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STUDIES ON THE REGULATORY PROPERTIES OF CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE

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Summary

The regulatory properties of chloroplast fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, (EC 3.1.3.11) were examined with a homogeneous enzyme preparation isolated from spinach leaves. The activation of the enzyme, that was earlier shown to occur via reduced thioredoxin, was found to be accompanied by a structural change that took place more slowly than the rate of catalysis. The recently found deactivation of the thioredoxin-activated enzyme by physiological oxidants such as oxidized glutathione and dehydroascorbic acid was also slow relative to catalysis. Under the conditions used, the activated enzyme showed a pH optimum of about 8.0, whereas the corresponding value for the non-activated form was pH 8.8.

The importance of the thioredoxin-linked mechanism of enzyme regulation that is effected through photoreduced ferredoxin and ferredoxin-thioredoxin reductase is discussed in relation to other light-controlled regulatory agents in chloroplasts.

Introduction

Chloroplast fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) (Fru-P_2ase), a key regulatory [1—4] enzyme of the reductive pentose phosphate cycle of photosynthetic CO_2 assimilation, catalyzes the hydrolytic cleavage of fructose 1,6-biphosphate to fructose 6-phosphate and P_i (Eqn. 1).

$$\text{Fructose 1,6-bisphosphate} + H_2O \xrightarrow[Mg^{2+}]{\text{Fru-P}_2\text{ase}} \text{Fructose 6-phosphate} + P_i \qquad (1)$$

Fructose-1,6-bisphosphatase, whose activity in chloroplasts is controlled by

light [1,3,4], is reductively activated by photoreduced ferredoxin and the two newly identified chloroplast proteins, thioredoxin and ferredoxin-thioredoxin reductase [5—7] (Eqns. 2—4).

$$H_2O + 2\ \text{Ferredoxin}_{oxidized} \xrightarrow[light]{\text{Chloroplasts}} 2\ \text{Ferredoxin}_{reduced} + \tfrac{1}{2}O_2 + 2H^+ \quad (2)$$

$$2\ \text{Ferredoxin}_{reduced} + \text{Thioredoxin}_{oxidized} + 2H^+ \xrightarrow[reductase]{\text{ferredoxin-thioredoxin}} \text{Thioredoxin}_{reduced} + 2\ \text{Ferredoxin}_{oxidized} \quad (3)$$

$$\text{Fru-}P_2\text{ase}_{inactive} \xrightarrow[thioredoxin]{\text{reduced}} \text{Fru-}P_2\text{ase}_{active} \quad (4)$$

Thioredoxin can also be reduced directly with dithiothreitol, a non-physiological dithiol, and in that case neither ferredoxin nor the ferredoxin-thioredoxin reductase is required for fructose-1,6-bisphosphatase activation [5,7]. Reduced thioredoxin activates the enzyme, as shown above in Eqn. 4.

It has recently been found that thioredoxin-activated fructose-1,6-bisphosphatase may be deactivated by one of several oxidants, such as oxidized glutathione [7]. On the basis of these findings, a regulatory system has been proposed whereby fructose-1,6-bisphosphatase is activated photochemically by reduced thioredoxin and decativated in the dark by oxidized glutathione. The activity of certain other regulatory enzymes of the reductive pentose phosphate cycle appears to be controlled by this or a related mechanism [5—8].

A recently found feature of at least three of the regulatory enzymes of the reductive pentose phosphate cycle in chloroplasts, namely ribulose-1,5-bisphosphate carboxylase [9], NADP:glyceraldehyde-3-phosphate dehydrogenase [10], and phosphoribulokinase [6] is that their rate of modification is slower than their rate of catalysis, i.e. they function as "hysteretic enzymes" [11]. The early observation that the lag phase exhibited by chloroplast fructose-1,6-bisphosphatase can be shortened by preincubation with photoreduced ferredoxin [3] raises the possibility that this enzyme is also hysteretic. However, the measurements necessary for a convincing demonstration of hysteresis have not been made. We have therefore designed experiments to this end and now report evidence that on activation with reduced thioredoxin chloroplast fructose-1,6-bisphosphatase undergoes a structural change that is slow relative to the rate of catalysis. The results further indicate that oxidant-induced deactivation of the thioredoxin-activated fructose-1,6-bisphosphatase resembles activation in being slower than the rate of catalysis.

