Enzyme Regulation in Crassulacean Acid Metabolism Photosynthesis

STUDIES ON THIOREDOXIN-LINKED ENZYMES OF KALANCHOE DAIGREMONTIANA

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ABSTRACT

Fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) were identified and purified from the Crassulacean acid metabolism (CAM) plant, Kalanchoe daigremontiana. FBPase and SBPase showed respective molecular weights of 180,000 and 76,000, and exhibited immunological cross-reactivity with their counterparts from chloroplasts of C4 (spinach) and C3 (corn) plants. Based on Western blot analysis, FBPase was composed of four identical 45,000-dalton subunits and SBPase of two identical 38,000-dalton subunits. Immunological evidence, together with physical properties, indicated that both enzymes were of chloroplast origin.

Kalanchoe FBPase and SBPase could be activated by thioredoxin f reduced chemically with dithiothreitol or photochemically by a reconstituted Kalanchoe ferredoxin/thioredoxin system. Both enzymes were activated synergistically by reduced thioredoxin f and their respective substrates.

Kalanchoe FBPase could be partially activated by Mg2+ at concentrations greater than 10 millimolar; however, such activation was considerably less than that observed in the presence of reduced thioredoxin and Ca2+, especially in the pH range between 7.8 and 8.3. In contrast to FBPase, Kalanchoe SBPase exhibited a specific requirement for a dithiol such as reduced thioredoxin irrespective of Mg2+ concentration. However, like FBPase, increased Mg2+ concentrations enhanced the thioredoxin-linked activation of this enzyme.

In conjunction with these studies, an NADP-linked malate dehydrogenase (NADP-MDH) was identified in cell-free preparations of Kalanchoe leaves which required reduced thioredoxin m for activity.

These results indicate that Kalanchoe FBPase, SBPase, and NADP-MDH share physical and regulatory properties with their equivalents in C3 and C4 plants. In contrast to previous evidence, all three enzymes appear to have the capacity to be photoactivated in chloroplasts of CAM plants, thereby providing a means for the functional segregation of glucon synthesis and degradation.

In contrast to C3 and C4 photosynthesis, relatively little is understood concerning the photoregulation of carbon assimilation in CAM plants. The principal study in this area is that of Gupta and Anderson (10) who measured the activity of enzymes in extracts of illuminated and darkened leaves of Kalanchoe, a CAM plant. These investigators concluded that, as in C3 plants, phosphoribulokinase, NADP-glyceraldehyde 3-P dehydrogenase, SBPase3 and NADP-MDH are activated by light in Kalanchoe preparations. The photoactivation of these enzymes was sensitive to inhibitors of photosynthetic electron transport, e.g. DCMU, and could be mimicked in the dark by treatment with DTT (1). Although FBPase was present, it was not activated either by light or DTT. In view of the evidence that FBPase is under photoregulation in C3 and C4 plants, the inability to observe its photoactivation in Kalanchoe is unexpected and, furthermore, is inconsistent with current models describing the regulation of photosynthetic carbon flow (5, 15).

In an attempt to resolve this puzzle, we have studied the properties of key enzymes of photosynthetic carbon assimilation, including FBPase. We now report that FBPase and SBPase isolated from Kalanchoe daigremontiana leaves share a number of properties in common with their C3 and C4 counterparts, including the capacity for photoregulation. As a part of this study, we were also able to show that the NADP-MDH of Kalanchoe, earlier reported to be under photoregulation (10, 21) is activated by reduced thioredoxin m. Preliminary reports of this work have been presented (13, 14).

MATERIALS AND METHODS

Plant Materials. Kalanchoe daigremontiana was greenhouse-grown as described in the preceding communication (15).

Reagents. Biochemicals were purchased from Sigma Chemical Co. All other reagents were purchased from commercial sources and were of the highest quality available. Thioredoxins and ferredoxin-thioredoxin reductase from spinach and Kalanchoe were prepared as described in the preceding communication (15). Spinach chloroplast FBPase antibody and corn leaf SBPase antibody were prepared in previous investigations (2, 19).

Assay Procedures. One-Step Colorimetric Assay for FBPase and SBPase. A colorimetric assay was employed for routine assay of Kalanchoe FBPase (27). Aliquots of fractions obtained during purification (0.1–0.2 ml) were assayed at 22°C in 1 × 7.5-cm test tubes containing (in μmol): Tricine-KOH buffer (pH 8.0), 50; MgSO4, 5; DTT, 2.5; sodium FBP, 3 (final volume, 0.5 ml). After 30 min, the reaction was stopped by the addition of 2.0 ml of the mixture used for the determination of F1 (27). After the samples

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1 This work was aided by a grant from the Competitive Research Grants Office, United States Department of Agriculture. This is the second of two communications; the accompanying article is Reference 15.

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3 Abbreviations: SBPase, sedoheptulose 1,7-bisphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FBP, fructose 1,6-bisphosphate; FTR, ferredoxin-thioredoxin reductase; FBPase, fructose 1,6-bisphosphatase; NADP-MDH, NADP-linked malate dehydrogenase; OAA, oxaloacetic acid; PMSF, phenylmethylsulfonyl fluoride; SBP, sedoheptulose 1,7-bisphosphate; PFK, phosphofructokinase; PAGE, polyacrylamide gel electrophoresis.
had stood at room temperature for 10 min, Amax was measured with a Gilford model 252 spectrophotometer. SBPase was assayed by the same procedure, except that the pH of the reaction mixture was 8.5, FBP was omitted, and 0.75 µmol SBP was included in the reaction mixture.

