

Activation of a Chloroplast Type of Fructose Bisphosphatase from *Chlamydomonas reinhardtii* by Light-Mediated Agents

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Chlamydomonas reinhardtii, Fructose-1,6-Bisphosphatase, Green Algae, Thioredoxin

A chloroplast type of fructose-1,6-bisphosphatase, a central regulatory enzyme of photosynthetic carbon metabolism, has been partially purified from *Chlamydomonas reinhardtii*. Unlike its counterpart from spinach chloroplasts, the algal FBPase showed a strict requirement for a dithiol reductant irrespective of Mg^{2+} concentration. The enzymes from the two sources resembled each other immunologically, in subunit molecular mass and response to pH. In the presence of dithiothreitol, the pH optimum for both the algal and spinach enzymes shifted from 8.5 to a more physiological value of 8.0 as the Mg^{2+} concentration was increased from 1 to 16 mM. At 1 mM Mg^{2+} , a concentration estimated to be close to physiological, the *Chlamydomonas* FBPase was active only in the presence of reduced thioredoxin and was most active with *Chlamydomonas* thioredoxin *f*. Under these conditions, the enzyme showed a pH optimum of 8.0. The data suggest that the *Chlamydomonas* enzyme resembles its spinach counterpart in most respects, but it has a stricter requirement for reduction and less strict reductant specificity. A comparison of the properties of the FBPases from *Chlamydomonas* and spinach will be helpful for elucidating the mechanism of the reductive activation of this enzyme.

Introduction

A key regulatory step in photosynthetic carbon assimilation is the conversion of Fru-1,6- P_2 to Fru-6-P via chloroplast fructose-1,6-bisphosphatase (FBPase), an enzyme that is active in the light and less active in the dark [1]. This differential activity is essential to maintaining the flow of carbon under light and dark conditions. If chloroplast FBPase were active in the dark, the futile cycle created would interrupt the export of carbon needed for survival of the plant [2, 3].

A number of studies have focussed on three light-mediated regulators of the activity of chloroplast FBPase: Mg^{2+} concentration, pH, and reduction via thioredoxin. None of these factors acts as the sole modulator of FBPase under physiological conditions. Although high Mg^{2+} concentrations activate FBPase, the activity of the enzyme at 1–3 mM Mg^{2+} – the

estimated concentration in illuminated chloroplasts [4] – is insufficient to account for the rate of photosynthesis [5]. Similarly, although the stroma becomes more alkaline upon illumination, changing approximately from pH 7.0 to 8.0 [6], the FBPase is not active below pH 8.4 at physiological Mg^{2+} concentrations [7]. When assayed at physiological pH and Mg^{2+} concentrations, the FBPase requires reduction to reach full activity [7]. *In vivo*, it is generally believed that FBPase is reduced by thioredoxin [1, 8–10].

Thioredoxin is part of a regulatory thiol redox chain with links light driven non-cyclic electron flow to the reduction of FBPase and other regulatory enzymes of chloroplasts [11–13]. Two types of thioredoxins have been identified in chloroplasts, thioredoxins *m* and *f*, that differ in their specificity for target enzymes. Thioredoxin *f* activates FBPase more effectively than thioredoxin *m*, and, under assay conditions with NaCl, thioredoxin *m* activates NADP-malate dehydrogenase (NADP-MDH) more effectively than thioredoxin *f* [14, 15]. Both thioredoxins are reduced by ferredoxin via the iron sulfur enzyme, ferredoxin-thioredoxin reductase (FTR) [11, 12, 16].

