Ferredoxin-activated Fructose Diphosphatase of Spinach Chloroplasts

RESOLUTION OF THE SYSTEM, PROPERTIES OF THE ALKALINE FRUCTOSE DIPHOSPHATASE COMPONENT, AND PHYSIOLOGICAL SIGNIFICANCE OF THE FERREDXIN-LINKED ACTIVATION*

(Received for publication, March 22, 1971)

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SUMMARY

The hydrolytic cleavage of Pi from fructose diphosphate by the fructose diphosphatase (FDPase) system of spinach chloroplasts was found to require (a) an alkaline FDPase component insensitive to inhibition by AMP or fructose diphosphate; (b) reduced ferredoxin; (c) a protein factor; and (d) Mg++. Reduced spinach ferredoxin could be replaced by reduced methyl viologen or dithiothreitol but not by reduced bacterial ferredoxin. Activation of the alkaline FDPase component by the nonphysiological substitutes required the protein factor. The alkaline FDPase component in isolated intact chloroplasts was also activated by light.

The protein factor component of the FDPase system has been partly purified and found to have a molecular weight of 40,000. The alkaline FDPase component, purified to homogeneity, had a molecular weight of 145,000 and contained 2% carbohydrate. Except for a remarkably high half-cystine content (16% by weight as compared to 2% for mammalian FDPases) which reflects an apparent abundance of cysteine, the amino acid composition of the chloroplast alkaline FDPase component resembled that of the mammalian FDPases.

In the absence of reduced ferredoxin and protein factor, the chloroplast alkaline FDPase component can be activated by high concentrations of Mg++. Like liver alkaline FDPase, the chloroplast enzyme component is also activated by cystamine or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

Ferredoxin-activated FDPase was inhibited completely by $5 \times 10^{-4} \text{M}$ EDTA but was insensitive to KCN, cuprizone, and diethylthiocarbamate (all at $1 \times 10^{-4} \text{M}$).

The activity of chloroplast FDPase during photosynthesis appears to be controlled photochemically through reduced ferredoxin. What, if any, physiological role can be ascribed to the activation of the enzyme by high concentrations of Mg++ and disulfide reagents, independently of reduced ferredoxin, is not clear.

Ferredoxins are iron-sulfur proteins noted for their strongly electronegative oxidation-reduction potentials. Ferredoxins are widely distributed in nature and function as electron carriers in all photosynthetic cells and in certain anaerobic nonphotosynthetic bacteria (1). Work with isolated chloroplasts has shown that ferredoxin is the endogenous catalyst of cyclic and noncyclic photophosphorylation—the two processes that provide the assimilatory power, made up of ATP and NADPH, needed for carbon dioxide assimilation (1, 2).

An additional role of ferredoxin came to light when Buchanan, Kalberer, and Arnon (3) found that photochemically reduced ferredoxin activated the hydrolytic cleavage of fructose 1,6-diphosphate to fructose 6-phosphate and Pi by a fructose diphosphatase present in an aqueous extract of chloroplasts (Equation 1).

Fructose 1,6-diphosphate + H₂O $\rightarrow$ reduced ferredoxin $\rightarrow$ chloroplast extract $\rightarrow$ fructose 6-phosphate + Pi

Since chloroplasts reduce ferredoxin only in the light, Buchanan et al. (3) suggested that a requirement of reduced ferredoxin for activation of the chloroplast FDPase—a key enzyme of the photosynthetic reductive pentose phosphate cycle (8)—could serve as a light-actuated mechanism for regulation of carbon dioxide assimilation in photosynthesis. Kinetic evidence for a light activation of FDPase in whole algal cells was reported by Bassham, Kirk, and Jensen (9) and Pedersen, Kirk, and Bassham (10).

The fructose 1,6-diphosphatase reaction in chloroplasts was previously considered to require a single enzyme, alkaline FDPase, analogous to mammalian alkaline FDPase—a “pacemaker” enzyme in gluconeogenesis (11). The present experiments show that the chloroplast FDPase is, however, more complex. It consists not only of the alkaline FDPase component but also of a protein factor and ferredoxin. Ferredoxin, in reduced form, and the protein factor are needed to activate the alkaline FDPase component (Equation 2) prior to its catalysis.

