Chloroplast Sedoheptulose 1,7-Bisphosphatase: Evidence for Regulation by the Ferredoxin/Thioredoxin System

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1. A substrate-specific sedoheptulose-1,7-bisphosphatase has been found in chloroplasts and separated from its fructose-1,6-bisphosphatase counterpart. Experiments with antibodies indicate that the two enzymes are structurally different.

2. Activity of the sedoheptulose-1,7-bisphosphatase enzyme was dependent on Mg^{2+} and a reductant. The most effective reductant tested was thioredoxin that was reduced either photochemically via ferredoxin with chloroplasts or chemically with dithiothreitol. Dithiothreitol added alone also activated the enzyme, but reduced glutathione or 2-mercaptoethanol did not. The thioredoxin-activated enzyme was deactivated by oxidized glutathione.

3. The results suggest that the new substrate-specific sedoheptulose-1,7-bisphosphatase depends on light for activity and resembles certain other regulatory enzymes of the reductive pentose phosphate cycle in its mode of regulation.

Introduction

Since elucidation of the path of carbon dioxide fixation in photosynthesis [1], evidence has accumulated that light is involved in the regulation of key enzymes of this pathway [2–4]. One way that light mediates the regulation of these enzymes is through the formation of a reductant [5–8]. The newly found ferredoxin-thioredoxin system of chloroplasts constitutes one mechanism that functions in this capacity [9, 10]. In this system, electrons from chlorophyll are transferred to ferredoxin and then to thioredoxin via ferredoxin-thioredoxin reductase. Reduced thioredoxin, in turn, reduces and thereby activates regulatory enzymes of the reductive pentose phosphate cycle which include: fructose-1,6-bisphosphatase (Fru-P_2ase) [10], phosphoribulokinase [11], and NADP-glyceraldehyde 3-phosphate dehydrogenase [12]. Thioredoxin reduced in this manner also activates enzymes not associated with the carbon reduction cycle, namely, NADP-malate dehydrogenase [13] and phenylalanine ammonia lyase [14]. In the dark, deactivation of thioredoxin-activated enzymes appears to be accomplished by chloroplast oxidants. One mechanism for deactivation involves the oxidation of the reduced enzymes by the oxidized form of glutathione (GSSG) [10]. The latter may be formed in chloroplasts from its reduced counterpart by oxidation either with H_2O_2 via the enzyme glutathione peroxidase [10] or with dehydroascorbate nonenzymically [15]. Dehydroascorbate, a compound that appears to be formed enzymically from ascorbate in chloroplasts [16], is also effective in the deactivation of enzymes activated by reduced thioredoxin [10].

Previous studies have also linked the second phosphatase enzyme of the carbon reduction cycle, sedoheptulose-1,7-bisphosphatase (Sed-P_2ase), to the ferredoxin-thioredoxin system [7]. Work with Sed-P_2ase is, however, not extensive. In particular, there remains the important unanswered question of whether there exists in chloroplasts an Sed-P_2ase that is separate from the Sed-P_2ase activity that was recently shown to be associated with the Fru-P_2ase enzyme after alkaline treatment [17]. We have, therefore, addressed ourselves to this and related questions and we now report experiments with isolated spinach chloroplasts that indicate: (i) that chloroplasts contain a substrate-specific Sed-P_2ase that is different from the Fru-P_2ase enzyme; (ii) that this new Sed-P_2ase is activated by thioredoxin reduced either photochemically with ferredoxin or chemically with dithiothreitol (DTT); and (iii) that, following activation by reduced thioredoxin, the Sed-P_2ase is deactivated by the oxidized form of glutathione.

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Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase; Sed-P_2ase, sedoheptulose-1,7-bisphosphatase; Sed-1,7-P_2, sedoheptulose-1,7-bisphosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; DTT, dithiothreitol; GSSG, oxidized glutathione; GSH, reduced glutathione; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; MES, 2(N-morpholino) ethane sulfonic acid; TRIS, tris(hydroxymethyl) aminomethane; P_i, inorganic phosphate; Ab, antibody.