Our understanding of chloroplast FBPase has increased greatly since the recognition of its importance in photosynthesis. It is interesting, though, that

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBPase, fructose-1,6-bisphosphatase; Fru-1,6- P_2 , fructose-1,6-bisphosphate; Fru-6-P, fructose-6-phosphate; FTR, ferredoxin-thioredoxin reductase; Pi, phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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while elucidation of its central role stemmed from studies of unicellular green algae, little information is available about FBPase from these organisms. We decided, therefore, to determine if FBPase from *Chlamydomonas reinhardtii*, a representative member of the unicellular green algae, is regulated by mechanisms similar to its counterpart from spinach chloroplasts. We have partially purified the chloroplast FBPase from *Chlamydomonas* and investigated its dependence on Mg^{2+} , pH and thioredoxin. We report here that, while resembling its spinach counterpart in most respects, the algal FBPase shows differences which could prove useful in elucidating the general mechanism of regulating photosynthetic FBPase.

Methods

Plant material

Chlamydomonas reinhardtii cc-124 mt- (Levine) cultures were obtained from Dr. Elizabeth Harris, Duke University. Cells were grown photoautotrophically in 20 l carboys for 7 to 10 days [17] and harvested by continuous centrifugation. The packed pellet was used fresh because freezing the cells seemed to decrease extractable FBPase activity.

Reagents and proteins

Biochemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Reagents used in electrophoresis and Western blot analysis were purchased from BioRad Laboratories (Richmond, Cal.). DEAE cellulose (DE-52) was purchased from Whatman, Inc.; all other column materials were purchased from Pharmacia LKB Biotechnology, Inc. Spinach chloroplast FBPase was prepared according to the method of Nishizawa *et al.* [18]. Spinach thioredoxins were purified according to Crawford *et al.* [19]. All antibodies were gifts from N. Crawford of this laboratory. *Chlamydomonas* thioredoxins were purified as detailed by Huppe *et al.* [20]. Dr. D. LeMaster (Yale University) kindly provided the thioredoxin from *E. coli*. Other reagents and supplies were purchased from commercial sources and were of highest quality available.

Purification procedure

Chlamydomonas FBPase was prepared by a modification of the method used for spinach chloroplast

FBPase [18]. To extract the enzyme, approximately 30 g of freshly harvested cells were resuspended to a total volume of 100 ml using 30 mM Tris-HCl buffer (pH 7.9). The cell suspension was mixed 1:1 (v/v) with 0.5 mm glass beads and blended 3 times for 1 min using a Biospec Products Bead Beater (Bartlesville, Okla.) fitted with an ice/water bath cooled container. The suspension was filtered through Miracloth to remove the beads. The filtrate was adjusted to pH 4.8 with 2 N formic acid. The precipitate formed was pelleted by centrifugation ($10,000 \times g$ for 10 min) and used below.

The pellet from the acid precipitation step was resuspended in 500 ml of Tricine-KOH (pH 7.6) to which 5 mM EDTA was added. After stirring overnight, the sample was centrifuged ($15,000 \times g$ for 10 min) and FBPase was recovered in the supernatant fraction. The sample was brought to 45% saturation with ammonium sulfate and the pellet was removed by centrifugation ($15,000 \times g$ for 10 min). Ammonium sulfate was added to the supernatant solution to 85% saturation and the pellet, containing the bulk of the FBPase activity, was collected by centrifugation as before. The pellet was resuspended and dialyzed against 50 mM sodium acetate buffer (pH 5.5) (Buffer A) for 10 h. The dialyzed sample was applied to a DE-52 column equilibrated with Buffer A and eluted with a 0–500 mM NaCl gradient. Fractions containing FBPase activity were pooled, concentrated by ultrafiltration and chromatographed on a Sepharose S-300 column (2.5×90 cm) equilibrated with Buffer A containing 200 mM NaCl. The active fractions were combined, concentrated by ultrafiltration and used without further treatment. The preparation could be stored at -20°C at this stage without significant loss of activity. When related to pure spinach chloroplast FBPase, we obtained the equivalent of 2 μg of FBPase per gram of *Chlamydomonas* wet cells paste (see below).

