48	3

substrates. Both enzymes specifically cleaved fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate yielding fructose 6-phosphate or sedoheptulose 7-phosphate, respectively, and inorganic phosphate. Neither enzyme hydrolysed glucose 1-phosphate, glucose 6-phosphate, glucose 1,6-bisphosphate, ribulose 5-phosphate, ribulose 1,5-bisphosphate, fructose 1-phosphate or fructose 6-phosphate in the inorganic phosphate assay at pH 8.5 or 6.0. The enzymes required Mg^{2+} for activity. Of other divalent cations tested, only Mn^{2+} could partially replace Mg^{2+} . At 0.5 mm- Mn^{2+} the reaction velocity for form A was 50 % and for form B 67 % of that measured with 0.5 mm- Mg^{2+} . Mn^{2+} concentrations higher than 0.5 mm inhibited both enzymes.

Kinetic data. Kinetic parameters were determined in initial velocity studies. Both enzymes exhibited normal Michaelis-Menten kinetics with respect to their substrates fructose bis-

Fig. 2

Fig. 3

Fig. 2. Influence of phosphoenolpyruvate on the fructose biphosphatase activity of the form B isoenzyme. Double-reciprocal plots of reaction velocity versus fructose biphosphate concentration are shown for fixed phosphoenolpyruvate concentrations of 0 (○), 0.05 mM (●), 0.10 mM (□) and 0.40 mM (■). Activities were determined at pH 8.5 using the spectrophotometric assay.

Fig. 3. Effect of phosphoenolpyruvate on the fructose biphosphatase activity of the form B isoenzyme at fixed AMP concentrations of 0 (○), 0.025 mM (●) and 0.10 mM (□). Plots of relative activity versus phosphoenolpyruvate concentrations are shown. The activities, measured by the spectrophotometric assay at pH 8.5, are expressed as a percentage of the activity with 0.1 mM-fructose biphosphate in the absence of both phosphoenolpyruvate and AMP, i.e. $0.008 \mu\text{mol min}^{-1}$.

phosphate and sedoheptulose biphosphate. The apparent K_m values obtained for fructose biphosphate and sedoheptulose biphosphate were $17 \mu\text{M}$ and $15 \mu\text{M}$, respectively, for both form A and form B. Form A was progressively inhibited by the substrates at concentrations higher than 0.1 mM. For both enzymes sedoheptulose biphosphate was a linear competitive inhibitor of the fructose biphosphatase activity (Fig. 1). The K_i values for sedoheptulose biphosphate were $16 \mu\text{M}$ for form A and $17 \mu\text{M}$ for form B and closely resembled the values of the Michaelis constants. These results strongly suggest that in both cases a single protein possessed fructose biphosphatase as well as sedoheptulose biphosphatase activity.

Sigmoid kinetics were observed with increasing concentrations of MgCl_2 in the presence of fixed concentrations of fructose biphosphate (results not shown). Hill coefficients (h) were calculated to be 1.7 for form A and 1.8 for form B, indicating positive cooperativity in the binding of Mg^{2+} . The $[\text{S}]_{0.5}$ for MgCl_2 was 0.26 mM and 0.36 mM for form A and form B, respectively.

Some regulatory properties. The effect of various metabolites on the activity of the isoenzymes was studied. The results indicated that the enzymes were differently regulated by purine and nicotinamide nucleotides and ribulose biphosphate (Table 3). Form A was inhibited by ATP but showed little sensitivity to AMP. A Hill plot of the inhibition data (not shown) yielded values of $[\text{I}]_{0.5}$ for ATP of 0.6 mM and an interaction coefficient of 0.8 indicating a weak, negative cooperativity in the binding of ATP. While ATP was only a moderate inhibitor for form B, AMP exerted a strong allosteric inhibition on this enzyme. An interaction coefficient of 3.4 and an $[\text{I}]_{0.5}$ value for AMP of $14 \mu\text{M}$ were calculated. NAD^+ and, particularly, NADH were also effective inhibitors of form B whereas form A was only slightly inhibited by these nucleotides. In contrast, only form A was significantly inhibited by ribulose biphosphate ($[\text{I}]_{0.5}$ 1.1 mM).

Initial velocity studies revealed that phosphoenolpyruvate was an activator of form B. Phosphoenolpyruvate did not effect the K_m value for fructose biphosphate but increased

the maximal velocity (Fig. 2) and had a K_a value of 40 μM . On the other hand, the activity of form A was inhibited 36 % by phosphoenolpyruvate at 1 mM. The AMP inhibition of form B could be partially overcome by phosphoenolpyruvate (Fig. 3). The degree of co-operative interactions among the AMP sites was greatly diminished by phosphoenolpyruvate. In the presence of 0.4 mM-phosphoenolpyruvate the Hill coefficient decreased to 2.0. In turn, the $[S]_{0.5}$ for phosphoenolpyruvate, i.e. the concentration of phosphoenolpyruvate giving 50 % activation, increased to 0.65 mM in the presence of 0.1 mM-AMP.

DISCUSSION

The hydrogen-oxidizing bacterium *Nocardia opaca* is able to form two distinct fructose biphosphatases: form A is an inducible enzyme formed only during autotrophic growth, suggesting that it functions in the Calvin cycle, whereas form B is a constitutive enzyme synthesized during heterotrophic growth on pyruvate or fructose as well as under autotrophic conditions. This latter enzyme plays a gluconeogenic role during heterotrophic growth.

