APPEARANCE OF SEDOHEPTULOSE 1,7-DIPHOSPHATASE ACTIVITY ON CONVERSION OF CHLOROPLAST FRUCTOSE 1,6-DIPHOSPHATASE FROM DIMER FORM TO MONOMER FORM

Bob B. Buchanan, Peter Schürmann[a], and Ricardo A. Wolosiuk[b]

Department of Cell Physiology
University of California
Berkeley, California 94720 U.S.A.

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Summary: Chloroplast fructose 1,6-diphosphatase isolated at pH 5.5 as the dimer dissociated to the monomer at pH 8.5. When the pH was adjusted from 8.5 back to 5.5, the newly formed monomer partly reassociated to form the dimer. The monomer lacked the fructose diphosphatase activity characteristic of the dimer (measured in the presence of a saturating concentration of Mg^{++}) but retained ferredoxin-dependent activity (measured in the presence of Mg^{++} plus protein factor and either reduced ferredoxin or dithiothreitol). In addition, the monomer acquired sedoheptulose 1,7-diphosphatase activity that was dependent on either reduced ferredoxin or dithiothreitol and the protein factor.

Fructose 1,6-diphosphatase (FDPase)[c] (E.C. 3.1.3.11) is a key regulatory enzyme of gluconeogenesis and photosynthesis that catalyzes the hydrolysis of fructose 1,6-diphosphate (Eq. 1). We previously reported (1,2) that homoge-

$$\text{Fructose 1,6-diphosphate} + H_2O \xrightarrow{Mg^{++}} \text{Fructose 6-phosphate} + P_i \qquad (1)$$

neous preparations of the chloroplast FDPase are activated by photoreduced ferredoxin in the presence of a partly purified chloroplast protein factor, as summarized in Eq. 2 and 3. The results also demonstrated that reduced fer-

$$2\ \text{Ferredoxin}_{ox} + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} 2\ \text{Ferredoxin}_{red} + 1/2\ O_2 + 2\ H^+ \qquad (2)$$

$$\text{FDPase}_{\text{inactive}} \xrightarrow[\text{Protein factor}]{\text{Reduced ferredoxin}} \text{FDPase}_{\text{active}} \qquad (3)$$

Present addresses: [a]Laboratoire de Physiologie végetale, Université de Neuchatel, Switzerland and [b]Departmento de Biologia, Fundacion Bariloche, Argentina.

[c]Abbreviations: FDPase, fructose 1,6-diphosphatase; SDPase, sedoheptulose 1,7-diphosphatase; DTT, dithiothreitol; P_i, inorganic phosphate; ox, oxidized; red, reduced.

redoxin can be replaced by the sulfhydryl reagent dithiothreitol (DTT) but not by physiological sulfhydryl compounds (Eq. 4).

$$\text{FDPase}_{\text{inactive}} \xrightarrow[\text{Protein factor}]{\text{DTT}} \text{FDPase}_{\text{active}} \qquad (4)$$

In a recent extension of these findings we reported (3) that the protein factor and reduced ferredoxin can also activate sedoheptulose 1,7-diphosphatase (SDPase) in the soluble extract of chloroplasts (Eq. 5). The activated enzyme

$$\text{SDPase}_{\text{inactive}} \xrightarrow[\text{Protein factor}]{\text{Reduced ferredoxin}} \text{SDPase}_{\text{active}} \qquad (5)$$

catalyzed the hydrolysis of sedoheptulose 1,7-diphosphate, a reaction that is necessary for photosynthetic CO_2 assimilation in chloroplasts (Eq. 6). Ander-

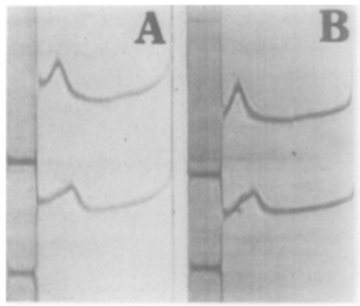
$$\text{Sedoheptulose 1,7-diphosphate} + H_2O \xrightarrow{Mg^{++}} \text{Sedoheptulose 7-phosphate} + P_i \qquad (6)$$

son reported previously that chloroplast SDPase is activated by DTT (4).