Materials and Methods

Plant material

Spinach plants (*Spinacea oleracea* var Resistoflay) were grown in nutrient solution in a greenhouse [18].

Reagents

Biochemicals were obtained from the Sigma Chemical Company (St. Louis, Mo). Other chemicals were obtained from commercial sources and were of the highest quality available.

Isolation of Sed-P_2ase

Spinach leaves were washed in distilled water, drained, placed in a plastic bag, and chilled to 4 °C. Subsequent operations were carried out at 4 °C. Chloroplasts were isolated from 600 g of leaves by blending for 2 min in 1200 ml of buffer containing: 0.35 M sucrose, 25 mM HEPES-NaOH (pH 7.6), and 0.35 mg/ml D-isoascorbic acid [18]. The homogenate was filtered through 8 layers of filtering silk. The filtrate was centrifuged for 1 min at $3000 \times g$ and the supernatant fluid was discarded. The precipitate, containing whole chloroplasts, was resuspended in 129 ml of 50 mM MES-NaOH buffer (pH 6.5) to give a chlorophyll concentration of 1.5 mg/ml. The suspension, containing ruptured chloroplasts, was centrifuged at $40{,}000 \times g$ for 15 min. The green precipitate, containing chloroplast membranes, was discarded. The supernatant fraction was brought to 90% saturation with solid ammonium sulfate and stirred for 30 min. The suspension was centrifuged at $40{,}000 \times g$ for 15 min and the supernatant fraction was discarded. The precipitate was resuspended in 6 ml of 50 mM MES-NaOH buffer (pH 6.5) (protein concentration, 25 – 30 mg/ml). The suspension was clarified by centrifugation at $100{,}000 \times g$ for 30 min. Six ml of the supernatant fraction in 2% sucrose was applied to a Bio-Gel A 1.5 m column (2.6×40 cm) equilibrated beforehand with 50 mM MES-NaOH buffer (pH 6.5). The column was eluted with the same buffer. Fractions (2.8 ml) were collected at a flow rate of 16 ml/h. Column fractions showing the highest Sed-P_2ase activity and the least overlap with the Fru-P_2ase activity peak were combined and concentrated by pressure dialysis with a Diaflo PM-30 membrane to give a protein concentration of 2.5 – 3.0 mg/ml. The concentrated enzyme was stored at – 10 °C.

Assay of Bio-Gel A 1.5 m fractions

Fru-P_2ase. Fifty μl of the indicated fractions were preincubated for 5 min with the following (μmol): Tris-HCl (pH 7.9), 50; $MgSO_4$, 5; and DTT, 2.5. The reaction was started with 3.0 μmol of fructose-1,6-bisphosphate (Fru-1,6-P_2). Final volume, 0.5 ml. Reaction time, 20 min; temperature, 25 °C. The reaction was stopped by adding 2 ml of the reagent used for phosphate (P_i) analysis (ref. [5] and see below).

Sed-P_2ase activity of alkaline-treated Fru-P_2ase. Conditions were as described above for Fru-P_2ase except that Tris-HCl (pH 8.8) was used and the reaction was started with 0.75 μmol of sedoheptulose-1,7-bisphosphate (Sed-1,7-P_2).

Substrate-specific Sed-P_2ase. Conditions were as described above for Fru-P_2ase except that the reaction was started with 0.75 μmol of Sed-1,7-P_2.

Thioredoxin. Conditions were as described above for Fru-P_2ase except that 8.5 μg of pure spinach Fru-P_2ase was added, 1 μmol of $MgSO_4$ was used, and fractions were preincubated for 10 min.