Methods

Purification of chloroplast fructose-1,6-bisphosphatase. Chloroplast fructose-1,6-bisphosphatase was purified according to our earlier procedure [5,12] that was modified to include a final DEAE-cellulose chromatography step. The pooled fructose-1,6-bisphosphatase fractions obtained after the described Sephadex G-100 chromatography step were applied to a DEAE-cellulose

column that had been equilibrated beforehand with a buffer solution containing 50 mM sodium acetate (pH 5.5) and 0.15 M NaCl. The column was washed with two volumes of this buffer and fructose-1,6-bisphosphatase was then eluted with a linear gradient between 0.15 and 0.5 M NaCl that was added to solutions containing 50 mM sodium acetate (pH 5.5) the enzyme so obtained had a specific activity of about 72 μmol/min per mg of protein and showed a single component in sedimentation velocity ultracentrifugation and in polyacrylamide gel electrophoresis.

Studies on the activation and deactivation of fructose-1,6-bisphosphatase. The reduced thioredoxin needed for fructose-1,6-bisphosphatase activation was formed from oxidized thioredoxin either photochemically with chloroplasts supplemented with ferredoxin and ferredoxin-thioredoxin reductase or by direct reduction with dithiothreitol. Fructose-1,6-bisphosphatase was preincubated with the reduced thioredoxin for the indicated times before addition to the reaction mixtures. Fructose-1,6-bisphosphatase activity was then followed either by measuring colorimetrically the P_i [12] or by measuring spectrophotometrically the fructose 6-phosphate [4] formed from fructose 1,6-bisphosphate. In the latter determination, fructose 6-phosphate is enzymically converted to 6-phosphogluconate and the $NADPH_2$ formed is measured spectrophotometrically at 340 nm.

Fructose-1,6-bisphosphatase deactivation was studied by adding the indicated oxidant to reaction mixtures containing the enzyme that has been activated by reduced thioredoxin. After the indicated periods of time, the residual activity was measured by the spectrophotometric method as described above.

Purification of other proteins. Ferredoxin was isolated from spinach leaves [13] or from dried cells of the blue-green alga *Spirulina maxima* [14] by previously described procedures. The ferredoxins from these two sources were interchangeable in the photoreduction of thioredoxin by the chloroplast system. Chloroplast thioredoxin (assimilation regulatory protein b (ARP_b)) and ferredoxin-thioredoxin reductase (ARP_a) were isolated as in previous studies [5,6]. Chloroplast ferredoxin:NADP reductase was isolated from spinach leaves by an earlier described method [15].

Other methods. Previously described methods were used for the isolation of once-washed chloroplasts, for the determination of chlorophyll, and for the estimation of protein [3].

Materials. Biochemicals were purchased from either Sigma Chemical Co. (St. Louis, Mo.) or Boehringer Corp. (Tutsing, G.F.R.). Chemicals were of analytical reagent grade.

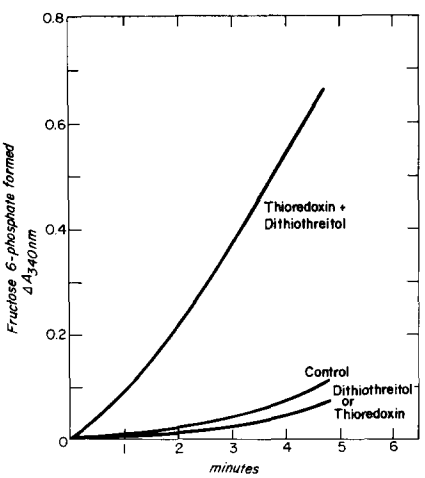
Results

In initial experiments, we observed that if catalysis is measured immediately after mixing the components required for the fructose-1,6-bisphosphatase reaction the activity showed a definite lag period. The duration of this lag depended on the Mg^{2+} concentration: with limiting (1 mM) Mg^{2+} (the amount routinely used for study of thioredoxin activation), the lag period lasted 3—4 min, whereas with saturating (16 mM) Mg^{2+} the lag period was reduced to

30—40 s. Fig. 1 shows that the length of the lag period observed with 1 mM Mg^{2+} was shortened from 4 min to about 1 min by preincubation of the fructose-1,6-bisphosphatase with thioredoxin and dithiothreitol. This decrease in lag period was accompanied by an increase in the rate of catalysis. Similar effects were observed when fructose-1,6-bisphosphatase was assayed at 16 mM Mg^{2+}: preincubation of the enzyme for 5 min with thioredoxin and dithiothreitol shortened the lag period from 40 to 15 s and increased the rate of catalysis by 10% (cf. ref. 12).