FDPase, fructose 1,6-diphosphatase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine.
of the hydrolytic cleavage of fructose 1,6-diphosphate (Equation 3).

\[ \text{FDPase}_{\text{inactive}} \rightarrow \text{reduced ferredoxin} \rightarrow \text{protein factor} \rightarrow \text{FDPase}_{\text{active}} \] (2)

\[ \text{Fructose 1,6-diphosphate} + \text{H}_2\text{O} \xrightarrow{\text{Mg}^{++}} \text{Fructose 6-phosphate} + \text{Pi} \] (3)

The alkaline FDPase component was earlier extensively purified from spinach leaves by Raeker and Schroeder (12) and Press, Biggs, and Greenberg (13); its crystallization has recently been reported by El-Badry and Bassham (14).

The chloroplast alkaline FDPase component resembles the mammalian FDPase from mammalian cells (15) in showing stimulation by cysteamine and DTTB. However, unlike the mammalian FDPase (11, 16–21), the alkaline FDPase component of the chloroplast FDPase system is not inhibited by AMP or fructose diphosphate (13).

A preliminary report of some of these findings has been published (22).

**METHODS**

Rabbit muscle aldolase, liver catalase, pig heart malic dehydrogenase, horse heart cytochrome c, ovalbumin, and bovine serum albumin were purchased from Sigma. Homogeneous alkaline FDPase from rabbit liver and plastocyanin from spinach leaves were gifts of Dr. B. Horecker (Albert Einstein College of Medicine) and Mr. R. Chain of this Department, respectively. A sample of homogeneous alkaline FDPase from spinach leaves was an earlier gift of Dr. J. Press (University of California at Davis).

Protein was estimated by a modified phenol reagent procedure (23). Chlorophyll was determined as described by Arnon (24). The carbohydrate content of chloroplast FDPase was determined by a naphthalene procedure (25). Zinc was determined by atomic absorption spectrophotometry.

Amino acid analyses on 22- and 72-hour acid hydrolysates (6 × HCl, 105°C) were carried out by Dr. H. Tatewara with the method of Spackman, Stein, and Moore (26), modified as described previously (27, 28). Total half-cystine (cysteine + cystine) residues were determined as cysteic acid after performic acid oxidation of the FDPase. Tryptophan was determined by a modified phenol reagent procedure (23). Chlorophyll was determined as described by Arnon (24).

Leaf Extract—Fresh market spinach leaves, 5 kg, were washed and frozen overnight at −20°C. The frozen leaves (in 1-kg batches) were blended in 1,400 ml of water for 3 min in a Waring Blender (gallon size, model CB-5). Ten milliliters of 1 M K$_2$HPO$_4$ (sufficient to give a final pH of 7) were added prior to blending. The homogenate was filtered through four layers of cheesecloth and the residue was discarded. The remaining particulate material was sedimented by centrifugation at 13,000 × g for 5 min and was also discarded.

**Assay and Purification of Alkaline FDPase Component of Chloroplast FDPase System**

For routine assay of the alkaline FDPase component, the reaction mixture contained (in micromoles) aside from the enzyme: Tris-HCl buffer, pH 8.5, 100; MgCl$_2$, 16; sodium EDTA, 0.1; and sodium fructose 1,6-diphosphate, 6, in a final volume of 1.0 ml. The assay, carried out in test tubes at room temperature for 10 min, was started by adding fructose diphosphate and stopped by 0.5 ml of 10% trichloroacetic acid. The Pi released was determined colorimetrically by a modified Fiske-SubbaRow procedure (34). One unit of enzyme activity is defined as that amount of enzyme which catalyzes the release of 1 μmole of Pi per min under the assay conditions.

Unless indicated otherwise, all purification steps of the FDPase component given below were carried out at 4°C.

**Leaf Extract**—Fresh market spinach leaves, 5 kg, were washed and frozen overnight at −20°C. The frozen leaves (in 1-kg batches) were blended in 1,400 ml of water for 3 min in a Waring Blender (gallon size, model CB-5). Ten milliliters of 1 M K$_2$HPO$_4$ (sufficient to give a final pH of 7) were added prior to blending. The homogenate was filtered through four layers of cheesecloth and the residue was discarded. The remaining particulate material was sedimented by centrifugation at 13,000 × g for 5 min and was also discarded.