**Two-Step Colorimetric Assay for FBPass and SBPass.** For determining the regulatory properties of *Kalanchoë* FBPass and SBPass, a two-step colorimetric assay system was developed. Enzyme, 1 to 10 µg, depending on sample purity and remaining activity, was preincubated at room temperature in 1 x 7.5-cm test tubes containing 0.1 M Tricine-KOH (pH 8.0) unless indicated otherwise with or without additional components (volume = 0.1 ml). After 10 min, the reaction was started by injecting 0.05 ml of the preincubation mixture into another (1 x 7.5 cm) test tube which contained the reaction mixture in a volume of 0.45 ml: Tricine-KOH buffer (pH 8.0), 45; MgSO4, 2.5; sodium FBP, 3. After 20 min, the reaction was stopped and free P was determined as above.

A similar two-step assay was employed for assay of SBPass. Enzyme (5–15 µg) was added to a preincubation mixture containing in a volume of 0.1 ml the following (in µmol): Tricine-KOH buffer (pH 8.5), 10; and DTT, 0.5. Other conditions were as above. After 10 min, the reaction was started by injecting 0.05 ml of the preincubation mixture into the reaction mixture composed of the following (in µmol): Tricine-KOH buffer (pH 8.5), 45; MgSO4, 5; SBP, 0.75.

**Spectrophotometric Assay of FBPass Activity.** For kinetic measurements of FBPass, fructose 6-P formation was determined spectrophotometrically by following the reduction of NADP in the presence of coupling enzymes (27). FBPass was preincubated for 10 min as described in the two-step colorimetric assay. To start the reaction, the preincubation sample (0.1 ml) was injected into the assay mixture in a 1-ml cuvette which contained in a volume of 0.9 ml: nonlimiting amounts of glucose 6-P dehydrogenase (1.5 units), P-glucose isomerase (2 units), and the following (in µmol): Tricine-KOH buffer (pH 8.0), 40; MgSO4, 5; sodium FBP, 6; and NADP, 0.25. NADPH formation was followed by measuring the change in A at 340 nm with a Gilford model 252 recording spectrophotometer equipped with a sample changer so that multiple assays could be monitored simultaneously.

**Assay of NADP-MDH.** NADP-MDH was assayed after activation by DTT-reduced thioredoxin m by following the OAAs dependent oxidation of NADPH (27). Enzyme (5–10 µg) was added to a preincubation mixture (0.2 ml) containing 20 µmol Tris-HCl buffer (pH 7.9), 2 µmol DTT, and 19 µg spinach thioredoxin m. After 5 min, the preincubation solution was transferred into a 1-cm cuvette (1-ml capacity) containing (in 0.75 ml) the following (in µmol): Tris-HCl buffer (pH 7.9), 100; NADPH, 0.125. The reaction was started by addition of 2.5 µmol OAAs. The change in A at 340 nm was measured in a Gilford model 252 spectrophotometer equipped with a sample changer.

**Preparative Procedures.** Except for the preparation of leaf extract, all steps were carried out at 4°C. Buffers were adjusted to the indicated pH at 20°C. Detailed procedures for the purification of FBPass, SBPass, and PFK are provided in the text.

**Preparation of FBPass.** FBPass was purified from leaf extracts by DEAE-cellulose and Sephacryl S-300 superfine (Pharmacia) column chromatography.

**Preparation of SBPass.** SBPass was purified in conjunction with FBPass. Following separation from FBPass by DEAE-cellulose column chromatography, SBPass was chromatographed on a Sephadex G-100 column (Pharmacia).

**Preparation of PFK.** PFK was purified from *Kalanchoë* leaf extracts by sequential chromatography on columns of DE52 (Whatman) and Sephacryl S-300 superfine (Pharmacia) by following the procedure of Cséke et al. (8). Leaves were harvested and treated as given in the preceding communication (15).

**Preparation of NADP-MDH.** Cell-free extracts from frozen leaves were prepared and clarified as described for preparation of ferredoxin-thioredoxin reductase in the preceding communication (15). The green clarified extract was applied to a DEAE-cellulose column (4 x 25 cm) which had been equilibrated beforehand with 30 mM Tris-HCl buffer (pH 7.9) containing 0.1% (v/v) 2-mercaptoethanol and 3 mM MgCl2 (hereafter called buffer C). The column was washed with 500 ml buffer C and eluted with a linear NaCl gradient (900 ml; 0–500 mM NaCl) in buffer C. During sample application and column wash, fractions of 20 ml were collected; subsequent fractions were 4.4 ml. Fractions were monitored by A280, and assayed for NADP-MDH as described above. Fractions enriched in NADP-MDH were pooled and concentrated to 20 ml by ultrafiltration with an Amicon YM-5 membrane. The concentrated DEAE-cellulose fractions were chromatographed on a Sephadex G-100 column (2.6 x 90 cm), equilibrated, and subsequently developed with buffer C supplemented with 200 mM NaCl. Fractions (3.5 ml) were collected and assayed, and those containing peak activity were combined and concentrated as above. Enzyme was stored in 50 mM sodium acetate buffer, pH 5.5, containing 0.1% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol.