Like its counterpart from cells of the yeast *Candida utilis* (5), the FDPase from chloroplasts is reported to be quite specific for its substrate fructose 1,6-diphosphate and to show no activity with sedoheptulose 1,7-diphosphate or other sugar diphosphates (6). We now report evidence that one form of the chloroplast FDPase lacks this constraint in activity. When the FDPase was converted from the dimer to the monomer form by increasing the pH from 5.5 to 8.5 (cf. ref. 1), the enzyme lost its specificity for fructose 1,6-diphosphate and acquired an SDPase activity. The enzyme monomer therefore resembled liver FDPase in showing both FDPase and SDPase activities (7). However, unlike liver FDPase, the chloroplast enzyme required for both activities the protein factor and either reduced ferredoxin or DTT.

METHODS

In light of the finding that chloroplast FDPase dissociates when the pH is greater than 8 (see below), we revised the procedure used earlier for purification of this enzyme (2). The first purification steps were unchanged (preparation of leaf extract, pH 4.5 precipitate, and 50-90% ammonium sulfate precipitate) but subsequent purification steps were changed in that: (i) the pH of the



A: Lower profile, Dimer (pH 5.5), s = 8.0

A: Upper profile, Monomer (pH 5.5 → 8.5), s = 4.0

B: Lower profile, Reassociated Dimer (pH 5.5 → 8.5), s = 8.0, major peak

B: Upper profile, Monomer (pH 5.5 → 8.5), s = 4.0

Figure 1. Sedimentation velocity pattern of the monomer and dimer forms of chloroplast FDPase. (A) lower profile, dimer (pH 5.5), s = 8.0; upper profile, monomer (pH 5.5 increased to 8.5), s = 4.0. (B) lower profile, reassociated dimer (pH 5.5 increased to 8.5; sample incubated 1 hr at 25° and pH then decreased to 5.5), s = 8.0, major peak; upper profile, monomer (pH 5.5 increased to 8.5), s = 4.0. Patterns were determined as described previously (2) with 0.4-ml samples containing 3.6 mg per ml of FDPase in either 0.1 M Na-acetate buffer (pH 5.5) or 0.1 M Tris-HCl buffer (pH 8.5). In (B) the newly formed monomer (in 0.1 M Tris-HCl buffer, pH 8.5) was dialyzed against 0.1 M Na-acetate buffer at pH 5.5 overnight to form the reassociated dimer (lower profile) or against 0.1 M Tris-HCl buffer at pH 8.5 to retain the monomer form (upper profile). Measurements were made at a rotor speed of 59,780 rpm; average temperature, 20°. Photographs were taken 32 min after full speed was reached.

buffer solution was decreased to minimize dissociation of the enzyme; and (ii) the final DEAE-cellulose chromatography step was omitted. Accordingly, the 50-90% ammonium sulfate precipitate was dissolved not in the buffer used earlier but in 50 mM sodium acetate buffer, pH 5.5. The redissolved precipitate was placed in steel centrifugation tubes, frozen solid, thawed, and then centrifuged (30 min, 48,200 g). The precipitate was discarded and the supernatant solution was fractionated by Sephadex G-100 chromatography as described previously except that the buffer used for equilibration and elution of the column was replaced by 50 mM sodium acetate, pH 5.5. Fractions containing FDPase activity were lyophilized and dissolved in 15 mM acetic acid to a protein content of 2 to 3 mg/ml. Fresh preparations of the enzyme showed one peak in the ultracentrifuge at pH 5.5 and were stable for several months at 0°.

The FDPase monomer was obtained by adding 100 µl of the purified FDPase dimer (4.0 mg/ml) in 0.015 M Na-acetate buffer (pH 5.5) to 10 µl of 1 M Tris-HCl buffer (pH 8.5). Except for stability studies, enzyme activities were assayed with samples that had been stored 6 to 12 hr at 4° after the pH was

increased to 8.5. Unless indicated otherwise, ultracentrifugation studies were carried out immediately after the increase in pH.

