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Regulatory Properties of a Fructose 1,6-Bisphosphatase from the Cyanobacterium *Anacystis nidulans*

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A fructose 1,6-bisphosphatase (EC 3.1.3.11) (FBPase) was purified over 100-fold from *Anacystis nidulans*. At variance with a previous report (R. H. Bishop, Arch. Biochem. Biophys. **196**:295–300, 1979), the regulatory properties of the enzyme were found to be like those of chloroplast enzymes rather than intermediate between chloroplast (photosynthetic) and heterotrophic FBPases. The pH optimum of *Anacystis* FBPase was between 8.0 and 8.5 and shifted to lower values with increasing Mg^{2+} concentration. Under the experimental conditions used by Bishop, we found the saturation curve of the enzyme to be sigmoidal for Mg^{2+} ions and hyperbolic for fructose 1,6-bisphosphate. The half-maximal velocity of the *Anacystis* FBPase was reached at concentrations of 5 mM $MgCl_2$ and 0.06 mM fructose 1,6-bisphosphate. AMP did not inhibit the enzyme. The activity of the FBPase was found to be under a delicate control of oxidizing and reducing conditions. Oxidants like O_2, H_2O_2, oxidized glutathione, and dehydroascorbic acid decreased the enzyme activity, whereas reductants like dithiothreitol and reduced glutathione increased it. The oxido-reductive modulation of FBPase proved to be reversible. Reduced glutathione stimulated the enzyme activity at physiological concentrations (1 to 10 mM). The reduced glutathione-induced activation was higher at pH 8.0 than at pH 7.0.

D-Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11), an enzyme which cleaves D-fructose 1,6-bisphosphate into D-fructose 6-phosphate and inorganic phosphate, occurs in both autotrophic and heterotrophic organisms. The FBPases of chloroplasts (4, 13, 22) and photosynthetic procaryotes (20) participate in the photosynthetic fixation of CO_2 (Calvin cycle), whereas the FBPases of mammals and heterotrophic bacteria take part in gluconeogenesis (11, 21). Corresponding to their respective functions, the regulatory properties of the chloroplast and heterotrophic FBPases are distinctly different (1, 2, 11, 15, 21, 23, 29, 32). One would expect that the functionally and probably also phylogenetically related FBPases of chloroplasts and cyanobacteria should have similar regulatory properties. Surprisingly, Bishop (2) has concluded in a recent paper that the regulatory properties of the FBPase isolated from the unicellular, photoautotrophic cyanobacterium *Anacystis nidulans* are "not typical of either class of FBPase from other organisms" and "the enzyme may not be involved in the light-dependent reductive regulatory mechanisms." We have reinvestigated the problem and could not confirm any of the major conclusions of Bishop (2). We have found that the properties of the *Anacystis* FBPase are characteristic of the chloroplast type (photosynthetic) FBPases. In this paper, we present a short summary of the data which are at variance with those of Bishop (2) and describe in some more detail the redox properties of the enzyme.

MATERIALS AND METHODS

Organism and growth conditions. *A. nidulans* 14625 (*Synechococcus* sp. strain ANPCC6301) cells were grown in 6-liter glass vessels under sterile conditions in the liquid medium "C" of Kratz and Myers (12). The cultures were illuminated with cool-white fluorescent light (36 W/m^2). Aeration of the cultures, kept at 37°C, was achieved by bubbling sterile air containing 5% CO_2. Cells were harvested by centrifugation in the midlog phase of growth (5×10^7 cells ml^{-1}), washed with 0.05 M Tris-hydrochloride buffer (pH 7.5), and used for the experiments. Sterility of the cultures was rigorously tested by plating samples on yeast-tryptone medium solidified by 1% agar.

Assay of FBPase. Two methods were used to determine FBPase activity, as follows. (i) A continuous spectrophotometric assay (assay I) measured the enzyme activity by means of a coupled system which converted the fructose 6-phosphate formed into glucose 6-phosphate by phosphohexose isomerase, and the amount of the glucose 6-phosphate formed was quantified by adding glucose 6-phosphate dehydrogenase and NADP. (ii) A second assay (assay II) was based on the determination of P_i liberated from fructose 1,6-bisphosphate. The respective compositions of