**Analytical Procedures.** Procedures for polyacrylamide gel electrophoresis and for the determination of Chl, protein, Pi, and NaCl were as described in the preceding communication (15).

Mol wt of FBPass and SBPass were estimated by gel filtration on calibrated Sephacryl S-300 and Sephadex G-100 columns (2.6 x 90 cm), respectively, equilibrated, and developed with 50 mM sodium acetate buffer (pH 5.5) containing 0.1% (v/v) 2-mercaptoethanol, 3 mM MgCl2, and 200 mM NaCl. Mol wt of NADP-MDH was estimated by chromatography on a calibrated Sephadex G-100 column essentially as described by Jacquot et al. (16). The elution buffer was buffer C supplemented with 200 mM NaCl.

**Western Blot Analysis of Electrophoresis Gel.** Gels were probed for specific proteins by using the Western blot procedures described in (20).

**RESULTS**

**STUDIES ON FRUCTOSE AND SEDOHEPTULOSE BISPHOSPHATASES**

**Preparation of FBPass and SBPass. Step 1: Preparation of Leaf Extract.** Cell-free extracts were prepared from frozen leaves (250 g) harvested in the late afternoon in order to minimize acid content as described in the preceding communication (15) except that PMSF was omitted from the extraction buffer. During the course of these experiments, it was observed that preparations made in the absence of 2-mercaptoethanol or PVP-40 and those in which the pH of the leaf extract was above 8.0 or below 5.0 retained little FBPass activity.

**Step 2: DEAE-Cellulose Column Chromatography.** The clarified amber leaf extract (900 ml) from step 1 was applied to a DEAE-cellulose column (5 x 25 cm) which had been equilibrated beforehand with 50 mM sodium acetate buffer (pH 5.5), containing 0.1% (v/v) 2-mercaptoethanol and 3 mM MgCl2 (hereafter designated buffer D). The column was washed with 500 ml buffer D and then eluted with a 0 to 500 mM NaCl linear gradient in 900 ml buffer D followed by 400 ml of 500 mM NaCl in buffer D. Fractions of 20 ml were collected during sample application and column wash. During elution of adsorbed proteins, 4.5-ml fractions were collected. Fractions were monitored for A280, and assayed for SBPass. Fractions were pooled by using the one-step colorimetric assay described in the experimental procedures.

After chromatography on DEAE-cellulose, three peaks of phosphatase activity were observed (Fig. 1): one specific for SBPass, another specific for FBPass, and a third minor peak of phosphatase activity which did not bind to the column matrix (not shown) and may contain the cytoplasmic equivalent of *Kalanchoë* FBPass.
Kalanchoë FBPase and SBPase were prepared for chromatography by DEAE-cellulose column chromatography.

Table 1. Molecular Weights of Holoenzyme and Subunits of FBPase and SBPase from Spinach, Corn, and Kalanchoë Leaves

<table>
<thead>
<tr>
<th>Holoenzyme Subunit</th>
<th>Apparent M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>No. of Subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBPase isolated from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach*</td>
<td>180,000</td>
<td>45,000</td>
</tr>
<tr>
<td>Corn*</td>
<td>184,000</td>
<td>46,000</td>
</tr>
<tr>
<td>Kalanchoë</td>
<td>180,000</td>
<td>46,000</td>
</tr>
<tr>
<td>SBPase isolated from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>76,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38,000</td>
</tr>
<tr>
<td>Corn*</td>
<td>76,000</td>
<td>38,000</td>
</tr>
<tr>
<td>Kalanchoë</td>
<td>74,000</td>
<td>38,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Taken from Nishizawa and Buchanan (19).
<sup>b</sup> Calculated from the data of Breazeale et al. (2).

The chromatographic step completely resolved SBPase from FBPase activity. SBPase eluted from the column at low salt concentrations (25–150 mM NaCl) whereas elution of FBPase required higher salt (250–350 mM NaCl) (cf. 19). Fractions containing peak activity of FBPase and SBPase (137–156 and 81–115, respectively) were pooled (157 and 90 ml, respectively) and each was concentrated to 20 ml by ultrafiltration with an Amicon YM-5 membrane.