Ferredoxin-thioredoxin reductase. Activity was determined for fractions showing Sed-P_2ase activity [10]. The complete system contained 500 μl of each fraction, chloroplast fragments (0.1 mg chlorophyll) heated for 5 min at 55 °C to destroy their oxygen evolving capacity, spinach ferredoxin (0.15 mg), spinach thioredoxin (0.125 mg), pure spinach Fru-P_2ase (17 μg), and the following (μmol): Tris-HCl buffer (pH 7.9), 100; $MgSO_4$, 1.0; sodium ascorbate, 10; Fru-1,6-P_2, 6; and 2,6-dichlorophenol indophenol, 0.1. Final volume, 0.5 ml; gas phase, nitrogen; light intensity, 20,000 lx. The reaction was carried out at 20 °C in Warburg vessels containing the Fru-1,6-P_2 in the sidearm. After 6 min equilibration with nitrogen, the vessels were preilluminated for 10 min, Fru-1,6-P_2 was added from the sidearm, and the reaction was continued in the light for 30 min. A dark treatment (complete, ferredoxin not reduced) was assayed for each light treatment. The reaction was stopped with 0.5 ml of 10% trichloroacetic acid, the precipitated protein was centrifuged off, and an aliquot was used for P_i determination. The activity was corrected for Fru-P_2ase and thioredoxin activity present in the fractions.

Purification of components of the ferredoxin-thioredoxin system

Previously devised procedures were used for purification of ferredoxin [19], thioredoxin [20], ferredoxin-thioredoxin reductase [20], and Fru-P_2ase [17].

Preparation and purification of specific Fru-P_2ase antibodies

New Zealand albino rabbits were used to raise antibodies for each of two different forms of the pure Fru-P_2ase enzyme [21]. To obtain control γ-globulin, the rabbits were bled prior to injection from the largest peripheral ear vein with a 20 gauge sterile needle. A total of 275 ml of blood was collected. Initially, rabbit A was injected in both rear foot pads (25 gauge sterile needle) with a mixture of 2 mg of untreated Fru-P_2ase in 0.4 ml 30 mM Na-acetate buffer (pH 5.0) combined with 1.2 ml Freund's Incomplete Adjuvant. In the same manner, rabbit B was injected with 2.3 mg of alkaline-treated Fru-P_2ase [prepared by adding 0.2 ml of 1 M Tris-HCl buffer (pH 8.8) to 10 mg of Fru-P_2ase in 0.2 ml of 30 mM Na-acetate buffer (pH 5.0)[17]] combined with 0.5 ml 100 mM Tris-HCl buffer (pH 8.8) in 1.5 ml of Freund's Incomplete Adjuvant. (Parallel experiments revealed that the Fru-P_2ase was dissociated under these conditions.) Rabbit A received, over a period of 22 days 5 more injections, each consisting of 1.0 mg of untreated Fru-P_2ase in 0.6 ml of Adjuvant. Rabbit B received the same injection regime, each injection consisting of a mixture of 1.0 mg of alkaline-treated Fru-P_2ase in 0.75 ml of Adjuvant. Bleeding for antibody serum began 17 days after the first injection and was continued for 17 days thereafter. A total of 390 ml of blood, containing antibody to untreated Fru-P_2ase, was collected from rabbit A. A total of 425 ml of blood, containing antibody to alkaline-treated Fru-P_2ase, was collected from rabbit B.

The freshly collected blood was allowed to clot at room temperature (approx. 3 h) and then left at 4 °C overnight. Subsequent operations were carried out at 4 °C. The clotted blood was centrifuged at $3000 \times g$ for 10 min. The precipitate, containing whole red blood cells, was discarded. The straw-colored supernatant fraction was adjusted to 40% saturation with solid ammonium sulfate and stirred for 30 min. The suspension was centrifuged for 20 min at $40{,}000 \times g$. The supernatant fraction was discarded and the precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.5). The suspension was dialyzed against 10 volumes of the same buffer for 12 h. The suspension was centrifuged to clarity at $40{,}000 \times g$ for 10 min. The protein concentration of the supernatant fraction was determined by the phenol reagent procedure [22] and the supernatant fraction was then applied to a DEAE-cellulose column (1 g wet weight DEAE-cellulose/50 mg protein) that was equilibrated and eluted with 10 mM potassium phosphate buffer (pH 7.7) [23]. Ten-ml fractions were collected. The major protein fractions determined by absorbance at 280 nm were concentrated by pressure dialysis with a Diaflo PM-30 membrane to a protein concentration of 70 mg/ml [22].