Procedures for routine assay of the FDPase, purification of the protein factor, ultracentrifugation, and estimation of protein were as described previously (2). Sedoheptulose 1,7-diphosphate and fructose 1,6-diphosphate were purchased from the Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Dissociation of the FDPase Dimer. In experiments designed to identify the factors that influence the previously demonstrated conversion of the isolated dimer form of the chloroplast FDPase (molecular weight, 145,000) to its monomer derivative (molecular weight, 72,500), we observed that treatments involving an increase in pH altered the sedimentation properties of the enzyme. Fresh preparations of the FDPase that showed at pH 5.5 only the dimer form (s = 8.0) (Fig. 1A, lower pattern) underwent complete dissociation at pH 8.5 to the earlier observed monomer form (s = 4.0) (Fig. 1A, upper pattern). The monomer form of the FDPase was also detected in the same preparations of the dimer that had been stored frozen at pH 5.5. However, the appearance of the monomer was slow under these conditions and it was only when the pH was raised above 8.0 that a rapid (less than 10 min) dimer-monomer transition was observed. The addition of either $MgCl_2$ (20 mM), DTT (100 mM), protein factor plus DTT (10^{-4} M), protease inhibitor (phenylmethyl-sulfonylfluoride) (10^{-4} M), or cystamine (1 mM) had no effect on the conversion of the FDPase dimer to the monomer form (cf. 2, 8). The results therefore support the conclusion of Lazaro et al (9) that the structure of the chloroplast FDPase changes with the pH, according to Eq. 7.

$$\text{FDPase}_{\text{dimer}} \underset{\text{pH 5.5}}{\overset{\text{pH 8.5}}{\rightleftharpoons}} 2\ \text{FDPase}_{\text{monomer}} \qquad (7)$$

The reversibility of this dissociation was indicated by the finding that, when dialyzed against a solution of 0.1 M sodium acetate buffer, pH 5.5, the newly formed FDPase monomer (at pH 8.5) partly reassociated to form the original dimer (Fig. 1B).

Activity of Dimer and Monomer Forms of FDPase. The enzyme activity of the FDPase monomer differed from that of the parent dimer in important respects. First, the activity measured in the presence of a saturating concentration of

Table 1. FDPase AND SDPase ACTIVITIES OF DIMER AND MONOMER FORMS OF CHLOROPLAST FDPase AT A LIMITING CONCENTRATION OF $MgCl_2$

Treatment	FDPase Activity (P_i released, μmoles/10 min)	SDPase Activity (P_i released, μmoles/30 min)
FDPase Dimer		
Control	0.00	0.00
+ Protein factor	0.18	0.00
+ DTT	0.06	0.00
+ Protein factor, DTT	1.88	0.06
FDPase Monomer		
Control	0.00	0.00
+ Protein factor	0.04	0.00
+ DTT	0.00	0.16
+ Protein factor, DTT	1.80	0.61

For assaying FDPase activity of the enzyme dimer, the control system contained FDPase dimer, 23 μg, and (μmoles): Tris-HCl buffer (pH 8.0), 100; $MgCl_2$, 0.7; fructose 1,6-diphosphate, 6. As indicated, DTT, 5 μmoles, and protein factor, 110 μg were added. Final volume, 1.0 ml; reaction time, 10 min. For assaying FDPase activity of the monomer, the same conditions were used except that the FDPase monomer (23 μg) replaced the dimer and the pH of the Tris-HCl buffer was 7.8.

For assaying SDPase activity of the enzyme dimer, the control system contained FDPase dimer, 23 μg, and (μmoles): Tris-HCl buffer (pH 8.0), 100; $MgCl_2$, 0.7; sedoheptulose 1,7-diphosphate, 1.5. As indicated, DTT, 5 μmoles, and protein factor, 110 μg, were added. Final volume, 1.0 ml; reaction time, 30 min. SDPase activity of the monomer was assayed under the same conditions except that the FDPase monomer, 23 μg, replaced the dimer and the pH of the Tris-HCl buffer was 8.8.