Step 3: Sephacryl S-300 Chromatography of FBPase. The concentration containing FBPase activity (20 ml) from step 2 was clarified by high speed centrifugation (100,000g, 30 min) and applied to a pump-assisted Sephacryl S-300 superfine column (2.6 x 90 cm), equilibrated, and developed with 50 mM sodium acetate buffer (pH 5.5) containing 0.1% (v/v) 2-mercaptoethanol and 200 mM NaCl (henceforth termed buffer DD). The NaCl was included to facilitate the complete separation of FBPase from low mol wt protein contaminants (e.g. thioredoxins) which in some preparations were found to co-migrate with FBPase when chromato-
sponding enzymes from C₃ and C₄ plants. Initial studies showed two enzymes to have an absolute requirement for Mg²⁺ for activity and, consistent with findings of Gupta and Anderson (10) for the CAM enzymes and of a number of investigators for the enzymes from other sources (see 28 for references), Kalanchoe SBPase activity was also dependent on a dithiol (i.e. DTT or reduced thioredoxin). When assayed in the presence of high Mg²⁺ (see below) and DTT, the preparations were substrate-specific (data not shown). Kalanchoe FBPane and SBPane catalyzed the respective hydrolyses of FBP and SBP specifically, and showed little phosphatase activity in the presence of other sugar phosphates tested or ATP. Unlike previous studies which indicated that alkaline treatment of spinach FBPane dissociated the enzyme and induced significant levels of SBPane activity (3), alkaline treatment of Kalanchoe FBPane did not induce significant SBPane activity. A similar resistance to alkaline treatment was also observed with corn FBPane (19).

The resolution of SBPane and FBPane activity during DEAE-cellulose chromatography and the inability to induce SBPane activity in the purified Kalanchoe FBPane preparation suggests that these activities reside on separate proteins (cf. 3). If so, differential affinity for specific antibodies should then be observed as was reported for the corn enzymes (19). Here, an antibody prepared against spinach FBPane cross-reacted specifically with corn FBPane, and a corn SBPane antibody cross-reacted with spinach SBPane. When tested for cross-affinity with the Kalanchoe enzymes, these antibodies reacted as expected. Thus, the antibody against spinach chloroplast FBPane selectively inhibited Kalanchoe FBPane greater than 90% but had little influence on Kalanchoe SBPane activity (data not shown). Conversely, the corn SBPane antibody, while having little effect on FBPane, substantially diminished Kalanchoe SBPane activity. These results support the conclusion that, like spinach and corn, Kalanchoe FBPane and SBPane activity reside on distinct proteins. Other work in our laboratory has shown that the spinach FBPane antibody reacts poorly with the cytoplasmic FBPane equivalent of spinach (N. Weeden, unpublished results). The cross-reaction of the spinach chloroplast FBPane antibody with Kalanchoe FBPane suggests that the latter represents the chloroplast form of the enzyme. Unfortunately, we were not able to obtain sufficient amounts of chloroplasts from Kalanchoe to test this point with isolated organelles. Nevertheless, other lines of evidence suggest that the Kalanchoe

![Image of Western blot analysis of FBPane and SBPane obtained from spinach from spinach (Sp), corn and Kalanchoe (Kal) following SDS-PAGE.](image-url)
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Table II. Effectiveness of Thioredoxins f and m in Activation of FBPase, SBPase, and NADP-MDH from Kalanchoë Leaves

<table>
<thead>
<tr>
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<th>FBPase</th>
<th>SBPase</th>
<th>NADP-MDH</th>
</tr>
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<tbody>
<tr>
<td>Thioredoxin f</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thioredoxin m</td>
<td>7</td>
<td>57</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>43</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity, 17.5 nmol P<sub>r</sub> released/min.
<sup>b</sup> Activity, 17 nmol P<sub>r</sub> released/min.
<sup>c</sup> Activity, 7.2 nmol NADPH oxidized/min.

FBPase and SBPase isolated in these experiments were of chloroplast origin. For example, the similarity of the mol wt of these proteins, relative to their equivalents from spinach chloroplasts, is in accord with a chloroplast origin (Table I). Chloroplast FBPase and SBPase from all three sources migrated as proteins with M<sub>r</sub> = 180,000 and 76,000, respectively (cf. 19, 30). To discern subunit constituents of Kalanchoë FBPase and SBPase, we applied a Western blot procedure (20) to analyze SDS-polyacrylamide gels for antigenic proteins. As seen in Figure 2, the major antigenic band of the Kalanchoë FBPase preparation migrated as a protein with a mol wt of 45,000 in agreement with values obtained with purified preparations of spinach and corn FBPase (19). In most Kalanchoë preparations, a second antigenic band was observed with an estimated mol wt of 35,000. The relative intensity of this band increased as the preparation aged with a corresponding loss in the 45,000-D band, suggesting that the lower mol wt band represents a degradation product of Kalanchoë FBPase. Marcus et al. (18) reported that treatment of spinach FBPase with the protease subtilisin yielded a similar FBPase derivative with a mol wt 4 KD smaller than the parent enzyme. The subtilisin derivative was active but its optimum pH was shifted to the alkaline region.

When applied to the Kalanchoë SBPase, Western blot analysis indicated a subunit mol wt of 38,000, irrespective of source. No SBPase degradation product was evident in preparations tested.

Regulatory Properties of Kalanchoë FBPase and SBPase. Confident that we had isolated the chloroplast forms of Kalanchoë FBPase and SBPase, we proceeded to investigate their regulatory properties. In this respect, we examined their response to mechanisms invoked for the photoregulation of equivalent enzymes in C<sub>3</sub> and C<sub>4</sub> plants, which are activated by reduced thioredoxin and respond to changes in pH, Mg<sup>2+</sup>, and effector substrates (i.e. FBP and SBP).