**pH 4.5 Precipitation**—The green supernatant fraction (10,700 ml) was adjusted to pH 4.5 with 1 N formic acid and centrifuged (5 min, 12,000 × g). The yellow supernatant fraction obtained in this step contained the bulk of the protein factor needed for activation of alkaline FDPase by reduced ferredoxin; the fraction was neutralized with 1 M Tris and fractionated as described below. The green precipitate containing the alkaline FDPase component was suspended in a HEPES-EDTA solution (0.025 M HEPES- NaOH buffer, pH 7.0, containing 2.5 × 10−4 M EDTA-Na) and then brought to a volume of 1,000 ml. The pH was adjusted to 6.5 with 0.1 N NH$_4$OH. The suspension was centrifuged for 10 min at 35,000 × g and the residue was discarded. Final volume of the supernatant fraction containing the FDPase was 700 ml.

**Ammonium Sulfate Fractionation**—Solid ammonium sulfate was added to the final resuspended pH 4.5 precipitate fraction to give 50% saturation. The solution was centrifuged (10 min, 13,000 × g) and the precipitate was discarded. Ammonium sulfate was added to the supernatant fraction for 80% saturation. The solution was centrifuged as above, the supernatant fraction was discarded, and the precipitate (containing the bulk of the FDPase activity) was dissolved in 0.03 M Tricine buffer, pH 8.0, to a final volume of 80 ml. This slightly turbid solution was centrifuged (15 min, 35,000 × g) to clarify.

**Sephadex G-100 Chromatography**—The 50 to 90% ammonium sulfate fraction was applied to a Sephadex G-100 column (5 × 90 cm) equilibrated beforehand and developed with 0.05 M Tricine, pH 8.0. Fractions of 10 ml were collected with a fraction collector. FDPase chromatographed just behind the excluded volume and was eluted before the bulk of applied protein. The slightly yellow fractions containing FDPase were pooled; and sufficient HEPES-NaOH buffer, pH 7.6, and NaCl were added to give a final concentration of 0.05 M HEPES, pH 7.6, and 0.2 M NaCl.

**DEAE Cellulose Chromatography**—The combined FDPase fractions were applied to a DEAE-cellulose column (5 × 45 cm) equilibrated beforehand with a solution containing 0.05 M HEPES-NaOH buffer, pH 7.6, and 0.15 M NaCl. The column was washed with a solution containing 0.05 M HEPES-NaOH buffer, pH 7.6, and 0.3 M NaCl until the eluate was free of protein, attached to a fraction collector, and eluted in 10-ml fractions with a solution containing 0.05 M HEPES-NaOH buffer,
pH 7.6, and 0.5 mM NaCl. FDPase was eluted just behind the solvent front; the active fractions were pooled and concentrated by vacuum dialysis. The FDPase component purified by this procedure was homogeneous. The enzyme was relatively stable and could be stored in HEPES-EDTA buffer at -20°C for several weeks with little loss of activity. Repeated freezing and thawing caused a slow loss of activity.

**Purification of Protein Factor Component of Chloroplast FDPase System**

The activity of the protein factor was determined by the increase of P_i release from the fructose diphosphate in the presence of photoeducted ferredoxin and homogeneous FDPase component (cf. Table IV).

The neutralized pH 4.5 supernatant fraction from Step 2 of the alkaline FDPase procedure was used as a source of the protein factor. Acetone, cooled to -20°C, was added with constant stirring to a final concentration of 75%; the solution was left 1 hour at -20°C to allow the precipitate to settle. The supernatant fraction was decanted and discarded; the precipitate (containing the protein factor) was collected by centrifugation (3 min, 10,000 × g) and suspended in 0.03 M Tris-HCl buffer, pH 8.0. The turbid solution was then dialyzed 24 hours against 0.03 M Tris-HCl buffer, pH 8.0.

Denatured protein was centrifuged off (15 min, 13,000 × g), and solid ammonium sulfate was added to the supernatant fraction to 50% saturation. The heavy precipitate was sedimented by centrifugation as before and discarded. The ammonium sulfate concentration in the supernatant solution was increased to 90% saturation and the precipitate (containing the protein factor) was collected by centrifugation as above and dissolved in 100 ml of 0.02 M Tris-HCl buffer, pH 8.0. The slightly turbid solution was centrifuged (5 min, 35,000 × g) to clarify.