the assay media were as follows. The first assay contained 100 mM Tris-hydrochloride buffer (pH 8.0), 0.1 mM fructose 1,6-bisphosphate, 5 mM $MgCl_2$, 0.5 mM NADP, 5 μg of commercial phosphohexose isomerase, 5 μg of commercial glucose 6-phosphate dehydrogenase, and an appropriate amount of enzyme in a final volume of 1 ml. The reaction was started by the addition of substrate, and the increase in absorbance was followed spectrophotometrically at 340 nm. A reference mixture was run lacking substrate. The rate of reaction was linear with time up to 10 min. The second assay contained 100 mM Tris-hydrochloride buffer (pH 8.0), 1 mM fructose 1,6-bisphosphate, 10 mM $MgCl_2$, 1 mM EDTA, and an appropriate amount of enzyme in a final volume of 1 ml. Control tubes were run without substrate. After incubation for 10 to 20 min, the reaction was terminated by the addition of 0.5 ml of 15% trichloroacetic acid, and the mixture was centrifuged at 10,000 rpm for 10 min. One-milliliter portions of the supernatant were analyzed for P_i by the method of Chen et al. (5). The rate of reaction measured by this assay was linear up to 30 min. Assay I was used in the kinetic experiments, and assay II was used in all experiments on the redox modulation of the enzyme.

Enzyme purification. Cultured cells (12 liters) were centrifuged. The sedimented cells were suspended in 0.05 M Tris-hydrochloride buffer containing 1 mM $MgCl_2$ and 1 mM mercaptoethanol at pH 7.5 (TMM buffer). The suspension, containing 10^{10} cells ml^{-1}, was subjected to ultrasonication in an MSE sonic disintegrator for a total of 20 min in 15-s intervals followed by 15-s cooling periods. The sonic extract was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 40,000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation, and the resulting precipitate was discarded. The precipitate obtained between 40 to 80% $(NH_4)_2SO_4$ saturation was retained and dissolved in 4 ml of 0.02 M TMM buffer (pH 7.5). The slightly turbid solution was centrifuged at 40,000 × *g* for 30 min. The supernatant was applied to a Sephadex G-100 column (2.5 by 100 cm; bed volume, 440 ml) previously equilibrated with 0.02 M TMM buffer (pH 7.5). Proteins were eluted from the column with the same buffer at a flow rate of 16 to 18 ml h^{-1}. The FBPase-rich fractions were pooled and applied to a DEAE-Sephacel column (2 by 30 cm) equilibrated with 0.02 M TMM buffer (pH 7.5). The column was washed with the above buffer, and the proteins were eluted with a NaCl gradient, composed from 150 ml of 0.3 M NaCl and 150 ml of 0.1 M NaCl, with a Varigrad apparatus. The active fractions were pooled and dialyzed against TMM buffer.

Assay of glutaredoxin. Glutaredoxin was assayed as described by Luthman et al. (see Table 1 in reference 17).

Assay of thioltransferase. The assay of thioltransferase (glutathione:disulfide oxidoreductase) was carried out by the method of Eriksson et al. (8) with slight modifications. The assay medium contained 50 mM Tris (pH 7.5), 3 μmol of reduced glutathione (GSH), 4 μmol of 5,5'-dithiobis(2-nitrobenzoate), 1 μmol of EDTA, and a suitable amount of enzyme in 1 ml. The change in absorbance at 412 nm was followed.

Protein content. The protein content was determined by the Lowry method (16).

RESULTS

Purification of FBPase. The enzyme was purified 135-fold. The specific activity of the purified enzyme preparation was 8.1 μmol min^{-1} mg^{-1} of protein. The FBPase preparation was not contaminated by nonspecific phosphatases. No enzyme activity or only traces of enzyme activity were found with the following substrates: *p*-nitrophenyl phosphate, glucose 6-phosphate, 6-phosphogluconate, fructose 6-phosphate, α-glycerophosphate, β-glycerophosphate, 5'-AMP, ATP, ADP, dAMP, and dUMP. The enzyme was stable and could be stored at −20°C for several weeks without significant loss of activity. Repeated freezing and thawing of the preparation, however, led to a gradual decrease in FBPase activity.

Experiments aimed at checking the published data. The following data, obtained with an enzyme preparation purified to a higher extent than that used by Bishop (2), were found to be at variance with the results published by Bishop. (i) Instead of a fixed pH optimum at pH 8.0 (2), we found a shift of the pH optimum to lower values with an increase in Mg^{2+} ion concentration (5 → 20 mM). This type of shift in pH (Fig. 1) is typical for the chloroplast FBPases (3, 22, 23, 32). (ii) The saturation curve of the enzyme