This is the first time that fructose biphosphatase isoenzymes have been identified in an autotrophic bacterium. The existence of two fructose biphosphatases in plant tissues, one located in the cytoplasm and the other in the chloroplasts, has recently been demonstrated for spinach leaves (Zimmermann *et al.*, 1976, 1978). Two forms of the enzyme were also found in mammalian tissues. The fructose biphosphatase isolated from muscle differs in its immunological properties and amino acid composition from the enzyme in kidney or liver (Enser *et al.*, 1969; Tejwani *et al.*, 1976).

The isoenzymes from *N. opaca* resemble the fructose biphosphatases from other organisms in many properties. They have high affinities for their substrates, require Mg^{2+} or Mn^{2+} for activity and exhibit single pH optima in the alkaline range and are thus similar to the fructose biphosphatase from the hydrogen bacterium *Alcaligenes eutrophus* (Abdelal & Schlegel, 1974) and from the photosynthetic bacterium *Rhodospseudomonas palustris* (Springate & Stachow, 1972a). Like the enzymes from several eukaryotic and prokaryotic sources (Van Tol *et al.*, 1972; Rosen, 1966; Mukkada & Bell, 1971), the *N. opaca* enzymes are stimulated by EDTA, but the chelating agent did not alter the pH optima. For ox liver fructose biphosphatase, activation by EDTA was due to the removal of heavy metal inhibitors (Nimmo & Tipton, 1975).

Like the fructose biphosphatases from rat liver (Bonsignore *et al.*, 1963), *R. palustris* (Springate & Stachow, 1972a) and *A. eutrophus* (Abdelal & Schlegel, 1974), but unlike the enzymes from *Candida utilis* (Rosen *et al.*, 1965) and *Escherichia coli* (Fraenkel *et al.*, 1966), the purified *N. opaca* enzymes functioned as both fructose biphosphatases and sedoheptulose biphosphatases. Identical pH optima, similar kinetic constants and the competitive inhibition of the fructose biphosphatase activities by sedoheptulose biphosphate strongly suggested that in both cases the activities were associated with the same protein. The sedoheptulose biphosphatase activity of the form B isoenzyme was not observed in crude fractions but appeared in subsequent stages of purification, indicating that *in vivo* form B acts only as a fructose biphosphatase. We are unable to explain this loss of substrate specificity during purification. The molecular weight of the enzyme (190000) did not change detectably during purification. However, although precautions against proteolysis were taken by using the proteolytic inhibitors phenylmethanesulphonyl-fluoride and EDTA, slight proteolytic damage cannot be excluded. The molecular weight of form B is significantly higher than that of form A (140000); the latter is similar to molecular weights reported for the enzyme from other organisms (130000 to 140000) (Springate & Stachow, 1972a; Horecker *et al.*, 1975).

The regulatory properties of the two isoenzymes from *N. opaca* differ in many respects. Form A, like the enzyme from most autotrophic bacteria (Springate & Stachow, 1972b; Joint *et al.*, 1972; Abdelal & Schlegel, 1974), is inhibited by ATP and relatively insensitive

to AMP. The physiological significance of the inhibition by ATP is unclear since the Calvin cycle must operate maximally when the energy supply is in excess. The inhibition of form A by ribulose 1,5-bisphosphate, which is the primary carbon dioxide acceptor molecule, can be interpreted as end-product inhibition of an early reaction of the Calvin cycle catalysed by fructose biphosphatase.

Form B is severely inhibited by AMP. Thus, although *N. opaca* does not contain phosphofructokinase and degrades fructose preferentially via the Entner–Doudoroff pathway (Probst & Schlegel, 1973), the gluconeogenic form B enzyme is regulated in the same way as the fructose biphosphatases from heterotrophic organisms which use the Embden–Meyerhof glycolytic pathway (Taketa & Pogell, 1965; Fraenkel *et al.*, 1966). The finding that phosphoenolpyruvate not only activated form B but also counteracted the inhibitory effect of AMP underlines the gluconeogenic role of this enzyme. This type of precursor activation of fructose biphosphatase by phosphoenolpyruvate has also been reported for the enzymes from *Flavobacterium thermophilum* (Yoshida & Oshima, 1971) and *Bacillus licheniformis* (Opheim & Bernlohr, 1975). The strong inhibitory effects of NAD^+ and NADH on form B are surprising since their significance in the regulation of the enzyme is not clear. So far this is the only fructose biphosphatase which has been reported to be inhibited by nicotinamide dinucleotides.

To summarize, the results suggest that the two fructose biphosphatase isoenzymes of *N. opaca* play different physiological roles. During autotrophic growth form A catalyses the essential hydrolysis of both fructose bisphosphate and sedoheptulose bisphosphate in the Calvin cycle. Form B has a gluconeogenic function under heterotrophic growth conditions. Interestingly, indirect evidence has been obtained that in this strain the genes coding for the key autotrophic enzymes hydrogenase, phosphoribulokinase and ribulose bisphosphate carboxylase are located on a plasmid (Reh & Schlegel, 1975; Ecker, 1977). It remains to be determined whether additional enzymes of the Calvin cycle, e.g. form A fructose biphosphatase, are also coded on extrachromosomal DNA.

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