In parallel experiments we observed that only reduced thioredoxin was required during the preincubation period for the thioredoxin-mediated activation of fructose-1,6-bisphosphatase and that Mg^{2+}, which is essential for catalysis, was not needed. It would appear that the activation of the fructose-1,6-bisphosphatase that is induced by high Mg^{2+} concentrations during routine assays [5] is a readily reversible process because the enzyme deactivated rapidly once the Mg^{2+} concentration was lowered from 20 mM during the preincubation period to 1 mM during the reaction period. It should be noted that, as in previous studies, conditions of the experiments described here were varied only during the preincubation period and in any set of experiments conditions were constant during the catalytic phase of the reaction [10].

Fig. 2 shows that the thioredoxin-mediated activation of fructose-1,6-bisphosphatase was relatively slow. After a 5 min preincubation of the enzyme with reduced thioredoxin, the final (linear) velocity was only about 40% of that observed after a 20 min preincubation.



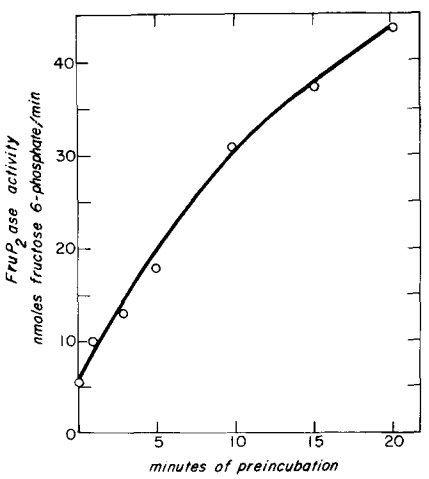


Fig. 1. Effect of preincubation with thioredoxin and dithiothreitol on the activity of chloroplast fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphatase (17 μg) was preincubated for 5 min in 0.1 ml of a solution containing 0.1 M Tris · HCl buffer (pH 7.9) and, as indicated, 10 mM dithiothreitol and 25 μg chloroplast thioredoxin. After preincubation, the mixture was injected into 0.9 ml of the assay solution (in a 1-cm cuvette of 1 ml capacity) that contained 1.3 units of glucose-6-phosphate dehydrogenase, 4 units of phosphoglucose isomerase, and the following (in μmol): $MgSO_4$, 1; sodium fructose 1,6-bisphosphate, 6; NADP (sodium salt), 1; and Tris · HCl buffer (pH 7.9), 40. Temperature, 25°C. The change in absorbance at 340 nm was followed with a Cary 14 spectrophotometer.

Fig. 2. Effect of time of preincubation on the activation of chloroplast fructose-1,6-bisphosphatase by reduced thioredoxin. Except for time of preincubation, experimental conditions were as described in Fig. 1.

Effect of dithiothreitol and thioredoxin on pH optimum of fructose-1,6-bisphosphatase

In this phase of the investigation we observed, in agreement with other investigators [2,16], that high concentrations of Mg^{2+} displaced the pH optimum for chloroplast fructose-1,6-bisphosphatase from the alkaline to the neutral region (Fig. 3). At 1 mM Mg^{2+} the enzyme exhibited maximal activity at pH 8.8 and showed no activity at pH values more acidic than 8.4. At 16 mM Mg^{2+}, the pH optimum broadened and decreased to about 8.0. Dithiothreitol added alone did not appreciably alter this pattern.

By contrast, dithiothreitol added in the presence of thioredoxin caused a large change in the pH profile at a low (4 mM) Mg^{2+} concentration that is conducive to studying enzyme activation. Fig. 4 shows that the pH optimum shifted from pH 8.6 with the control containing only dithiothreitol to pH 8.2 with the sample containing dithiothreitol plus thioredoxin. In addition, reduced thioredoxin greatly increased catalytic activity of the enzyme in the pH range from 7.4 to 8.0.