In each case, the reaction (at 25°) was started by the addition of sugar diphosphate and was stopped by the addition of 4 ml of the reaction mixture previously used for P_i analysis (2).

Mg^{++} and in the absence of protein factor, DTT, or ferredoxin (henceforth designated "control activity") that is characteristic of the FDPase dimer was not observed with the monomer form. FDPase activity was restored to the monomer only on the addition of protein factor and either reduced ferredoxin or DTT

Table 2. FDPase AND SDPase ACTIVITIES OF DIMER AND MONOMER FORMS OF CHLOROPLAST FDPase AT A SATURATING CONCENTRATION OF $MgCl_2$

Treatment	FDPase Activity (μmoles of P_i per min per mg of enzyme)	SDPase Activity (μmoles of P_i per min per mg of enzyme)
FDPase Dimer		
Control	36	0.0
+ Protein factor	36	0.0
+ DTT	52	0.0
+ Protein factor, DTT	72	0.5
+ Reduced glutathione	36	0.0
FDPase Monomer		
Control	0	0.0
+ Protein factor	0	0.0
+ DTT	10	2.0
+ Protein factor, DTT	23	5.0
+ Reduced glutathione	0	0.0

For assaying FDPase activity of the enzyme dimer, the conditions were as in Table 1 except that 12 μg of FDPase dimer and 10 μmoles of $MgCl_2$ were used with a reaction time of 2.5 min. For assaying FDPase activity of the monomer, the conditions were as in Table 1 except that 12 μg of FDPase monomer and 10 μmoles of $MgCl_2$ were used with a reaction time of 5 min. As indicated, reduced glutathione, 5 μmoles, was added.

For assaying SDPase activity of the enzyme dimer, the conditions were as in Table 1 except that 12 μg of FDPase dimer and 10 μmoles of $MgCl_2$ were used with a reaction time of 15 min. For assaying SDPase activity of the monomer, the conditions were as in Table 1 except that 12 μg of FDPase monomer and 10 μmoles of $MgCl_2$ were used with a reaction time of 15 min. As indicated, reduced glutathione, 5 μmoles, was added.

(optimum pH, 7.8). In addition, and perhaps more importantly, the monomer acquired SDPase activity (optimum pH, 8.8). SDPase activity was observed at both limiting (Table 1) and saturating (Table 2) concentrations of Mg^{++}. In both cases, SDPase activity required the protein factor and either reduced fer

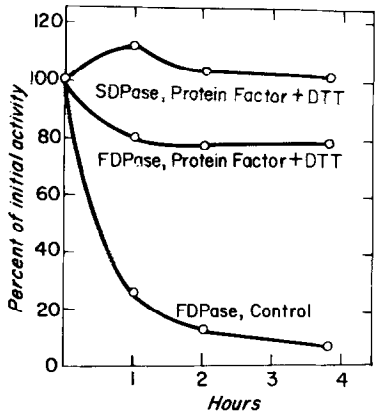


Figure 2. Stability of FDPase and SDPase activities of chloroplast FDPase monomer. Except for using Tris-HCl buffer at pH 8.5 in each assay and a reaction time of 6 min, conditions were as described for the corresponding treatments in Table 2. The initial activity was (μmoles/6 min): FDPase, control, 2.7; FDPase, protein factor plus DTT, 3.4; SDPase, protein factor plus DTT, 0.4.