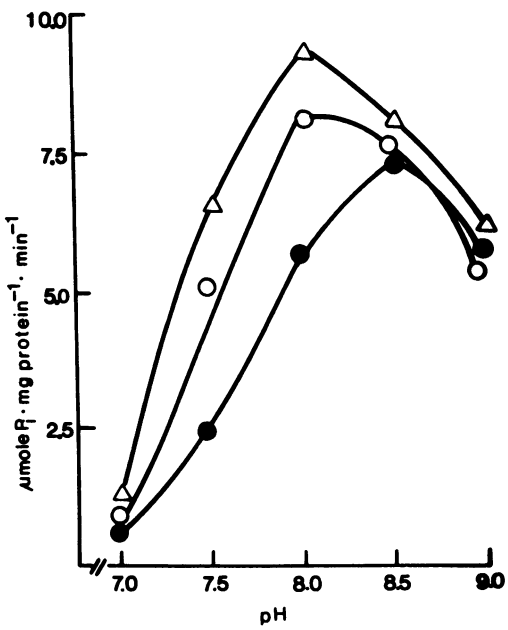

FIG. 1. Dependence of the activity of purified *Anacystis* FBPase on pH and Mg^{2+} ion concentration: 5 mM (●), 10 mM (○) and 20 mM (△) $MgCl_2$, respectively, in Tris-hydrochloride buffer.

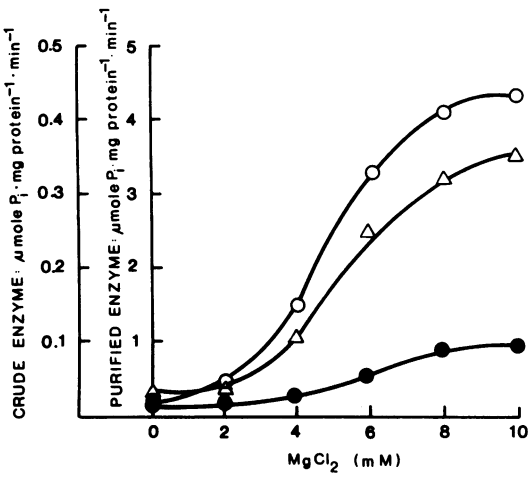

FIG. 2. Effect of $MgCl_2$ concentration on FBPase activity in a purified (●) and a crude (▲) preparation as well as in a purified preparation in the presence of 5 mM DTT (○).

for Mg^{2+} was not hyperbolic but sigmoidal at pH 8.0 both in crude and purified preparations (Fig. 2), as has also been observed with the chloroplast FBPases (3, 6, 22, 23, 31). (iii) The half-maximal velocity of the enzyme for Mg^{2+} was around 5 mM $MgCl_2$, a value much higher than claimed by Bishop (2). (iv) The enzyme activity was inhibited by fructose 1,6-bisphosphate concentrations above 3 to 4 mM (not shown), a phenomenon which occurs with the chloroplast FBPase as well (3). (v) Most importantly, the crude FBPase was only slightly (20%) inhibited, and the purified enzyme not at all, by AMP in the physiological concentration range (0.01 to 0.2 mM) at pH 7.0 or at pH 8.0 (not shown). This property is shared by all photosynthetic FBPases (3, 23, 31). (vi) In sharp contrast with Bishop (2) and an earlier paper by Duggan and Anderson (7), treatment of the *Anacystis* FBPase with the strong reductant dithiothreitol (DTT) dramatically increased the FBPase activity in crude preparations and, at higher concentrations, activated the purified enzyme as well (Fig. 3). Activation by DTT is a general characteristic of the chloroplast FBPase (1, 22, 31).

In accordance with Bishop (2), we found the substrate (fructose 1,6-bisphosphate) saturation curve of the *Anacystis* FBPase to be hyperbolic (not shown). The K_m for fructose 1,6-bisphosphate was 0.06 mM at pH 8.0, a value indeed significantly lower than that reported for the chloroplast FBPases (1, 3, 15).

Redox properties of the *Anacystis* FBPase. The enzyme could be deactivated by treatment with H_2O_2, oxidized glutathione, or dehydroascorbic acid in concentration ranges in which the chloroplast FBPases are also deactivated (25, 30). The results obtained with freshly prepared *Anacystis* crude extract are shown in Fig. 4. Similar results were obtained with DTT- or GSH-treated, activated enzyme (not shown). The H_2O_2-induced inhibition was, on a percentage basis, higher with the DTT-treated enzyme than with the freshly isolated, untreated one (not shown), in agreement with recent data of Charles and Halliwell (4) with a chloroplast FBPase (not