The 50 to 90% ammonium sulfate fraction was applied to a Sephadex G-100 column (5 x 90 cm) equilibrated beforehand with a solution containing 0.1 M potassium phosphate buffer, pH 7.3, and 0.1 M NaCl was calibrated with liver catalase, pig heart male dehydrogenase, horse heart cytochrome c, ovalbumin, bovine serum albumin, and plastocyanin as standards. Chloroplast FDPase was applied to the calibrated column and was eluted between aldolase and bovine serum albumin, corresponding to a molecular weight of approximately 130,000.

The procedure of Davis (39) was used in acrylamide gel electrophoresis with 7.5% gels and Tris-glycine, pH 8.3, as electrode buffer. A current of 2 to 3 mA per gel was applied for 2 hours at room temperature. Gels were stained in Coomassie blue (0.5% solution in 12.5% trichloroacetic acid) (40).

The reaction of FDPase with p-mercuribenzoate was followed spectrophotometrically as described by Boyer (41). p-Mercuribenzoate (10⁻⁶ M) was added in 5-μl amounts to a cuvette containing 0.3 mg of FDPase in 3 ml of 0.1 M Tris-HCl buffer, pH 8.0. After 5 min, absorbance at 290 nm was measured against a cuvette containing FDPase but no p-mercuribenzoate. Additional incubation did not increase the amount of p-mercuribenzoate reacting with the FDPase.

The reaction of FDPase with DTNB was carried out spectrophotometrically as described by Ellman (42). A cuvette contained 0.3 mg of FDPase in 3 ml of 0.1 M Tris-HCl buffer, pH 7.3 (in the presence or absence of 8 M urea) and 20 μl of a 0.4% DTNB solution. Absorbance at 412 nm was read immediately after mixing and again after 12 hours at room temperature, when the reaction was complete.

**RESULTS AND DISCUSSION**

**Chemical and Physical Properties of Alkaline FDPase Component of Chloroplast FDPase System**

Alkaline FDPase, protein factor, and ferredoxin are the three protein components of the FDPase system of chloroplasts. Early in this investigation the FDPase and protein factor components were identified in the aqueous extract of isolated chloroplasts. However, for preparation on a large scale, procedures were devised for purification of the FDPase and protein factor from spinach leaves. Table I shows a summary of the purification and yield of the alkaline FDPase component from spinach leaves in each of the purification steps described under “Methods.”

The freshly purified FDPase component appeared to be homogeneous. The purified protein showed a single peak in the ultracentrifuge (Fig. 1, lower patterns in A and B) and in Sephadex G-200 chromatography. The FDPase component traveled in polyacrylamide gel as one main band trailing by diffuse protein material (Fig. 2). This slow moving material, present in all preparations, was probably due to change in the homogeneous enzyme during electrophoresis—an interpretation supported by the observation that under certain conditions (particularly after

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**Table I**

| Fraction       | Total protein | Total activity | Yield | Specific activity |
|----------------|---------------|----------------|-------|------------------|
| Leaf extract   | 48,100 mg     | 2,900 units    | 100%  | 0.06 units/mg    |
| pH 4.5 precipitate | 8,400 mg     | 3,200 units    | 110%  | 0.38 units/mg    |
| 50-90% (NH₄)SO₄ | 1,750 mg      | 2,720 units    | 94%   | 1.54 units/mg    |
| Sephadex G-100 | 384 mg        | 2,160 units    | 75%   | 5.62 units/mg    |
| DEAE-cellulose | 84 mg         | 2,050 units    | 71%   | 24.4 units/mg    |

**Summary of purification of chloroplast alkaline FDPase component from spinach leaves**
the enzyme was concentrated by vacuum or pressure dialysis) the FDPase component dissociated into subunits.

The subunits formed during concentration dialysis were separated from the parent alkaline FDPase component of the FDPase system by Sephadex G-200 chromatography. The subunit fraction showed a single peak in the ultracentrifuge (Fig. 1, upper patterns in A and B) and, based on both gel filtration and ultracentrifugation (see below), had a molecular weight half that of the original enzyme. This finding indicates that the active alkaline FDPase component is composed of two subunits of about equal molecular weight; the isolated subunit fraction had no FDPase activity.