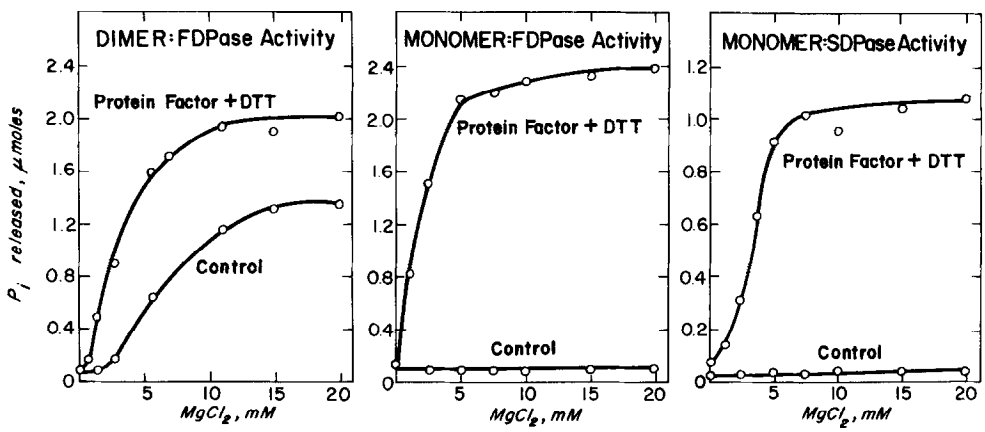


Figure 3. Effect of concentration of $MgCl_2$ on FDPase and SDPase activities of the dimer and monomer forms of chloroplast FDPase. Except for variation of $MgCl_2$ concentration, conditions were as described for the corresponding treatments in Table 2.

redoxin or DTT. As was observed previously (2), DTT could not be replaced by reduced glutathione or other sulfhydryl agents. SDPase activity was consistently absent in the FDPase dimer. Both the dimer and monomer forms lacked phosphatase activity with other phosphorylated substances (glucose 1,6-diphosphate, ribulose 1,5-diphosphate, fructose 1-phosphate, ATP).

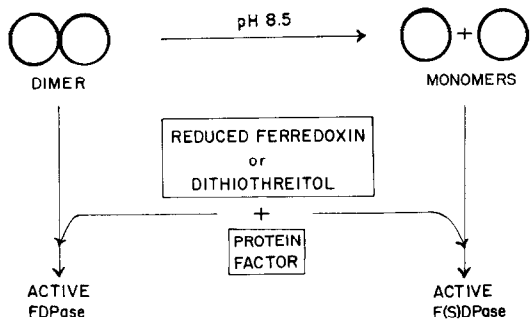


Figure 4. Effect of pH on the ferredoxin- or DTT-mediated regulation of FDPase or SDPase [F(S)DPase] activities of chloroplast FDPase.

SDPase activity was detected immediately after increasing the pH of the isolated dimer from 5.5 to 8.5 and, like the FDPase activity observed in the presence of protein factor and DTT, was stable for at least 24 hr. By contrast, control FDPase activity disappeared rapidly after conversion of the dimer to the monomer form (Fig. 2) and remained feeble irrespective of the concentration of Mg^{++} during the assay (Fig. 3). Other experiments showed that the monomer displayed no control activity over the pH range from 7.5 to 9.5.

It is pertinent to note that the addition of protein factor and DTT to the FDPase dimer decreased the $S_{0.5}$ for $MgCl_2$ from 6 to 3 mM and increased the maximum velocity of the enzyme from 30 to 47 μmoles of P_i per min/mg of enzyme (cf. Fig. 3). There was correspondingly little change in the $S_{0.5}$ for fructose 1,6-diphosphate. Similar results were obtained in the presence of ferredoxin and protein factor.

CONCLUDING REMARKS

The present results show that when the pH is shifted from 5.5 to 8.5 the isolated chloroplast FDPase dimer dissociated to the monomer form. On dissociation, the FDPase monomer acquired an SDPase activity that required protein factor and either reduced ferredoxin or DTT. The monomer rapidly lost control FDPase activity but retained the FDPase activity that, like the new SDPase activity, depended on protein factor and either reduced ferredoxin or DTT (Fig. 4).

It is not yet possible to relate the SDPase activity associated with the FDPase monomer to the SDPase activity observed previously in chloroplast extracts. The requirement of both activities for protein factor and either reduced ferredoxin or DTT suggests that they may be related. If so, it is possible that the light-induced increase in pH that has been reported to occur in the stroma portion of chloroplasts (pH 7.2 → pH 8.0) (10) could influence the association state of the FDPase enzyme and hence its catalytic activity during CO_2 assimilation in photosynthesis.

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