Purified antibody samples were tested against their antigens with the Ouchterlony double diffusion assay [21]. The purified δ-globulin fractions (70 mg/ml) showed single precipitation lines against the injected Fru-P_2ase samples (5 mg/ml) up to a dilution of 1 : 8 for the untreated enzyme and 1 : 4 for its alkaline-treated counterpart.

Analytical methods

Methods earlier described were used for determining chlorophyll [24] and P_i [5]. Unless otherwise indicated, protein was determined by measurement of absorbance at 280 nm.

Results and Discussion

Isolation of Sed-P_2ase

In examining different procedures to resolve components of the ferredoxin-thioredoxin system, we observed that chromatography on Bio-Gel A 1.5 m serves effectively to this end. We therefore used this technique to determine whether chloroplasts contain a specific Sed-P_2ase in addition to Fru-P_2ase. The column profile of a chloroplast extract preparation, showing Sed-P_2ase and other components in question, is given in Fig. 1. It may be noted that an Sed-P_2ase activity was separated from the Fru-P_2ase enzyme. The pH found to be best for this separation was pH 6.5. Under these conditions, Sed-P_2ase was also separated from thioredoxin but not from ferredoxin-thioredoxin reductase. In Fig. 1, both Sed-P_2ase and Fru-P_2ase were assayed at pH 7.9.

The stability of Sed-P_2ase isolated on Bio-Gel A 1.5 m was low. Storing the enzyme at 4 °C without

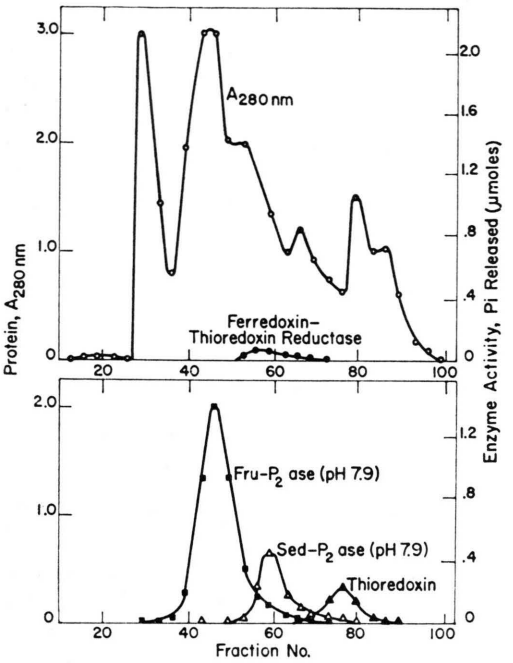

Fig. 1. Separation of chloroplast Sed-P_2ase and Fru-P_2ase by Bio-Gel Al. 5 m column chromatography.

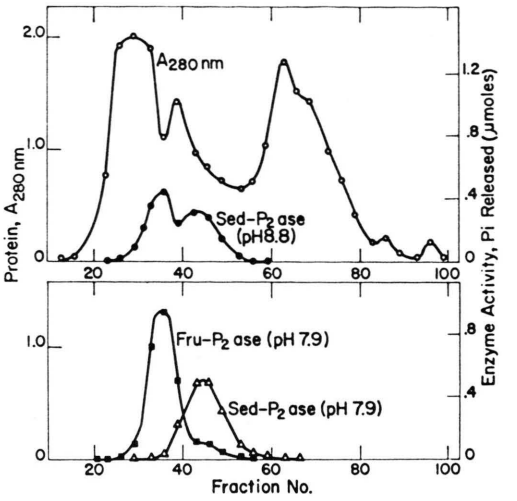

Fig. 2. Relation of Sed-P_2ase to Fru-P_2ase linked Sed-P_2ase in chloroplasts.

further treatment resulted in total loss of activity within approximately 48 h. Attempts to increase stability were only partially successful. It was found that concentration of the combined column fractions, by ultrafiltration (PM-30 Diaflo membrane), increased the stability of the enzyme at 4° and -10 °C. When stored at -10 °C, the concentrated enzyme was stable about 1 month.

Fig. 2 shows that when the Bio-Gel fractions were assayed at pH 8.8 rather than pH 7.9 two distinct peaks of Sed-P_2ase activity were observed, one that was found previously to be associated with Fru-P_2ase and unmasked at pH 8.8 [17], and a second form that was independent of Fru-P_2ase. The latter activity about 2-fold over that observed with DTT cordingly, we chose this component for further investigation.