The sedimentation velocity pattern (determined with schlieren optics) of the fresh alkaline FDPase component (lower patterns) and the isolated subunits (upper patterns) in Fig. 1 were measured 11 min (A) and 27 min (B) after the centrifuge reached full speed. The log of the displacement of the boundary of each protein under these conditions was linear with time. The data obtained with four different levels of both FDPase and subunit fraction showed that the sedimentation coefficient was dependent on the protein concentration; extrapolation to zero protein gave an $s_{20, w}$ of 8.3 for the FDPase component (and of 4.2 for the subunit fraction).

The sedimentation equilibrium technique showed a pattern typical of a homogeneous preparation and gave a molecular weight of 145,000 for the active undissociated FDPase component (Fig. 3) based on an average partial specific volume of 0.700. (The partial specific volume was determined experimentally by sedimentation equilibrium in D$_2$O and H$_2$O and found to be 0.697; a value of 0.706 was calculated from amino acid composition.) The molecular weight of the subunit fraction would therefore be 73,000. Values of 130,000 and 139,000 for the active FDPase component were obtained by the Sephadex G-200 chromatography technique and by amino acid analysis. A molecular weight of 145,000 is substantially lower than 195,000 obtained by Preiss (quoted by Preiss and Kosuge (43)) for his alkaline FDPase preparation.

Table II shows the amino acid composition of the alkaline FDPase component from chloroplasts in comparison with the enzyme from rabbit muscle and liver. Each of the three proteins is characterized by a high content of glutamate, aspartate, and glycine, a low content of histidine, and the absence of tryptophan. A striking feature of the chloroplast component (which distinguishes it from the mammalian enzymes) is the unusually high half-cystine content (210 residues per mole of enzyme, accounting for 16% of the enzyme by weight as compared to 2% for the mammalian enzymes). This feature is especially noteworthy when considered in the light of sulfhydryl group titration data. The chloroplast alkaline FDPase component contained only 10 p-mercuribenzoate-reactive —SH groups per mole (a value not increased by treating the enzyme with 8 M urea) and three to four DTNB-reactive —SH groups per mole (a value increased to 10 by treating the enzyme with 8 M urea). The data point to an unusually large cystine content of the alkaline FDPase component.

Homogeneous preparations of the chloroplast alkaline FDPase component were characterized by a single peak in the ultraviolet at 278 nm and showed no absorption in the visible region. An absorbance at 278 nm of 1.0 (1-cm light path) was equal to 1.3 mg of protein determined by the phenol reagent method.

Three different homogeneous preparations of the alkaline FDPase component were characterized by a single peak in the ultraviolet at 278 nm and showed no absorption in the visible region. An absorbance at 278 nm of 1.0 (1-cm light path) was equal to 1.3 mg of protein determined by the phenol reagent method.

Three different homogeneous preparations of the alkaline FDPase component were characterized by a single peak in the ultraviolet at 278 nm and showed no absorption in the visible region. An absorbance at 278 nm of 1.0 (1-cm light path) was equal to 1.3 mg of protein determined by the phenol reagent method.
Amino acid composition of chloroplast alkaline FDPase component in comparison with FDPases from liver and muscle

| Amino acid residue | Number of residues per mole in FDPase of: |
|--------------------|------------------------------------------|
|                    | Spinach chloroplasts | Rabbit liver* | Rabbit muscle |
| Lysine             | 65                  | 117           | 80            |
| Histidine          | 12                  | 14            | 10            |
| Arginine           | 30                  | 34            | 43            |
| Aspartic acid      | 98                  | 128           | 103           |
| Threonine          | 28                  | 70            | 78            |
| Serine             | 78                  | 70            | 76            |
| Glutamic acid      | 216                 | 81            | 118           |
| Proline            | 43                  | 52            | 48            |
| Glycine            | 166                 | 100           | 104           |
| Alanine            | 64                  | 106           | 111           |
| Half-cystine       | 210                 | 20            | 21            |
| Valine             | 67                  | 104           | 104           |
| Methionine         | 14                  | 34            | 21            |
| Isoleucine         | 57                  | 70            | 58            |
| Leucine            | 73                  | 94            | 119           |
| Tyrosine           | 36                  | 52            | 63            |
| Phenylalanine      | 25                  | 40            | 32            |
| Tryptophan         | 0                   | 0             | 0             |

* Data of Fernando, Pontremoli, and Horecker (44).