Thioredoxin-Mediated Activation of Kalanchoë FBPase and SBPase. We first investigated whether Kalanchoë FBPase and SBPase were activated by reduced thioredoxin. In these experiments, we took advantage of the ability of thioredoxins to be reduced chemically by the nonphysiological reductant, DTT (5), thus providing a simple assay system. Due to the instability of Kalanchoë thioredoxins, these experiments were carried out mainly with thioredoxins obtained from spinach (cf. 19), although, as noted below, thioredoxins obtained from Kalanchoë were similarly effective. As with spinach and corn FBPase, Kalanchoë FBPase activity was stimulated 15-fold by incubation with DTT-reduced thioredoxin (see below). Kalanchoë SBPase was similarly activated by reduced thioredoxin but this enzyme exhibited much greater activation by DTT alone than did FBPase. Hypersensitivity to DTT treatment has also been reported with the spinach SBPase (2). With both the SBPase and FBPase from Kalanchoë, monothiols, such as 2-mercaptoethanol, were without effect (5).

While demonstrating thioredoxin-mediated activation of latent FBPase and SBPase activities, these experiments give no indication as to the thioredoxin functional in this process. It is well established that multiple forms of chloroplast thioredoxins exist in leaves of C<sub>3</sub> and C<sub>4</sub> plants (see preceding communication, 15). Thioredoxin m activates NADP-MDH whereas thioredoxin f stimulates the activity of SBPase, FBPase, and several other enzymes of photosynthetic and secondary metabolism. As reported in the preceding communication (15), multiple forms of thioredoxins have also been identified in Kalanchoë leaves. When tested for thioredoxin specificity, Kalanchoë FBPase and SBPase were specifically activated by thioredoxin f (Table II). These results indicate that Kalanchoë FBPase and SBPase, like their C<sub>3</sub> and C<sub>4</sub> equivalents, are thioredoxin f-linked enzymes. Other experiments revealed the two Kalanchoë bifosphatases were hysteresis enzymes (cf. 9).

Mg<sup>2+</sup>-Dependent Activation of Kalanchoë FBPase and SBPase. FBPase from both spinach and corn are activated by Mg<sup>2+</sup> concentrations greater than 10 mM in the absence of reduced thioredoxin f. Kalanchoë FBPase activity was also strongly stimulated by increased [Mg<sup>2+</sup>] (Fig. 3). Significantly, however, much higher concentrations of Mg<sup>2+</sup> were required to activate Kalanchoë FBPase relative to the enzyme from C<sub>3</sub> and C<sub>4</sub> sources (cf. 19, 23). Furthermore, the degree of activation was much less than that observed in the presence of reduced thioredoxin. In these experiments, the inclusion of reduced thioredoxin resulted in a 2-fold increase in the V<sub>max</sub> and a decrease in the apparent K<sub>m</sub> for Mg<sup>2+</sup> from 18 to 3.5 mM. Double reciprocal plots of the data presented in Figure 3 exhibited linear kinetics only in the presence of reduced thioredoxin, indicative of a thioredoxin-mediated shift from sigmoidal to hyperbolic kinetics (i.e. from allosteric to Michaelis-Menten).

Like its equivalent in C<sub>3</sub> and C<sub>4</sub> plants, Kalanchoë SBPase was
also activated by high Mg\(^{2+}\). *Kalanchoë* SBPase required 10 mm Mg\(^{2+}\) for maximal activity and was found to exhibit an apparent \(K_m\) for Mg\(^{2+}\) of 4 mm. In the absence of a di thiol, *Kalanchoë* SBPase was inactive, irrespective of the Mg\(^{2+}\) concentration up to 30 mm (see below).

Effect of pH on *Kalanchoë* FBPase and SBPase. Catalytic activities of *Kalanchoë* FBPase and SBPase were highly sensitive to changes in pH (Fig. 4). The pH optimum of both enzymes was found to be pH 8.4 to 8.8, irrespective of the preincubation condition. At pH values reputed to be physiological in illuminated chloroplasts, pH 7.8 to 8.3, (see 5 for references), the activity of both enzymes was dependent on the presence of a dithiol (i.e. DTT or reduced thioredoxin f).

While demonstrating that reduced thioredoxin effects increases in FBPase and SBPase activity at apparent physiological pH values, the above results give little indication as to the optimum pH for activation by reduced thioredoxin f. To gain information on this point, we preincubated *Kalanchoë* FBPase at different pH values in the presence or absence of reduced thioredoxin (pH for the assay of activity was consistently pH 8.0). These experiments, carried out in parallel with spinach FBPase, indicate that alkaline conditions are required for the thioredoxin-mediated activation of both enzymes. Thioredoxin-mediated activation of both spinach and *Kalanchoë* FBPase was optimal at pH values greater than pH 8.5 and negligible at pH values lower than 8.0 (Fig. 5). In these experiments, it was observed that inclusion of FBP, a positive effector of spinach FBPase, also strongly enhanced activation of *Kalanchoë* enzyme (see below).