Thiol-mediated activation of Sed-P_2ase

The Sed-P_2ase activity associated with either the new enzyme or with the Fru-P_2ase enzyme could not be detected without preincubation with DTT, indicating that the enzymes must be activated by reduction in order to function. The requirement for DTT was specific and could not be satisfied with either reduced glutathione (GSH) or 2-mercaptoethanol (Table I). The table shows that thioredoxin

Table I. Effect of thiol reagents on activity of Sed-P_2ase in the presence and absence of thioredoxin.

Treatment	P_i released [μmol]
DTT	0.11
DTT, Thioredoxin	0.27
GSH	0.0
GSH, Thioredoxin	0.0
2-Mercaptoethanol	0.0
2-Mercaptoethanol, Thioredoxin	0.0

Sed-P_2ase (42 μg) was preincubated for 10 min with 250 μg thioredoxin (as indicated) and the following (μmol): Tris-HCl buffer (pH 7.9), 50; $MgSO_4$, 5; and DTT, 2.5, or GSH, 5, or 2-mercaptoethanol, 1.4. Final volume, 0.5 ml. After preincubation, 1.25 μmol of Sed-1,7-P_2 was added to start the reaction. Reaction time, 10 min; temperature, 25 °C. The reaction was stopped by adding 2 ml of the reagent used for P_i analysis.

added in the presence of DTT increased Sed-P_2ase activity about 2-fold over that observed with DTT alone. With other preparations, an up to 3-fold activation by dithiothreitol-reduced thioredoxin was seen (compare Figs 3 and 4). As with other regulatory enzymes of the reductive pentose phosphate cycle, activation of Sed-P_2ase was slow relative to the rate of catalysis [11, 12, 25 – 27]. Accordingly, the enzyme was preincubated with reduced thioredoxin prior to initiation of catalysis in a reaction mixture containing no substrate. Optimal activation was observed after a 10-min preincubation of the enzyme with DTT and thioredoxin. Enzymes whose rates of modification are slower than their catalysis

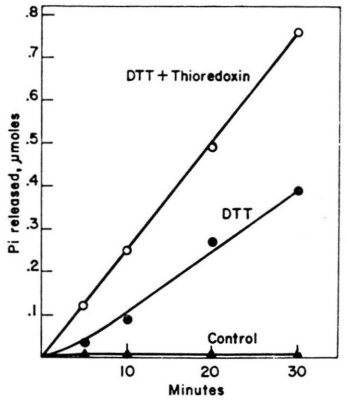

Fig. 3. Effect of DTT and thioredoxin on chloroplast Sed-P_2ase. Except for varying the reaction time, conditions were as given in Table I.

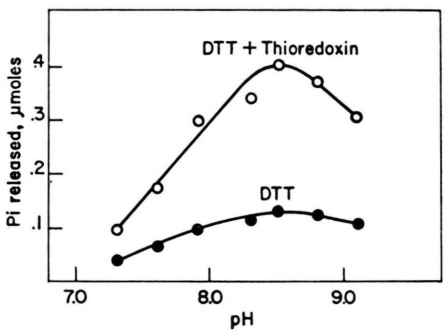

Fig. 4. Effect of pH on chloroplast Sed-P_2ase. Except for varying the pH, conditions were as given in Table I.

have been called hysteretic enzymes [28, 29]. It appears that the activation of these enzymes is accompanied by a structural change.

The lag in catalysis was apparent when the enzyme was preincubated with DTT in the absence of thioredoxin (Fig. 3). Addition of thioredoxin to the preincubation mixture eliminated the lag phase and resulted in an increased rate of reaction. The initial velocity of the thioredoxin-activated enzyme was about three times greater than the initial velocity of the unactivated enzyme (0.65 μmol P_i/mg total protein per min *vs* 0.22 μmol P_i/mg total protein per min) and about one and a half times greater than the final (linear) velocity of the unactivated enzyme (0.65 μmol P_i/mg total protein per min *vs* 0.38 μmol P_i/mg total protein per min). The relatively high activity observed with DTT alone distinguishes the Sed-P_2ase from other thioredoxin-linked enzymes of the carbon reduction cycle, all of which show limited increase in activity by DTT added in the absence of thioredoxin [10 – 12].