Effect of AMP on alkaline FDPase component from spinach chloroplasts in comparison with the enzyme from rabbit liver. Except for adding AMP, as indicated, the enzymes were assayed in 12 mM MgCl₂ at pH 8.0 as described under “Methods.” One hundred per cent activity corresponds to 1.8 and 2.1 amoles of Pi released by 0.05 mg of the chloroplast and liver enzymes, respectively.

FDPane component contained 2% carbohydrate (with glucose as a standard). As determined by atomic absorption analysis, these same preparations contained less than 0.4 eq of zinc per mole of enzyme.

Effect of AMP, Mg++, and Light on Alkaline FDPase Component of Chloroplast FDPase System

In 1954, Krebs (45) discussed the importance of FDPase in regulating gluconeogenesis in animal cells. Nine years later, three laboratories (16-18) independently made the important observation that relatively low concentrations of AMP inhibit mammalian FDPase—a finding that has led to the conclusion (11, 19-21) that the activity of FDPase in gluconeogenesis is regulated by the level of AMP. Such a role for AMP in sugar formation in photosynthesis can, however, be excluded. Fig. 4 shows that the chloroplast alkaline FDPase component, unlike the liver enzyme, is not inhibited by AMP. Insensitivity of the chloroplast alkaline FDPase component to AMP was also observed by Preiss et al. (13).

Apart from differential sensitivity to AMP, plant and mammalian (or microbial) FDPases differ in the level of Mg++ required for maximal activity. The FDPases from rat liver (40), rabbit liver (47), and nonphotosynthetic microorganisms (48, 49) require, depending on the source, 0.7 to 5.0 mM Mg++ for maximal activity, whereas the enzyme from photosynthetic cells requires about a 10-fold greater concentration of Mg++. Spinach leaf alkaline FDPase, dependent on pH, was reported to require 5 to 50 mM Mg++ (12, 13), and the Euglena gracilis enzyme was reported to require 50 mM Mg++ (50).

It is not known whether chloroplasts can maintain the high concentrations of Mg++ reported to be required by the alkaline FDPase component. A possible explanation is that light induces an accumulation in chloroplasts of Mg++ sufficient to activate the alkaline FDPase component. Such a dependence on light might also explain the photoactivation of the FDPase component observed in isolated intact chloroplasts (Table III; activity of the alkaline FDPase component was nearly doubled by illuminating the chloroplasts for 10 min prior to breaking osmotically and assaying the enzyme. The flux of Mg++ required for activation would, however, be in a direction opposite to that observed experimentally: recent studies from several laboratories have shown that Mg++ is expelled from chloroplasts on illumination and is reabsorbed in the dark (51-53). We searched, therefore, for a mechanism for activation of the chloroplast alkaline FDPase other than a light-induced enhancement of the intrachloroplastic level of Mg++. This search was made at a low level of Mg++ (0.67 mM)—which cannot itself activate the FDPase component.
The complete system contained alkaline FDPase component, 0.04 mg; protein factor, 0.15 mg; spinach ferredoxin, 0.12 mg; spinach chloroplast fragments, P₇₀₀, equivalent to 0.1 mg of chlorophyll; and (in pmole) Tris-HCl buffer, pH 8.0, 100; neutralized reduced glutathione, 5; sodium fructose 1,6-diphosphate, 6; sodium ascorbate, 10; 2,6-dichlorophenol indophenol, 0.1; EDTA-Na, 0.1; and MgCl₂ 1. Final volume, 1.5 ml; gas phase, argon. The reaction (carried out at 20° in Warburg flasks) was started by adding fructose diphosphate and chloroplast fragments from the side arm and was continued for 30 min under illumination (10,000 lux). The reaction was stopped and (after centrifuging off the precipitate) Pi was estimated as in Table III.