Analytical procedures

Activity assays

FBPase activity was determined by a colorimetric Pi assay in which the enzyme is activated by either dithiothreitol (DTT)-reduced thioredoxin or high (16 mM) Mg^{2+} concentration [18]. With thioredoxin, the enzyme was activated in the following solution (0.5 ml final vol.): 100 mM Tris-HCl (pH 7.9),

2.5 mM DTT, 1.0 mM MgSO_4 , and thioredoxin as indicated. After 10 min, 6 mM FBP was added to start the reaction. The reaction proceeded for 20 min and then was stopped by the addition of 2 ml of the solution used for analyzing phosphate (Pi mix). After allowing 10 min for color development, the absorbance was read at 660 nm. Control assays without thioredoxin were run simultaneously. Unless otherwise noted, the activities reported reflect the difference in absorbance between the experimental and control assays. Also unless indicated otherwise, assays in which FBPase was activated by Mg^{2+} were performed in the same solution, except that thioredoxin was eliminated and MgSO_4 concentrations were varied up to 16 mM.

Because the *Chlamydomonas* FBPase was not pure, its content was estimated by measuring its activity in relation to that of pure spinach FBPase in an assay containing 2.5 mM DTT and 16 mM Mg^{2+} at pH 8.0. Unless otherwise noted, we used, as indicated, either 3 μg of spinach FBPase or an amount of *Chlamydomonas* FBPase that showed comparable activity.

Gel electrophoresis and Western blots

Gradient gels (10–17.5%) were prepared according to Laemmli [21] and, following development, were stained with Coomassie Blue R-250 (1.0%, w/v, dissolved in 40%, v/v, methanol and 10%, v/v, acetic acid).

SDS-PAGE gels were routinely transferred to nitrocellulose in a solution containing 25 mM Tris (Trizma), 192 mM glycine and 20% (v/v) methanol (pH 8.3) by applying an electric field of 60 V for 4 h [22]. After transfer was complete, the nitrocellulose was blocked with a solution containing 20 mM Tris-HCl buffer (pH 7.6), 500 mM NaCl and 1% bovine serum albumin (BSA) (Buffer B) for 1 h at 25 °C. Approximately 7.7 μg of an antibody affinity purified against spinach FBPase [13] was diluted into Buffer B and incubated with the blot for 4 h at 25 °C. Blots were washed 4 times for 20 min per wash with 100 ml of modified Buffer B in which 3% non-fat powdered milk replaced the BSA and to which 0.05% Tween-20 was added. The secondary antibody, goat anti-rabbit IgG coupled with horseradish peroxidase, was diluted 1 to 3000 in Buffer B and incubated with the blot for 1 h. After washing as before, cross-reactivity was visualized by reacting the

blot in 120 ml of a solution containing 20 mM Tris-HCl (pH = 7.6), 150 mM NaCl and 17% methanol; 60 μl of 30% hydrogen peroxide and 60 mg of BioRad HRP Color Development reagent were added to the solution just prior to use.

Results

Purification of the fructose-1,6-bisphosphatase

Chlamydomonas FBPase, purified over 200-fold by modification of a standard procedure, behaved similarly to the spinach chloroplast FBPase during purification [18]. The enzyme precipitated with the green membrane fraction upon acidification of the cell extract and was then solubilized with buffer containing EDTA. This behavior differs from the FBPase of *Euglena*, another unicellular alga, which remained in the supernatant fraction after acid precipitation ([23]; P. Schürmann, personal communication). The degree to which FBPase associates with thylakoid membranes during extraction has been used to indicate its possible association with membranes *in vivo* [24, 25]; the behavior of these two algal FBPases during purification suggests that they associate differently with photosynthetic membranes. As the *in vitro* activity of the FBPase is affected by the hydrophobicity of its environment, such membrane association may be significant in contributing to the *in vivo* activity of the enzyme [15, 26].