Activation by Photochemically Reduced Thioredoxin. The results described above and in the preceding communication (15) suggest that the thioredoxin-linked activation FBPase and SBPase is of physiological significance in *Kalanchoë*. Further support for this conclusion stems from the experiments described below on FBPase and SBPase activation by photochemically reduced thioredoxin f. In these experiments, components of the ferredoxin-thioredoxin system partially purified from *Kalanchoë* were recombined to form a reconstituted system for enzyme photoactivation. A photoactivation of both the FBPase and SBPase was observed with this system (Table III). Absence of any component of the ferredoxin-thioredoxin system, or incubation in the dark, resulted in little or no activation of either enzyme. The low *Kalanchoë* SBPase activity shown in Table III is attributed, in part, to limited enzyme and substrate present in the assay mixture. Significantly, the photoactivation of *Kalanchoë* SBPase was enhanced by the addition of Ca\(^{2+}\) (50 nmol) to the reaction mixture (cf. 28) (see below).

Effect of Enzyme Effectors. The sugar bisP substrates of both FBPase and SBPase have been reported to enhance the activation of these enzymes from several different types of chloroplasts (11, 19, 26–28). When the *Kalanchoë* enzymes were tested in this regard, the appropriate sugar bisP stimulated the thioredoxin-mediated activation by 2- to 3-fold (data not shown). The substrate effect occurred irrespective of the thioredoxin concentration and, as mentioned previously, was optimal at alkaline pH values. Maximum activation of *Kalanchoë* FBPase occurred when the FBP concentration of the preincubation mixture was between 3 and 6 mm. Similar concentrations of SBP were effective in the activation of *Kalanchoë* SBPase. These experiments suggest that, like their spinach and corn equivalents, *Kalanchoë* FBPase and SBPase, are ‘turned on’ by reduced thioredoxin and are further modulated by the level of their respective substrates.

In conjunction with these experiments, we also investigated whether *Kalanchoë* FBPase responded to other enzyme effectors (P-glyceric acid, glyceraldehyde 3-P, fructose 6-P, P-enolpyruvate, OAA, malate). These compounds were without effect on the thioredoxin-activated enzyme, suggesting that CAM photosynthesis places no unique regulatory demands on the FBPase.

Effect of Divalent Cations. It has been reported that the substrate-dependent activation of both FBPase and SBPase (obtained from spinach and corn) has an absolute requirement for divalent cations such as Ca\(^{2+}\) (28). Paradoxically, higher Ca\(^{2+}\) concentrations inhibit the catalytic activity of FBPase (6). Partly because of this observation, Rosa (24) has questioned the role of Ca\(^{2+}\) in the effector-dependent modulation of FBPase. She suggested that the Ca\(^{2+}\) requirement for effector-mediated activation of FBPase may be an artifact due to the assay system in earlier studies and presented evidence suggesting that Mg\(^{2+}\) alone could mediate the FBP-dependent activation of FBPase. In view of the controversy in this area, we decided to reinvestigate the Ca\(^{2+}\) requirement on effector-mediated activation of these enzymes. As the concentra tion of Ca\(^{2+}\) required for FBPase activation is low (40 \(\mu\)M; Ref.

![Fig. 4. A, FBPase. Effect of pH and reduced thioredoxin on *Kalanchoë* FBPase activity. Conditions were as given in Table II for assay of FBPase except the pH was varied as indicated. B, SBPase. Effect of pH and reduced thioredoxin on *Kalanchoë* SBPase activity. Conditions were as given in Table II for assay of SBPase except the pH was varied as indicated.](image-url)
Fig. 5. Effect of pH on the thioredoxin-linked activation of FBPase obtained from spinach and Kalanchoë. Conditions were as given for the two-step colorimetric assay described in “Materials and Methods” except Tricine-KOH buffer concentrations in the preincubation and reaction mixture were doubled and, as indicated, the pH of the preincubation mixture was varied. The pH of the reaction mixture was 8.0. Kalanchoë and spinach FBPase (1.2 and 19 µg, respectively) were used. Thioredoxin f obtained from the same source as the target enzyme were employed (Kalanchoë thioredoxin f, 1 µg; spinach thioredoxin f, 8 µg) for the activation of FBPase.

Table III. Photoactivation of Kalanchoë FBPase and SBPase by A Reconstituted Ferredoxin/Thioredoxin System

The reaction was carried out in Warburg-Krippahl vessels. The complete system contained in the main compartment Kalanchoë FBPase (5 µg) or SBPase (5.8 µg); freshly prepared, twice-washed spinach chloroplast membrane fragments equivalent to 100 µg of Chl; Kalanchoë leaf ferredoxin (15 µg); Kalanchoë ferredoxin-thioredoxin reductase (34 µg); Kalanchoë thioredoxin f (5 µg); and the following (in µmol): Tricine-KOH buffer (pH 8.0 for assay of FBPase and pH 8.5 for assay of SBPase), 100; MgCl₂, 7.5. Fructose 1,6-bisP (6 µmol) or SBP (1.5 µmol) was added to the side arm. Final volume, 1.5 ml. Temperature, 20°C. After 10 min equilibration with N₂, the vessels were preilluminated for 10 min at 1000 µE m⁻² s⁻¹ of 400 to 700 nm light, sugar bisP substrate was added from the sidearm and the reaction was continued for 45 min. In the dark complete treatment, the vessels were kept in the dark throughout the preillumination and reaction periods. The reaction was stopped by the addition of 0.5 ml of 12% TCA, the precipitated protein was centrifuged down, and a 0.5-ml aliquot was used for the determination of Pi.