Structural modification of chloroplast fructose-1,6-bisphosphatase following activation by reduced thioredoxin

The possibility of a structural change associated with the thioredoxin-linked activation of fructose-1,6-bisphosphatase was investigated by measuring the change in sensitivity to trypsin digestion (Table I). In this experiment, fructose-1,6-bisphosphatase was (i) preincubated for 10 min with the indicated components (dithiothreitol and thioredoxin), (ii) preincubated for an additional 5 min with trypsin, and (iii) injected into a reaction mixture containing trypsin inhibitor and assayed for fructose-1,6-bisphosphatase activity. In this particular

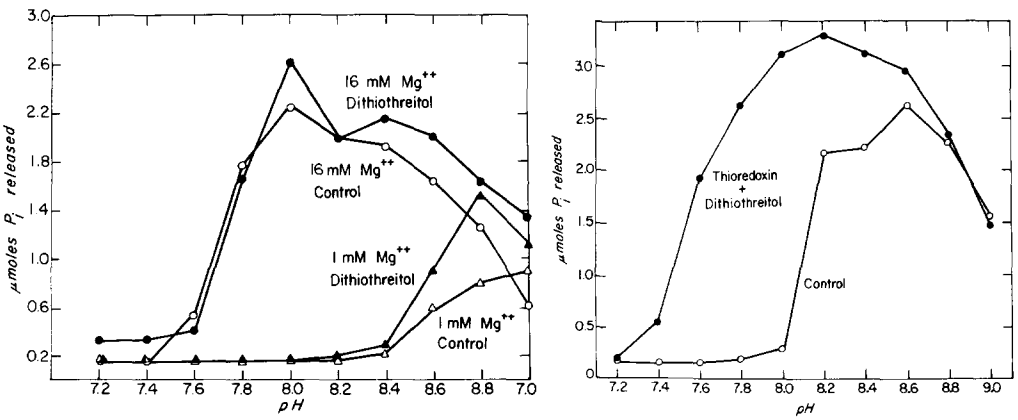


Fig. 3. Effect of Mg^{2+} concentration and dithiothreitol on the pH optimum of chloroplast fructose-1,6-bisphosphatase. The reaction mixture contained 7.5 µg of enzyme and the following (in µmol): Tris · HCl buffer, 100; EDTA-Na, 0.1; $MgCl_2$, 1 or 16; sodium fructose 1,6-bisphosphate, 6; and, as indicated, dithiothreitol 5. Final volume, 1.0 ml. Temperature, 25° C. The reaction was started by adding fructose 1,6-bisphosphate and after 5 min was stopped by adding 4 ml of a mixture of the reagents used for P_i analysis.

Fig. 4. Effect of reduced thioredoxin on the pH optimum of chloroplast fructose-1,6-bisphosphatase. Except for the indicated addition of 25 µg of thioredoxin and a final concentration of 4 mM $MgCl_2$, experimental conditions were as described in Fig. 3.

TABLE I

EFFECT OF TRYPSIN DIGESTION ON CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE

Fructose-1,6-bisphosphatase, 17 μg, was activated with reduced thioredoxin by following the preincubation conditions given in Fig. 1. Trypsin, 10 μg, was added, and the reaction was continued for another 5 min. The mixture was then injected into the assay solution and activity was measured as described in Fig. 1 except that the assay solution contained 10 mM $MgSO_4$ and 50 μg of soybean trypsin inhibitor.

Preincubation conditions	Relative fructose-1,6-bisphosphatase activity (nmol fructose 6-phosphate/min)
Dithiothreitol, 15 min	100 *
Dithiothreitol, 10 min	100
Dithiothreitol, 10 min and trypsin, 5 min	75
Dithiothreitol plus thioredoxin, 15 min	100 **
Dithiothreitol plus thioredoxin, 10 min	76
Dithiothreitol plus thioredoxin, 10 min and trypsin, 5 min	24

* Actual value, 58 nmol of fructose 6-phosphate formed per min.
** Actual value, 102 nmol of fructose 6-phosphate formed per min.

experiment, enzyme activity was measured under conditions supporting maximal fructose-1,6-bisphosphatase activity (i.e. at a saturating Mg^{2+} concentration in the presence of thioredoxin and dithiothreitol) so that any trypsin-effected change in the non-activated enzyme could be ascertained. As seen in Table I, trypsin treatment greatly lowered the activity of the fructose-1,6-bisphosphatase that had been activated by thioredoxin plus dithiothreitol and had little effect on the fructose-1,6-bisphosphatase control incubated with dithiothreitol alone. These results suggest that on activation by reduced thioredoxin fructose-1,6-bisphosphatase undergoes a structural change that renders the enzyme more susceptible to trypsin digestion. Parallel experiments indicated that this change is not accompanied by an appreciable change in the molecular weight of the fructose-1,6-bisphosphatase. Sedimentation velocity ultracentrifugation experiments, performed as described earlier [12] revealed that incubation of fructose-1,6-bisphosphatase (1.4—3.6 μg/ml) with dithiothreitol (5 mM) plus chloroplast thioredoxin (1.4 mg/ml) or with dithiothreitol alone caused no change in the molecular weight of the enzyme. When analyzed in sodium acetate buffer at pH 5.5 the control as well as the incubated enzyme sedimented with an $s_{20,w}$ of 8, indicating a molecular weight of 145 000. When changing to 0.1 M Tris · HCl buffer at pH 8.8, thus forming the subunits of the enzyme, the control as well as the incubated enzyme sedimented with an $s_{20,w}$ of 4 indicating a mol. wt. of 72 500 [17,12,18].