Chlamydomonas FBPase was purified by successive chromatography on DEAE cellulose and Sephacryl S-300 (both at pH 5.5). Unlike the spinach FBPase, which is essentially homogeneous after these steps, the *Chlamydomonas* FBPase was approximately 50% pure, with numerous minor contaminants evident on a Coomassie stained SDS-PAGE gel (data not shown). The enzyme, however, was sufficiently pure at this stage to be used for the experiments below.

Subunit size

The *Chlamydomonas* FBPase subunit was identified and its size estimated by comparative analysis using SDS-PAGE and Western blot procedures. Fig. 1 shows a Western blot probed with affinity purified antibody to FBPase from spinach chloroplasts. The antibody reacted with a single band in the *Chlamydomonas* preparation that corresponded to the subunit

Western Blot Analysis with Antibody to Spinach Chloroplast FBPase



Fig. 1. Identification of the subunit of spinach (S) and *Chlamydomonas* (C) FBPases with antibodies to spinach FBPase. The lanes contained 1 μ g spinach FBPase or an amount of *Chlamydomonas* FBPase with equivalent activity.

size of the spinach enzyme. This finding suggests that the two FBPases are comprised of a subunit of similar mass, estimated to be 40 kDa [5, 18]. The difference in intensity between the spinach and the *Chlamydomonas* subunits indicates that while there is significant homology between the two enzymes there are also structural differences. Further details of these differences await future experiments.

Requirement for reduction

We found no FBPase activity in *Chlamydomonas* extracts unless the dithiol reductant, DTT, or reduced thioredoxin was included in the assay. Unlike the spinach enzyme which was active at Mg^{2+} concentrations greater than 4 mM without DTT, *Chlamydomonas* FBPase required DTT at all Mg^{2+} concentrations tested (Fig. 2). When DTT was present, *Chlamydomonas* FBPase was active at lower Mg^{2+} concentrations than the spinach enzyme. This response to DTT is similar to the sedoheptulose-1,7-bisphosphatase (SBPase) from spinach which is activated by DTT alone under certain conditions [27]. The *Chlamydomonas* FBPase, however, was considerably more active in the presence of thioredoxin

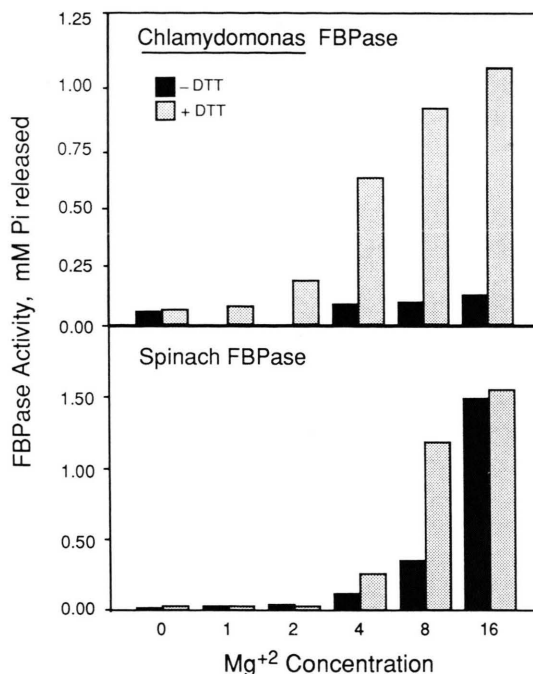


Fig. 2. Requirement of *Chlamydomonas* FBPase for a dithiol reductant. Values reflect the total (uncorrected) Pi released.

(Table I). The finding of an FBPase which requires reduction for activity, even when saturating concentrations of Mg^{2+} are present, supports the conclusion that changes in Mg^{2+} and pH cannot be the exclusive factors controlling the activity of this enzyme *in vivo* [28, 29].

At physiological Mg^{2+} concentrations, estimated to be 1–3 mM in the light [4], the algal FBPase and its spinach counterpart, were more active when reduced thioredoxin *f* was present during incubation (Table I). Regulation by reduced thioredoxin in-

Table I. Thioredoxin requirement for activation of *Chlamydomonas* and spinach chloroplast FBPases in the presence of DTT.