Optimal pH

The thioredoxin-linked activation of Sed-P_2ase depended on the pH of the preincubation and reaction mixtures (Fig. 4). Maximum activity of the enzyme was observed at a pH of 8 to 9. Activity decreased rapidly as the pH approached neutrality. Chloroplast Fru-P_2ase and other regulatory enzymes of the reductive pentose phosphate cycle show similar responses to pH: alkaline conditions are required for maximal activity [11, 12, 25 – 27].

Cation requirement

The Sed-P_2ase enzyme was dependent on Mg^{2+} (Fig. 5). Mn^{2+} could partially substitute for Mg^{2+}, particularly at low concentrations. Maximum activities were, however, not observed with Mn^{2+}, and a slight excess of Mn^{2+} had a pronounced inhibitory effect. Excess Mg^{2+} did not inhibit the activity of the enzyme. The effects of these ions on the Sed-P_2ase are similar to those observed for Fru-P_2ase [30]. However, unlike chloroplast Fru-P_2ase, saturating levels of Mg^{2+} or Mn^{2+} could not activate the Sed-P_2ase enzyme without DTT.

Substrate specificity of Sed-P_2ase

Chloroplast Sed-P_2ase appears to be substrate-specific (Table II). The activity of the enzyme with Sed-1,7-P_2 was four times greater than the activity with any other sugar phosphate tested. The fact that other substrates were somewhat active, notably Fru-1,6-P_2, may reflect contamination of the preparation with Fru-P_2ase (see Fig. 1).

Deactivation of Sed-P_2ase

Table III shows that GSSG, added at intervals during the preincubation period, deactivated Sed-P_2ase that had been previously activated with DTT-reduced thioredoxin. A 62% deactivation was ob-

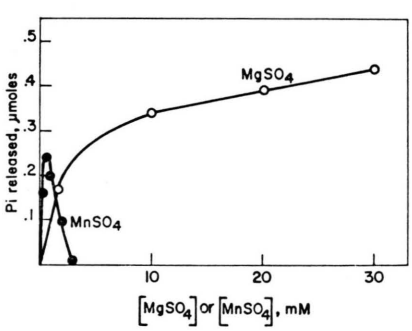

Fig. 5. Effect of $MgSO_4$ or $MnSO_4$ concentration on chloroplast Sed-P_2ase. Except for varying the cation concentration, conditions were as given in Table I.

Table II. Substrate specificity of Sed-P_2ase.

Substrate	P_i released [μmol]
Sedoheptulose-1,7-bisphosphate	0.18
Fructose-1,6-bisphosphate	0.04
Glucose-1,6-bisphosphate	0.02
Ribulose-1,5-bisphosphate	0.01
Fructose-1-phosphate	0.02
Fructose-6-phosphate	0.03
Glucose-1-phosphate	0.01

Conditions were as described in Table I except for use of: 37 μg Sed-P_2ase; 125 μg thioredoxin; and reaction time of 20 min. After preincubation the substrates were added to start the reaction in the following amounts (μmol): Sed-1,7-P_2, 0.75; Fru-1,6-P_2, 3.0; glucose-1,6-bisphosphate, 0.75; ribulose-1,5-bisphosphate, 0.25; fructose-1-phosphate, 0.75; fructose-6-phosphate, 0.75; and glucose-1-phosphate, 0.75.

Table III. Deactivation of Sed-P_2ase with GSSG added during preincubation with thioredoxin.

Treatment	P_i released [μmol]	Deactivation [%]
Control (no GSSG)	0.06	0
GSSG added after 10 min	0.05	12
GSSG added after 7 min	0.04	29
GSSG added after 5 min	0.03	41
GSSG added after 3 min	0.02	62

Conditions were as described in Table I except for use of: 37 μg Sed-P_2ase; 125 μg thioredoxin; and reaction time of 5 min. At the times indicated, 2.5 μmol of GSSG was added to the preincubation mixture.

served when GSSG was added after the enzyme had been preincubated for 3 min in the presence of DTT and thioredoxin. The GSSG-mediated deactivation of Sed-P_2ase is similar to that of thioredoxin-activated Fru-P_2ase [10] and phosphoribulokinase [11].