Deactivation of chloroplast fructose-1,6-bisphosphatase activated by reduced thioredoxin

The ferredoxin-thioredoxin system proposed for the regulation of chloroplast fructose-1,6-bisphosphatase constitutes a mechanism for the light-actuated regulation of several enzymes in chloroplasts [5,6]. An important component of this regulatory mechanism is the manner in which the activated enzymes are converted by certain oxidants back to a less active state [7]. In this connection, we have studied the effect of several oxidants on the fructose-1,6-bisphospha-

tase activated by reduced thioredoxin and have found that the enzyme is relatively non-specific in its oxidant requirement.

Fig. 5 shows that oxidized glutathione, tetrathionate ($Na_2S_4O_6$), and dehydroascorbic acid were all effective in the dark deactivation of the fructose-1,6-bisphosphatase that had been activated with photoreduced ferredoxin. The respective decreases in activity that were found after oxidant addition were 40% for oxidized glutathione, 48% for $Na_2S_4O_6$, and 72% for dehydroascorbic acid. NADP added with ferredoxin:NADP reductase had little effect on fructose-1,6-bisphosphatase activity under these conditions.

A study of the time-course of deactivation revealed that fructose-1,6-bisphosphatase deactivated relatively slowly after addition of an oxidant (Fig. 6). These experiments (with fructose-1,6-bisphosphatase that had been activated by dithiothreitol-reduced thioredoxin) showed that the longer the activated enzyme was exposed to the oxidant (in this case oxidized glutathione) the lower its activity. This finding indicates that the rate of deactivation of fructose-1,6-bisphosphatase, like activation, is slower than the rate of catalysis.

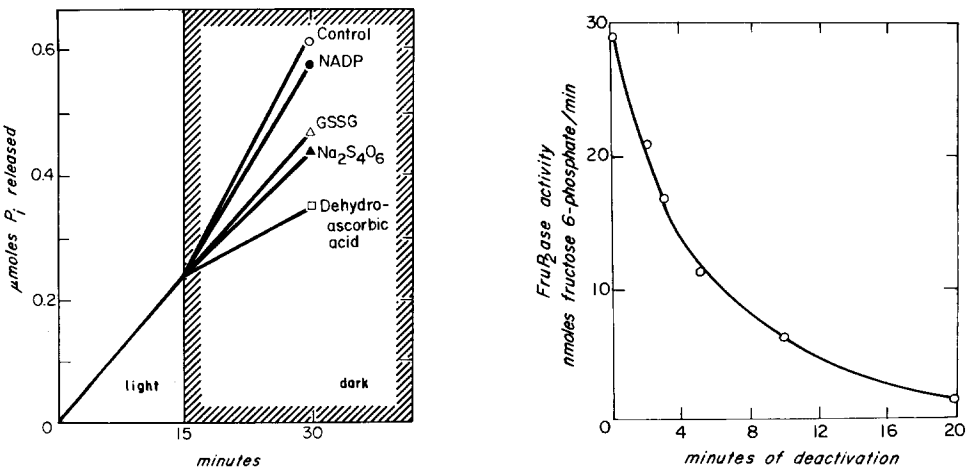


Fig. 5. Effect of different oxidants on the activity of chloroplast fructose-1,6-bisphosphatase activated by photoreduced thioredoxin. The reaction was carried out in Warburg vessels fitted with double side arms. The complete system contained (in one side arm) 6 μmol of sodium fructose 1,6-bisphosphate (in the other side arm) the indicated oxidant (37.5 μmol of oxidized glutathione (GSSG), 10 μmol of $Na_2S_4O_6$, 10 μmol of dehydroascorbic acid or 1 μmol of NADP plus 70 μg of ferredoxin:NADP reductase), and (in the main compartment) 17 μg of fructose-1,6-bisphosphatase, heated (5 min, 55°C) spinach chloroplast fragments (P_1S_1) equivalent to 0.1 mg of chlorophyll, 0.12 mg of *Spirulina maxima* ferredoxin, 0.14 mg of ferredoxin-thioredoxin reductase, 25 μg of thioredoxin, and the following (in μmol): Tris · HCl buffer (pH 7.9), 100; $MgSO_4$, 1.0; sodium ascorbate, 10; 2,6-dichlorophenolindophenol, 0.1. Final volume, 1.5 ml. Vessels were equilibrated with nitrogen for 6 min and were incubated for 10 min in the light (20 000 lux). The reaction was started by adding fructose 1,6-bisphosphate from the side arm and was continued for 15 min under illumination. The lights were then extinguished, the indicated oxidant was added from the second side arm, the reaction was continued for an additional 15 min in the dark. Temperature, 20°C. The reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid and, after the precipitate was centrifuged off, the reaction mixtures were analyzed for P_i.