Table IV. Comparative Effects of FBP, Ca²⁺, and EGTA on the Thioredoxin-Linked Activation of FBPase from Kalanchoë and Spinach

Conditions were as given in “Materials and Methods.” Kalanchoë FBPase that was purified through the DEAE-cellulose step (25 µg) or spinach FBPase (16 µg) were used. As indicated, the preincubation mixture (0.1 ml) contained DTT (1.5 µmol), spinach thioredoxin preparation (19 µg), fructose 1,6-bisP (0.3 µmol), EGTA (50 nmol), and CaSO₄ (5 or 50 nmol). For assay of Kalanchoë FBPase, the reaction mixture was supplemented with 50 nmol EGTA.

<table>
<thead>
<tr>
<th>Preincubation Condition</th>
<th>Kalanchoë FBPase</th>
<th>Spinach FBPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>DTT, thioredoxin</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>DTT, thioredoxin, FBP</td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td>DTT, thioredoxin, FBP, Ca²⁺</td>
<td>100*</td>
<td>65</td>
</tr>
<tr>
<td>(5 nmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTT, thioredoxin, FBP, EGTA</td>
<td>46</td>
<td>3</td>
</tr>
<tr>
<td>DTT, thioredoxin, FBP, EGTA, Ca²⁺ (5 nmol)</td>
<td>52</td>
<td>3</td>
</tr>
<tr>
<td>DTT, thioredoxin, FBP, EGTA, Ca²⁺ (50 nmol)</td>
<td>99*</td>
<td>100*</td>
</tr>
</tbody>
</table>

* Activity, 8 nmol NADP reduced/min.
* Activity, 41 nmol NADP reduced/min.

was sensitive to EGTA treatment, and could be overcome by increasing the Ca²⁺ content of the preincubation solution. In parallel experiments, Mg²⁺ (5 mM) was ineffective in reversing the inhibition effected by EGTA.

During these experiments, we observed that the time required for FBPase activation by Ca²⁺ and FBP appears to be a function of preincubation conditions. After preincubation at pH 8.5 in the presence of reduced thioredoxin, FBPase exhibited an increased sensitivity to FBP in which the lag time required for FBP-dependant activation decreased to less than 1 min (data not shown). Unlike at more acidic pH, effector-mediated activation after pre-
The activation of *Kalanchoe* leaf NADP-MDH by reduced thioredoxin. The complete system contained *Kalanchoe* leaf extract (5 μg protein), spinach thioredoxin preparation containing both m- and f-type activities (16 μg), DTT (1.5 μmol), and Tris-HCl buffer (pH 7.9; 20 μmol). After 5-min preincubation, activity was measured as described in “Materials and Methods.”

Treatment at pH 8.5 was relatively insensitive to EGTA treatment. This increase in sensitivity to FBP under alkaline conditions may account for the dramatic increase in apparent thioredoxin-mediated activation of FBPase observed in this pH range in the absence of added Ca^{2+}. The data thus demonstrate that the pH for activation of FBPase strongly influences its response to FBP substrate and Ca^{2+}. These findings are also consistent with the conclusion that Ca^{2+} is more effective as activator of chloroplast FBPase than is Mg^{2+} (11, 24).

**STUDIES ON NADP-MDH**

Thioredoxin-Linked Regulation of NADP-MDH. We initiated studies on *Kalanchoe* NADP-MDH by investigating whether this enzyme is activated via reduced thioredoxin like the corresponding enzyme of C_{3} and C_{4} plants (cf. 16, 25). As shown in Figure 6, *Kalanchoe* leaf extracts catalyzed the OAA-dependent oxidation of NADPH, an activity indicative of NADP-MDH. The activity was latent, requiring reduced thioredoxin before becoming apparent. In the absence of reduced thioredoxin, less than 10% of the maximum NADP-MDH activity was observed. Under optimal conditions, the NADP-MDH activity was 116 μmol NADPH oxidized/mg protein·h. In parallel experiments, the NADH-linked oxidation of OAA, which was not affected by reduced thioredoxin, was 50 times higher than the activity with NADPH.

To remove contaminating thioredoxins and malic enzyme and, thereby, to characterize the thioredoxin-dependent activation of *Kalanchoe* NADP-MDH, we partially purified the enzyme by chromatography on DEAE-cellulose and Sephadex G-100 columns. Elution profiles of both chromatographic steps showed a single peak of NADP-MDH activity which, like crude preparations, required reduced thioredoxin for activity. The activity of purified NADP-MDH was enhanced 20-fold by incubation with reduced thioredoxin m, similar to results obtained with NADP-MDH from other plants (data not shown). Activation was observed with thioredoxin m isolated from either spinach or *Kalanchoe*.