[Mg^{2+}]	FBPase activity [μ M Pi/min]*			
	<i>Chlamydomonas</i> +Trx	<i>Chlamydomonas</i> -Trx	Spinach +Trx	Spinach -Trx
0	2.0	1.6	1.0	0.6
1	7.0	2.2	10.0	1.0
2	14.3	5.2	20.0	2.8
4	37.8	17.5	71.3	7.5

* As indicated, 12.5 μ g of a mixture of spinach thioredoxin *m* and *f* (Trx) was added.

volves the reduction of a disulfide bridge which induces a conformational change that supports catalysis [7, 11, 12, 26]. Binding studies and sequence data obtained for the spinach FBPase suggest that the thioredoxin regulatory site is distinct from the catalytic site [30–32]. High Mg^{2+} concentrations cause sufficient conformational change in the spinach enzyme so catalysis can occur without reduction. That *Chlamydomonas* FBPase required reduction for activation, even at high Mg^{2+} concentrations, suggests that the catalytic site of the algal enzyme may be more deeply buried than that of the spinach FBPase. The relation of the reduction requirement to the mechanism of activation in *Chlamydomonas* FBPase awaits further information on its sequence and on the conformational changes that accompany its activation.

Effect of pH

Fig. 3 details the effect of pH on *Chlamydomonas* and spinach chloroplast FBPases which were incubated in the presence of 2.5 mM DTT (without

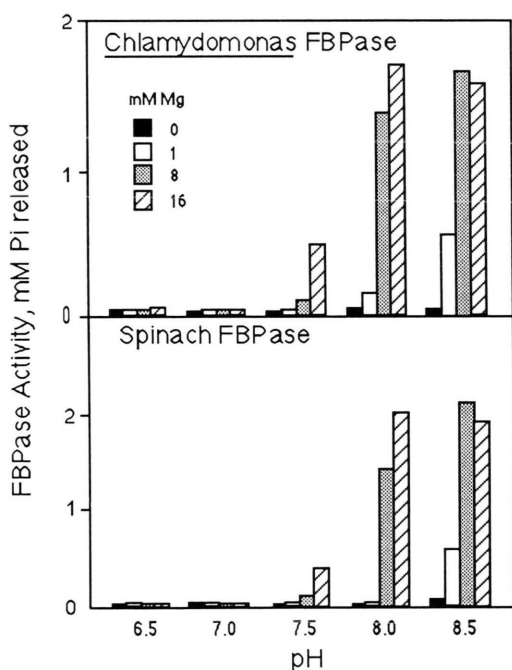


Fig. 3. Effect of pH and Mg^{2+} concentration on the activity of *Chlamydomonas* and spinach FBPases measured in the presence of DTT. Assays were conducted as described in Analytical Procedures except that the pH and Mg^{2+} concentrations were varied as indicated. Values reflect the total (uncorrected) Pi released.

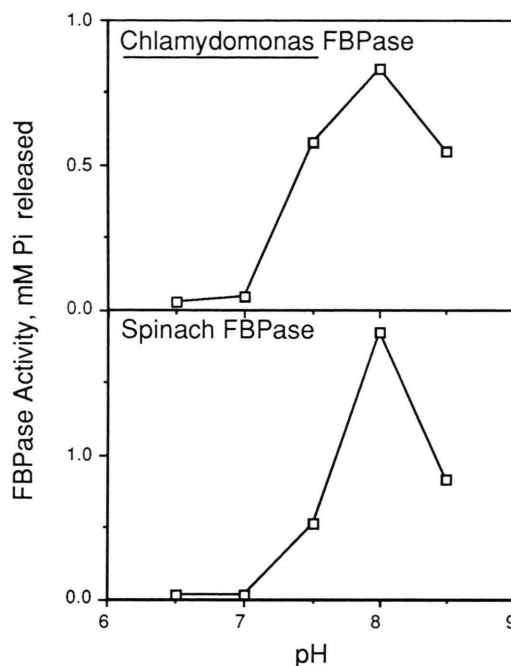


Fig. 4. Effect of pH on the activity of *Chlamydomonas* and spinach FBPases measured in the presence of reduced thioredoxin. A mixture of spinach thioredoxin *m* and *f*, 12.5 μ g, was added in each case.