Fig. 6. Effect of time on the deactivation of chloroplast fructose-1,6-bisphosphatase activated by dithiothreitol-reduced thioredoxin. Fructose-1,6-bisphosphatase was activated for 10 min under the conditions in Fig. 1, oxidized glutathione (2.5 μmol) was added, and the incubation was continued for the indicated times. Fructose-1,6-bisphosphatase activity was then measured as described in Fig. 1.

Discussion

Chloroplast fructose-1,6-bisphosphatase [5] (and its sedoheptulose-1,7-bisphosphatase counterpart [19,12]) are activated photochemically by reduced ferredoxin in the presence of thioredoxin and ferredoxin-thioredoxin reductase, two newly identified proteins that are native to chloroplasts [6,7]. Only one of these proteins, thioredoxin, is required when dithiothreitol replaces reduced ferredoxin as the reductant for activation [6]. Activation by dithiothreitol-reduced thioredoxin has been observed with enzymes of the reductive pentose phosphate cycle other than fructose-1,6-bisphosphatase, namely, the regulatory forms of NADP:glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase [6]. Recent evidence suggests that the thioredoxin mechanism may also apply for the regulation of NADP: malate dehydrogenase [20], an enzyme that is not a part of the reductive pentose phosphate cycle.

The present results show that, like ribulose-1,5-bisphosphate carboxylase [9] and NADP:glyceraldehyde-3-phosphate dehydrogenase [21,10], the fructose-1,6-bisphosphatase reaction consists of two phases: (i) an activation phase in which the enzyme is converted from an inactive to an active form; and (ii) a catalytic phase in which substrates are converted to products. With each of those enzymes, the activation phase is appreciably slower than the catalytic phase and, in the case of the fructose-1,6-bisphosphatase, this is particularly apparent at a limiting concentration of Mg^{2+}.

The current studies show that fructose-1,6-bisphosphatase can be activated (i) by reduced thioredoxin independently of Mg^{2+} or (ii) by a high concentration of Mg^{2+}. The enzyme activated by reduced thioredoxin may be deactivated by such oxidants indigenous to chloroplasts as oxidized glutathione [7,22] and, as seen above, deactivation resembles activation in being slow relative to the rate of catalysis. By contrast, deactivation of the Mg^{2+}-activated enzyme (effected by lowering the Mg^{2+} concentration) was relatively fast and could not be measured with the techniques described here. In view of recent evidence [23] that the light-induced increase in the concentration of Mg^{2+} in the stroma of chloroplasts is low (1—3 mM), it seems doubtful that Mg^{2+} alone could account for the photoactivation of fructose-1,6-bisphosphatase that is observed under physiological conditions. In this connection, it is noteworthy that lag periods were recently reported for both the activation and the deactivation of fructose-1,6-bisphosphatase in a blue-green alga [24] and in whole chloroplasts [4].

In addition to demonstrating the hysteretic nature of chloroplast fructose-1,6-bisphosphatase, the present evidence shows that the enzyme undergoes a structural change on activation with reduced thioredoxin which does not appear to involve a large change in molecular weight. However, there is at present little other information on this point.

Finally, it should be noted that the present results support the earlier expressed view [6] that the ferredoxin-thioredoxin regulatory system functions in conjunction with other mechanisms of enzyme regulation in chloroplasts, namely the light-induced shifts in stromal pH [25] and in Mg^{2+} concentration [23,26—28] and the light generation of enzyme effectors [9,10,29—32]. An independent line of evidence that the ferredoxin-thioredoxin system functions

in vivo is provided by the demonstration that dithiothreitol enhanced markedly the rate of CO_2 fixation in the dark in a model chloroplast system [33].

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