**STUDIES ON PHOSPHOFRUCTOKINASE**

PFK of C_{3} plants has been shown to respond to several metabolites in a manner consistent with a function in the photoregulation of this enzyme (17). A recent report suggests that products of photosynthetic electron transport may be involved in photo-inhibition of PFK in intact chloroplasts (12). Evidence was obtained independently that one such product, NADPH, may function in the photoregulation of PFK. At concentrations calculated to be present in illuminated chloroplasts (about 0.5 mm), NADPH inhibited spinach PFK activity by 75% (8). Because no studies of this type had been performed with CAM preparations, we tested the effect of NADPH on *Kalanchoe* PFK as described in (8). As with the spinach enzyme, NADPH concentrations greater than 0.25 mm significantly inhibited *Kalanchoe* PFK, i.e. 0.5 and 1 mm NADPH effected respective 40 and 60% inhibition of the enzyme (cf. 8). Also analogous to the case with spinach, fructose-2,6-bisP was without effect on *Kalanchoe* PFK (7, 8).

**DISCUSSION**

The present results provide evidence that FBPase, SBPase, NADP-MDH, and PFK have the capacity to be photoregulated in *Kalanchoe*. Each of these enzymes was found to share a number of structural and regulatory properties with its chloroplast equivalent of spinach and corn, including properties relevant to modulation by light. *Kalanchoe* FBPase and SBPase were activated by thioredoxin f, and reduced either chemically by DTT or photochemically by means of a reconstituted *Kalanchoe* ferredoxin/thioredoxin system. These enzymes could also be activated by high Mg^{2+} concentrations, but, significantly, substantially higher Mg^{2+} concentrations were required relative to the corresponding enzymes of C_{3} and C_{4} plants. In addition, in the present case, the activation due to high Mg^{2+} concentration was strongly enhanced by diethiothreitol (e.g. reduced thioredoxin). In related experiments, *Kalanchoe* NADP-MDH was shown to be activated by reduced thioredoxin m in a manner analogous to the spinach and corn enzymes.

Like their counterparts in spinach and corn chloroplasts, *Kalanchoe* FBPase and SBPase were activated synergistically by reduced thioredoxin and their sugar bisP substrates. The substrate-dependent activation of *Kalanchoe* FBPase at physiological pH required divalent cations, of which Ca^{2+} was most effective. Related experiments indicate that *Kalanchoe* FBPase and SBPase have certain physical and chemical properties in common (mol wt, immunological activity, Mg^{2+} requirement) with their spinach and corn equivalents. Thus, it seems likely that FBPase and SBPase and their mode of regulation are similar in representative higher plants showing different types of photosynthesis (C_{3}, C_{4}, and CAM).

Subsequent experiments have given new information on the photoregulation of FBPase. Thioredoxin-mediated activation of FBPase, obtained from both *Kalanchoe* and spinach, was optimum at alkaline pH values (pH > 8.2). At these pH values, substrate-mediated activation of FBPase was enhanced and, in contrast to lower pH, no clear role for Ca^{2+} could be demonstrated. These experiments suggest that stromal pH plays a critical role in the manner by which reduced thioredoxin brings about FBPase activation. Therefore, it becomes of interest to learn whether light effects an increase in stromal pH of *Kalanchoe* chloroplasts similar to that found in spinach.

The studies reported here and in the companion article (15) jointly provide evidence for specific mechanisms involved in the segregation of synthetic and degradative pathways of CAM photosynthesis. The results suggest that glucan metabolism of CAM plants is regulated by the same mechanisms proposed to function in the regulation of C_{3} and C_{4} photosynthesis (i.e. increased pH, Mg^{2+}, reduced thioredoxins, and metabolites such as NADPH). These mechanisms enable light to enhance synthetic activity via an activation of key enzymes (e.g. FBPase and SBPase of the reductive pentose-P pathway and gluconeogenesis). In conjunction with the stimulation of biosynthesis, degradative pathways such as glycolysis are blocked due to PFK photoinhibition. In the dark,
these increases in specific ions, metabolites, and reductants abate, thereby effecting a reversal of the carbon flux and allowing degradative processes to occur. It remains to be seen how these processes, which take place in the chloroplast, interface with essential processes taking place in the cytoplasm, e.g. the dark synthesis of malate, which distinguish CAM from other types of plants.

LITERATURE CITED

1. ANDERSON LE, HM CHEN, VK GUPTA 1979 Modulation of chloroplast fructose 1,6-bisphosphatase activity by light. Plant Physiol 64: 491-494
2. BREAZEALE VD, BB BUCHANAN, RA WOLOSIUK 1978 Chloroplast sedoheptulose 1,7-bisphosphatase: Evidence for regulation by the ferredoxin/thioredoxin system. Z Naturforsch Teil C 33: 521-528
3. BUCHANAN BB, P SCHURMANN, RA WOLOSIUK 1976 Appearance of sedoheptulose 1,7-diphosphatase activity on conversion of chloroplast fructose 1,6-diphosphatase from dimer form to monomer form. Biochem Biophys Res Commun 69: 970-978
7. CIŚKA C, NF WEEDEN, BB BUCHANAN, K UYEDA 1982 A special fructose bisphosphatase functions as a cytoplasmic regulatory metabolite in green leaves. Proc Natl Acad USA 79: 4322-4326

301-302
22. PLA A, J LOPEZ-GORGE 1981 Thioreredoxin/fructose 1,6-bisphosphatase affinity in the activation by the ferredoxin/thioredoxin system. Biochim Biophys Acta 636: 100-105