thioredoxin) and varying concentrations of Mg^{2+} . Neither the spinach nor the *Chlamydomonas* enzyme was active below pH 7.5 at any Mg^{2+} concentration tested, and at pH 7.5, the FBPases were significantly active only at 16 mM Mg^{2+} — a concentration well above that considered physiological. At the more physiological concentration of 1 mM Mg^{2+} , activity was observed only when the pH was raised to 8.5, and even then, was only about 30% of that found with 16 mM Mg^{2+} . As has been reported for FBPase from spinach chloroplasts [7, 33] and cyanobacteria [34], the pH optimum shifted to more acidic values as the concentration of Mg^{2+} increased. It should be noted that while the pH optimum at 16 mM Mg^{2+} was close to the estimated pH in chloroplasts in the light (pH 8.0) [6], the pH optimum was considerably higher (pH 8.5 or higher) at more physiological levels of Mg^{2+} (1 mM).

Fig. 4 summarizes the pH dependency of FBPase assayed at 1 mM Mg^{2+} in the presence of DTT-reduced thioredoxin *f*. The pH optimum with thioredoxin activated enzyme was about 8.0 for the FBPases from both *Chlamydomonas* and spinach — *i.e.*,

values near the physiological pH of illuminated chloroplasts (cf. [7]).

Thioredoxin specificity

Spinach chloroplast FBPase shows a high degree of specificity for *f*-type thioredoxins [15, 35]. To determine the thioredoxin specificity of the *Chlamydomonas* FBPase, we compared its activity following activation with several different thioredoxins (Table II). As expected, the *f*-type thioredoxins from either *Chlamydomonas* or spinach were most effective in FBPase activation. The *Chlamydomonas* FBPase, however, showed a limited, but significant activation by *m*-type and *E. coli* thioredoxins under conditions in which spinach FBPase was not affected. In agreement with the antibody evidence, these findings suggest that there are structural differences between the algal and spinach enzymes.

It has been found that thioredoxin specificity of spinach chloroplast FBPase can be modified by including FBP and Ca^{2+} during activation [35]. Under these conditions, high concentrations of *m*-type and *E. coli* thioredoxins were effective in activation, although less so than thioredoxin *f*. Our data show that even without the inclusion of FBP or Ca^{2+} , *Chlamydomonas* FBPase can be activated, albeit less so, by thioredoxins other than the *f*-type.

Table II. Activation of *Chlamydomonas* and spinach chloroplast FBPase by various thioredoxins*.

Thioredoxin	FBPase activity [$\mu\text{M Pi/min}$]	
	<i>Chlamydomonas</i>	Spinach
<i>Chlamydomonas f</i>	67.8	45.7
<i>Chlamydomonas m1</i>	12.1	1.8
<i>Chlamydomonas m2</i>	12.2	1.8
Spinach <i>f</i>	55.4	95.6
Spinach <i>m</i>	8.6	3.9
<i>E. coli</i>	13.6	3.6
No thioredoxin	6.0	1.6

* The thioredoxins used were more than 95% pure; in each case, 2.5 μg protein was added.