Light-mediated activation of Sed-P_2ase

The ability of photochemically reduced ferredoxin to replace DTT as the reductant for Sed-P_2ase activation is shown in Table IV. The activity of the light-activated complete system was approximately four times greater than the activity of the dark control in which ferredoxin was not reduced. Light activation was dependent on ferredoxin, thioredoxin, and Sed-P_2ase. For reasons not fully understood, activation was increased when ferredoxin-thioredoxin reductase was omitted from the complete system. This enzyme was present in the Sed-P_2ase preparation (see Fig. 1). It is possible, therefore, that the higher

Table IV. Requirements for light-activation of Sed-P_2ase.

Treatment	P_i released [μmol]
Light, complete	0.19
Light, ferredoxin omitted	0.04
Light, thioredoxin omitted	0.08
Light, ferredoxin-thioredoxin reductase omitted	0.25
Light, Sed-P_2ase omitted	0.03
Light, complete, GSSG added	0.03
Dark, complete (ferredoxin not reduced)	0.05

The complete system contained 110 μg Sed-P_2ase, fresh chloroplast fragments (10 μg chlorophyll), ferredoxin (60 μg), thioredoxin (250 μg), ferredoxin-thioredoxin reductase (200 μg) and the following (μmol): Tris-HCl buffer (pH 7.9), 30; $MgSO_4$, 5; and Sed-1,7-P_2, 1.25. From the sidearm after preillumination, 5 μmol of GSSG was added to the treatment indicated above. Final volume, 0.5 ml; gas phase, nitrogen; light intensity, 20,000 lux. The reaction was carried out at 20 °C in Warburg-Krippahl vessels containing Sed-1,7-P_2 in the sidearm. After 6 min equilibration with nitrogen, the vessels were preilluminated for 20 min, Sed-1,7-P_2 was added from the sidearm, and the reaction continued for 30 min. In the last treatment (dark, complete, ferredoxin not reduced), the vessels were kept in the dark throughout the preillumination and reaction periods. The reaction was stopped with 0.2 ml of 10% trichloroacetic acid, the precipitated protein was centrifuged off, and an aliquot was used for P_i determination.

Sed-P_2ase activity observed in the absence of ferredoxin-thioredoxin reductase could result from the presence of an inhibitor in the reductase preparation. It might be noted that separate experiments indicated that this inhibitor was not the 2-mercaptoethanol added to stabilize the ferredoxin-thioredoxin reductase preparation.

Effect of specific antibodies on activities of Fru-P_2ase and Sed-P_2ase

The finding that the Sed-P_2ase and Fru-P_2ase enzymes can be separated is in accord with the conclusion that these activities reside on different proteins. It is possible, however, that the two proteins are related structurally. The Sed-P_2ase enzyme could be a subunit component of the Fru-P_2ase that in some manner lost Fru-P_2ase activity. If such were the case, antibodies raised against the Fru-P_2ase (or its component subunits) would be expected to inhibit the Sed-P_2ase. The results in Table V are directed to this point. Antibodies against untreated Fru-P_2ase effectively inhibited the activity of both the pure Fru-P_2ase and the Fru-P_2ase obtained by Bio-Gel chromatography of chloroplast extract. Inhibition of Fru-P_2ase activity was observed ir-

Table V. Effect of specific antibodies (Ab) on the activity of chloroplast Fru-P_2ase and Sed-P_2ase.