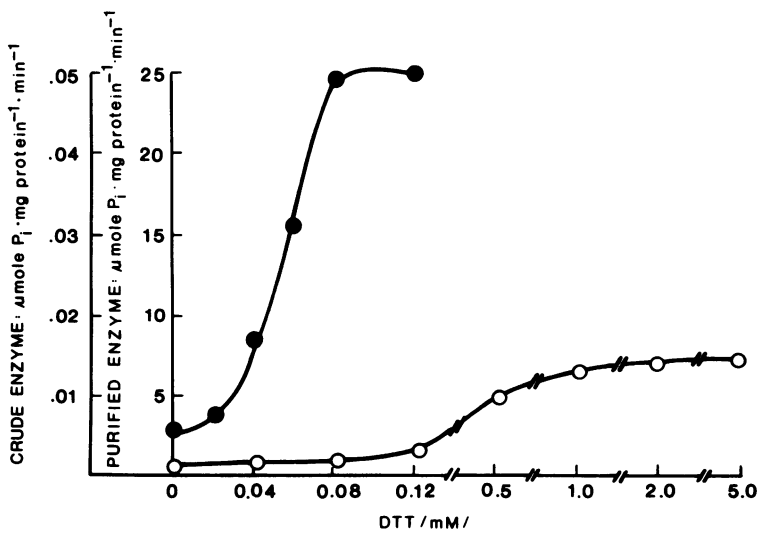

FIG. 3. Effect of DTT on the activity of FBPase in crude (●) and purified (○) preparations, respectively. DTT was present in the medium during assay.

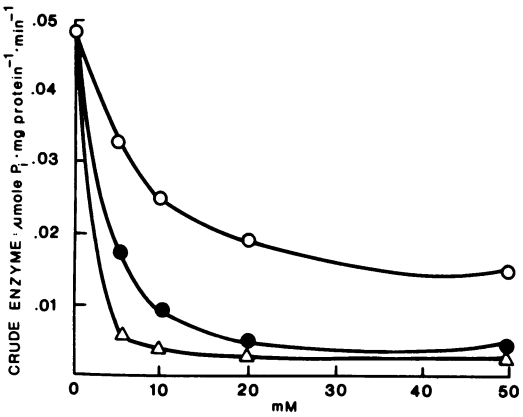

FIG. 4. Effect of oxidizing agents on the FBPase activity in crude enzyme preparations from *Anacystis*: oxidized glutathione (●), dehydroascorbic acid (○), H_2O_2 (△). The samples were pretreated for 20 min with the oxidants at the concentrations indicated in the figure. The pretreated sample was diluted 10-fold, and enzyme activity was measured at this 10-fold lower oxidant level.

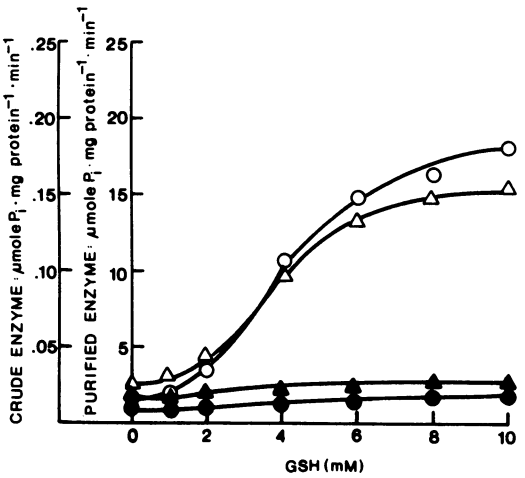

FIG. 5. Effect of different GSH concentrations on the activity of *Anacystis* FBPase in crude (△, ▲) and purified (○, ●) preparations at pH 8.0 (△, ○) and pH 7.0 (▲, ●). GSH was present in the medium during assay.

shown). The enzyme in freshly prepared crude extracts, after inactivation by treatment with H_2O_2, could be reactivated by both DTT and GSH (Table 1).

Effect of GSH. Of the above results, the reactivation of the oxidized *Anacystis* FBPase by GSH deserves special attention because activation by GSH has been reported neither for the chloroplast nor for the heterotrophic FBPases. Buchanan et al. (3), as well as Halliwell and Foyer (10), explicitly stated that in their experiments GSH had not activated the chloroplast FBPase. The mammalian FBPase, activated by oxidized glutathione via thiol-disulfide exchange, is deactivated by GSH (21).