**Table IV**

Requirements for activation of chloroplast alkaline FDPase component by ferredoxin

| Treatment                                      | Pi released (pmoles) |
|------------------------------------------------|----------------------|
| Complete                                       | 4.0                  |
| Reduced glutathione omitted^a                   | 1.4                  |
| Ferredoxin omitted                             | 0.1                  |
| Alkaline FDPase component omitted              | 0.1                  |
| MgCl₂ omitted                                  | 0.1                  |
| Protein factor omitted                         | 0.0                  |
| Fructose diphosphate omitted                   | 0.0                  |
| Complete, ferredoxin not reduced (dark)        | 0.1                  |

^a In a parallel experiment, the complete and the minus glutathione treatments showed, respectively, 2.0 and 0.4 pmole of P₁ released; 2-mercaptoethanol added in place of glutathione showed 3.3 pmole of P₁ released in a light-dependent reaction.

Activation of Chloroplast Alkaline FDPase Component by Photoreduced Ferredoxin

A possible mechanism for the activation of the alkaline FDPase component observed with intact chloroplasts is that light functions by way of a photochemical reductant. The finding that reduced ferredoxin sharply activated the homogeneous alkaline FDPase component (Table IV) constituted the first evidence that such a mechanism might be valid. Ferredoxin, photoreduced by chloroplasts, enhanced the release of P₁ from fructose diphosphate 40-fold. The release of P₁ required, in addition to reduced ferredoxin, MgCl₂, fructose diphosphate, the alkaline FDPase component, and a new component, the protein factor. The reaction was stimulated by an —SH reagent (reduced glutathione or 2-mercaptoethanol) supplied in addition to reduced ferredoxin. Native spinach ferredoxin could not be replaced by bacterial ferredoxin (from *Clostridium pasteurianum*) but could be partially replaced by the nonphysiological dye methyl viologen (Table IV). The FDPase component was shown earlier to be activated in the dark when ferredoxin was reduced with hydrogen gas in the presence of hydrogenase (3). Reduced NADP or NAD could not replace light or hydrogen gas.

The protein factor, a component of the soluble protein fraction of chloroplasts, has been partially purified. It is heat-sensitive (5 min, 80°) and, based on gel filtration, has a molecular weight of 40,000. Other properties are unknown.

A homogeneous chloroplast alkaline FDPase preparation (supplied by Dr. J. Preiss) behaved like our own preparation in the ferredoxin-dependent release of P₁ from fructose diphosphate. A homogeneous preparation of the enzyme from rabbit liver, however, showed no response to reduced ferredoxin under the conditions in Table IV.

**Effect of Some Inhibitors on Ferredoxin-activated FDPase from Chloroplasts**—Ferredoxin-activated FDPase was not affected by KCN or the copper chelating agents (54) diethyldithiocarbamate or cuprizone (at 1 X 10⁻⁴ M) but was 97% inhibited by 5 X 10⁻⁴ M EDTA. A lower level of EDTA (7 X 10⁻⁵ M) increased 2- to 4-fold the release of P₁ from fructose diphosphate by certain preparations. Racker and Schroeder (12) and Preiss et al. (13) previously reported a stimulation of FDPase activity with EDTA.

**Effect of Fructose Diphosphate Concentration on Ferredoxin-activated FDPase**—A fructose diphosphate concentration greater than 0.1 mM (16, 18) to 1.0 mM (17) has been shown to inhibit mammalian FDPase—an effect believed to be important in regulation of that enzyme. The activity of the ferredoxin-activated FDPase of chloroplasts, by contrast, increased with fructose diphosphate concentration up to 8 mM (Fig. 5). The release of P₁ from fructose diphosphate, in the absence of reduced ferredoxin, was not appreciably affected by the fructose diphosphate concentration.

**Effect of Mg²⁺ Concentration on Ferredoxin-activated FDPase**—Because chloroplast alkaline FDPase is activated by high con-
Fig. 6. Effect of MgCl₂ concentration on ferredoxin-activated FDPase. Except for varying the MgCl₂ concentration, conditions were as described in Table IV.