	Inhibition [% *]		
	Control γ-globulin	Ab *vs* pure Fru-P_2ase (untreated)	Ab *vs* pure Fru-P_2ase (alkaline-treated)
Fru-P_2ase enzyme			
Fru-P_2ase activity:			
Untreated pure enzyme	7.7	96.2	73.1
Untreated Bio-Gel fraction	2.4	92.7	68.3
Alkaline-treated pure enzyme	8.3	62.5	75.0
Alkaline-treated Bio-Gel fraction	0.0	19.1	71.4
Sed-P_2ase activity:			
Alkaline-treated pure enzyme	0.0	69.2	76.9
Alkaline-treated Bio-Gel fraction	0.0	44.4	77.8
Specific Sed-P_2ase enzyme:			
Sed-P_2ase activity:			
Bio-Gel fraction	0.0	5.9	5.9

* Percent inhibition $= \left(\frac{\text{control activity} - \text{serum activity}}{\text{control activity}}\right) \times 100$.

Conditions:

Fru-P_2ase activity (untreated pure enzyme). 1.7 μg of Fru-P_2ase was preincubated for 5 min with 12.5 μg thioredoxin, 17.5 μg serum (control γ-globulin, Ab *vs* untreated pure Fru-P_2ase, or Ab *vs* alkaline-treated pure Fru-P_2ase) and the following (μmol): Tris-HCl buffer (pH 7.9), 50; $MgSO_4$, 5; and DTT, 2.5. Final volume, 0.5 ml. The reaction was started with the addition of 3 μmol of Fru-1,6-P_2. Reaction time, 10 min; temperature, 25 °C.

Fru-P_2ase activity (alkaline-treated pure enzyme). Conditions as indicated for Fru-P_2ase (untreated pure enzyme) except that the enzyme was incubated for 10 min with Tris-HCl buffer (pH 8.8) and $MgSO_4$ before preincubation and the reaction time was 20 min.

Sed-P_2ase activity (alkaline-treated pure Fru-P_2ase). Conditions as indicated for Fru-P_2ase (alkaline-treated pure enzyme) except that the reaction was started with 0.75 μmol of Sed-1,7-P_2 and proceeded for 20 min.

Fru-P_2ase activity (untreated Bio-Gel fraction). Conditions as indicated for Fru-P_2ase (untreated pure enzyme) except for the use of: 100 μl of enzyme fraction (1.1 mg total protein per ml), 260 μg of serum, and a reaction time of 20 min.

Fru-P_2ase activity (alkaline-treated Bio-Gel fraction). Incubation conditions and reaction time as indicated for Fru-P_2ase (alkaline-treated pure enzyme). Other conditions as indicated for Fru-P_2ase (untreated Bio-Gel fraction).

Sed-P_2ase activity (alkaline-treated Bio-Gel Fru-P_2ase fraction). Conditions as indicated for Fru-P_2ase (alkaline-treated Bio-Gel fraction) except that the reaction was started with 0.75 μmol of Sed-1,7-P_2 and proceeded for 40 min.

Specific Sed-P_2ase activity. Conditions as indicated for Sed-P_2ase (alkaline-treated Bio-Gel Fru-P_2ase fraction) except that the specific Sed-P_2ase Bio-Gel peak activity fraction was used.

respective of whether or not the preparation was dissociated by subjection to alkaline treatment. The Sed-P_2ase activity of the alkaline-treated pure Fru-P_2ase and of the Bio-Gel column Fru-P_2ase fractions was also inhibited by these antibodies. By contrast, neither of these antibodies had a significant effect on the activity of the new specific Sed-P_2ase enzyme. Clearly, this enzyme appears to be functionally, and perhaps structurally, different from the Fru-P_2ase.

Concluding Remarks

In conclusion, the present findings indicate that a substrate-specific Sed-P_2ase enzyme is present in chloroplasts [30]. As with certain other regulatory enzymes of chloroplasts, Sed-P_2ase appears to be activated (reduced) photochemically via the ferredoxin-thioredoxin system and deactivated (oxidized) in the dark via a chloroplast oxidant such as oxidized glutathione. The ferredoxin-thioredoxin system is, however, not the only means by which light could control the activity of this enzyme which, according to certain results, may be rate-limiting in photosynthetic carbon dioxide assimilation [31]. Light may also exercise control of this and other regulatory enzymes of the carbon reduction cycle by effecting changes in stromal pH and Mg^{2+} concentration that are conducive to full enzyme activity during illumination [32 – 37].

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