The data in Table II also show that both spinach and *Chlamydomonas* FBPases were better activated by the autologous rather than the heterologous thioredoxin *f*. We further investigated this specificity by comparing FBPase activation at various concentrations of the two thioredoxins (Fig. 5). Although

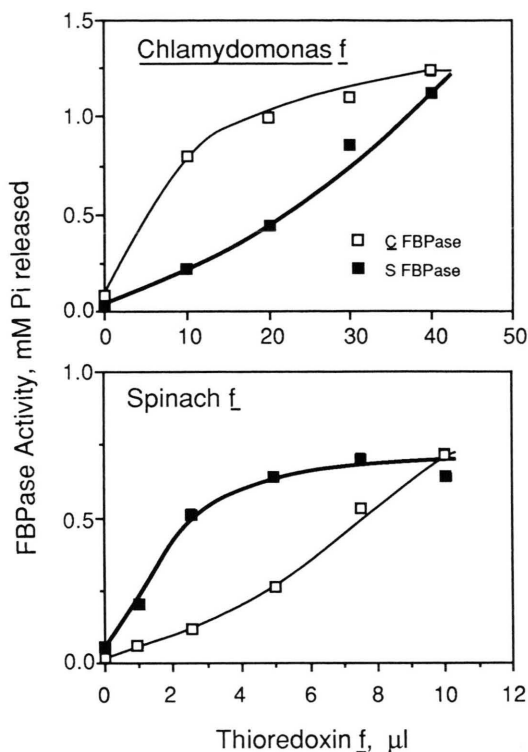


Fig. 5. Response of *Chlamydomonas* (C) and spinach (S) FBPases to autologous and heterologous thioredoxin *f*. Thioredoxin samples were partially purified and contained no thioredoxin *m* activity. Values reflect the total (uncorrected) Pi released.

FBPase from both sources was activated by the heterologous thioredoxin, the amount required for half-maximal activation was higher and the response curves differed, being sigmoidal with the heterologous interaction and hyperbolic with the autologous protein. Similar responses were observed with the activation of spinach FBPase by thioredoxins *f* from corn and spinach [15]. The significance of this difference may relate to the mechanism of interaction between the thioredoxin and its target site. Further information on this interaction will likely be gained from structural analysis of the thioredoxins and target enzymes from other plants.

Conclusion

FBPase, partially purified from *Chlamydomonas*, was structurally and antigenically related to its spinach chloroplast FBPase counterpart. The *Chlamydomonas* FBPase differed from the spinach enzyme by requiring a dithiol reductant (reduced thio-

redoxin or a non-physiological equivalent such as DTT) for activation in the presence of high (8 to 16 mM) Mg^{2+} concentrations. At 1–3 mM Mg^{2+} , a concentration considered to be physiological, the enzyme was active only in the presence of reduced thioredoxin, preferably of the *f*-type.

The response of *Chlamydomonas* FBPase to pH resembled that observed previously for the enzyme from other sources. *Chlamydomonas* FBPase was not active below pH 7.5 under any conditions tested. Its pH optimum showed a shift downward with increasing concentrations of Mg^{2+} , and, when activated by reduced thioredoxin, was about 8.0.

Chlamydomonas FBPase differed significantly from its spinach counterpart in thioredoxin specificity. Although the *f*-type thioredoxins were considerably more effective, *Chlamydomonas* FBPase was activated to some extent by *E. coli* and *m*-type thioredoxins. The specificity for thioredoxin *in vitro* has been shown to depend on assay conditions [35]. Why certain conditions are necessary for the spinach

enzyme, but not the *Chlamydomonas* enzyme may prove interesting in understanding the interaction of thioredoxin with its target site. This is particularly true in view of the large heterogeneity between *m* and *f* thioredoxins [36].

In summary, while the *Chlamydomonas* FBPase resembles its spinach counterpart in most respects, it apparently has a stricter requirement for reduction and a less strict specificity for thioredoxin. The interplay of reduction, Mg^{2+} concentration and pH for activation reveals that no single factor can serve as the sole regulator of this enzyme. These findings suggest that comparative studies on the regulation and structure of chloroplast FBPases will be helpful to our understanding of this important enzyme.

Acknowledgements

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