Figure 5 shows that in our hands both the crude and the purified *Anacystis* FBPase preparations were activated by GSH at physiological concentrations. Results recently reported by Schmidt (24) also indicate an activation of the FBPase of *A. nidulans* (*Synechococcus* sp. strain 6301) by GSH, albeit only at fairly high concentrations. Our measurements were done at two pH values (pH 7.0 and 8.0) which correspond to the pH of the stroma of darkened and illuminated chloroplasts, respectively (13). It is noteworthy (Fig. 5) that significant activation was observed only at pH 8.0.

TABLE 1. Inactivation of FBPase in crude extracts by H_2O_2 and its reactivation by GSH and DTT

Treatment[a]		Enzyme activity (nmol P_i μg^{-1} of protein h^{-1})
H_2O_2	Reductant	
None	None	4.5
10 mM	None	1.7
10 mM	GSH (5 mM)	17.8
10 mM	DTT (0.1 mM)	38.8

[a] Samples after preincubation with H_2O_2 for 20 min were diluted 10-fold and treated, or not treated, during the subsequent assay (20 min) with a reductant.

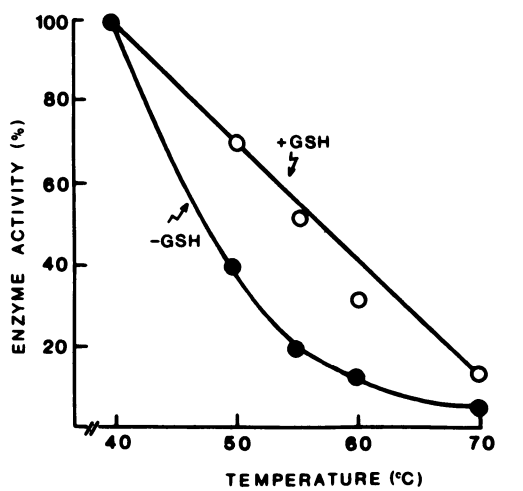

FIG. 6. Stabilizing effect of GSH against heat inactivation of a purified FBPase preparation from *Anacystis*. The enzyme, before assay, was exposed for 5 min to the temperatures indicated.

GSH not only activated but also stabilized the enzyme; it protected the FBPase against heat inactivation (Fig. 6).

DISCUSSION

The results show that the properties of the *Anacystis* FBPase are very close to those of the chloroplast FBPases. The enzyme is not a functional mixture in properties between autotrophic and heterotrophic FBPases, as claimed by Bishop (2). The reason for the discrepancy between the results of Bishop (2) and ours is not clear, but it is pertinent to stress that the *Anacystis* cultures are easily contaminated by heterotrophic bacteria. We have checked rigorously all preparations, crude or purified, for sterility.

A property of the *Anacystis* enzyme which, to the best of our knowledge, has been described neither for the chloroplast nor for the heterotrophic FBPases is that both the crude and the purified FBPase preparations from *Anacystis* were activated by GSH at physiological concentrations (glutathione is present in various organisms in concentrations up to 10 mM; 10, 26).

As to the possible mode of action of GSH, the following observations are pertinent: (i) millimolar concentrations of GSH activated both the crude and purified *Anacystis* FBPase; (ii) a major effect was obtained only at alkaline pH. These observations suggest an activation mechanism based on thiol-disulfide exchange in which mixed disulfide bonds of the enzyme are involved (for the conditions of such a reaction see references 19 and 28). The reaction observed has a strong nonenzymatic component (cf. the results obtained with the purified FBPase preparation), but we also found thiol-transferase (glutathione:disulfide oxidoreductase) activity in the *Anacystis* extracts which can be involved in the catalysis of thiol-disulfide exchange (9, 18, 26). Glutaredoxin (17) activity was, however, not found in the same extracts.

The thiol-protein mixed disulfide exchange reaction requires the reduced form of glutathione. This is available because glutathione is kept predominantly in the reduced state in all cell types by NADPH-dependent glutathione reductases (10, 26).

The role of the thioredoxin system in the redox modulation of chloroplast FBPase is well established (3, 13, 27, 30). The FBPase of *A. nidulans* (*Synechococcus* sp. strain 6301) is also modulatable by thioredoxin (24). Modulation by thioredoxin may indeed be the major mechanism of the redox (light/dark) modulation of FBPase in all photosynthetic organisms (31). However, alternative possibilities (regulation via ferralterin, an iron-sulfur protein) have also been reported for chloroplasts (14). The results described in the present paper and unpublished data (the glyceraldehyde 3-phosphate dehydrogenase of *Anacystis* is even more strongly activated by GSH than the *Anacystis* FBPase) suggest that in cyanobacteria regulatory mechanisms dependent on GSH and a light-induced change in pH also deserve serious attention.

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