**Table V**

Requirements for activation of chloroplast alkaline FDPase component by dithiothreitol

The complete system contained alkaline FDPase component, 0.04 mg; protein factor, 0.25 mg; and (in μmoles) Tris-HCl buffer, pH 8.0, 100; MgCl₂, 1; EDTA-Na, 0.1; sodium fructose 1,6-diphosphate, 6; dithiothreitol, 5; as indicated, 0.25 mg of bovine serum albumin was added. Final volume, 1.5 ml; gas phase, air. The reaction was carried out in test tubes at 23° and was started by adding fructose diphosphate. Reaction time, 30 min. The reaction was stopped and P₁ was determined as in Table III.

| Treatment                                      | P₁ released (μmoles) |
|------------------------------------------------|----------------------|
| Complete                                       | 3.7                  |
| Protein factor omitted                         | 0.3                  |
| MgCl₂ omitted                                  | 0.1                  |
| Alkaline FDPase component omitted              | 0.0                  |
| Dithiothreitol omitted                         | 0.0                  |
| Fructose diphosphate omitted                   | 0.0                  |
| Protein factor added, bovine serum albumin added| 0.3                  |

Concentrations of Mg²⁺ (5 mM or greater), the extent of the ferredoxin-linked activation is strictly dependent on the Mg²⁺ concentration (Fig. 6). A consistent stimulation in P₁ release was observed at all levels of MgCl₂ tested but the effect of reduced ferredoxin was most pronounced at 0.67 to 2 mM MgCl₂.

**Activation of Chloroplast Alkaline FDPase Component by Dithiothreitol and —S—S— Reagents**

The nonphysiological —SH reagent dithiothreitol (55) could replace reduced ferredoxin in activation of the chloroplast alkaline FDPase component (Table V). P₁ release from fructose diphosphate by the dithiothreitol-activated FDPase component occurred in the dark without chloroplasts and required MgCl₂ and the protein factor. The requirement for the protein factor in activation of the FDPase component by dithiothreitol was specific—an equivalent amount of bovine serum albumin or of various protein fractions obtained in purification of the alkaline FDPase component was without effect. Cysteine, reduced glutathione, 2-mercaptoethanol, and sodium dithionite did not replace dithiothreitol in activation of the alkaline FDPase component. Dithiothreitol has been shown to activate other chloroplast enzymes: ribulose 5-phosphate kinase (56), NADP-linked glyceraldehyde 3-phosphate dehydrogenase (57), and pyruvate, P₁ dikinase (58).

Cystamine and DTNB, —S—S— compounds found by Pontremoli et al. (15) to activate liver FDPase, also activated the homogeneous chloroplast alkaline FDPase component. Fig. 7 shows that cystamine enhanced 50-fold the release of P₁ from fructose diphosphate; the protein factor had no effect on the FDPase component under these conditions. When cystamine was replaced by an equivalent amount of DTNB, a 10-fold increase in P₁ release was observed. Activation by —S—S— reagents (via disulfide exchange) provides a model system for regulation of the FDPase reaction, first for mammalian cells (15) and now for chloroplasts. The physiological significance of this type of activation is, however, an open question.

In sum, the present experiments provide evidence that the FDPase system of chloroplasts is activated by light. Ferredoxin, a component of the system, is reduced photochemically and, in collaboration with another component, the protein factor, reduced ferredoxin activates a third component, the alkaline FDPase. Once activated, alkaline FDPase catalyzes the formation of fructose 6-phosphate (and P₁) from fructose diphosphate—a key reaction in the reductive pentose phosphate cycle of photosynthesis.

The mechanism of activation of the alkaline FDPase component is not known, but it may involve a reduction by reduced ferredoxin of specific —S—S— groups that cannot be reduced by more electropositive agents such as reduced glutathione or 2-mercaptoethanol (Equations 4 and 5).

\[
2 \text{Ferredoxin}_\text{red} + \text{H}_2\text{O} \xrightarrow{\text{light}} \text{Ferredoxin}_\text{ox} + \frac{1}{2} \text{O}_2
\]
The abundance of cysteine residues in the alkaline FDPase and the replaceability of reduced ferredoxin by dithiothreitol or reduced methyl viologen are in accord with such a mechanism.

Acknowledgment—We gratefully acknowledge the support and advice of Professor D. I. Arnon throughout this investigation.

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Ferredoxin-activated Fructose Diphosphatase of Spinach Chloroplasts: RESOLUTION OF THE SYSTEM, PROPERTIES OF THE ALKALINE FRUCTOSE DIPHOSPHATASE COMPONENT, AND PHYSIOLOGICAL SIGNIFICANCE OF THE FERREDOXIN-LINKED ACTIVATION
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*J. Biol. Chem.* 1971, 246:5952-5959.

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