Oxidation−Reduction and Activation Properties of Chloroplast Fructose 1,6-Bisphosphatase with Mutated Regulatory Site[†]

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ABSTRACT: The concentration of Mg^{2+} required for optimal activity of chloroplast fructose 1,6-bisphosphatase (FBPase) decreases when a disulfide, located on a flexible loop containing three conserved cysteines, is reduced by the ferredoxin/thioredoxin system. Mutation of either one of two regulatory cysteines in this loop (Cys155 and Cys174 in spinach FBPase) produces an enzyme with a $S_{0.5}$ for Mg^{2+} (0.6 mM) identical to that observed for the reduced WT enzyme and significantly lower than the $S_{0.5}$ of 12.2 mM of oxidized WT enzyme. E_m for the regulatory disulfide in WT spinach FBPase is −305 mV at pH 7.0, with an E_m vs pH dependence of −59 mV/pH unit, from pH 5.5 to 8.5. Aerobic storage of the C174S mutant produces a nonphysiological Cys155/Cys179 disulfide, rendering the enzyme partially dependent on activation by thioredoxin. Circular dichroism spectra and thiol titrations provide supporting evidence for the formation of nonphysiological disulfide bonds. Mutation of Cys179, the third conserved cysteine, produces FBPase that behaves very much like WT enzyme but which is more rapidly activated by thioredoxin *f*, perhaps because the E_m of the regulatory disulfide in the mutant has been increased to −290 mV (isopotential with thioredoxin *f*). Structural changes in the regulatory loop lower $S_{0.5}$ for Mg^{2+} to 3.2 mM for the oxidized C179S mutant. These results indicate that opening the regulatory disulfide bridge, either through reduction or mutation, produces structural changes that greatly decrease $S_{0.5}$ for Mg^{2+} and that only two of the conserved cysteines play a physiological role in regulation of FBPase.

Fructose-1,6-bisphosphatase (FBPase)[1] hydrolyzes fructose-1,6-bisphosphate to fructose-6-phosphate and phosphate. Two isoforms of this enzyme are present in plants: a cytosolic and a chloroplastic form. The cytosolic FBPase is involved in gluconeogenesis and regulated by AMP and divalent cations. The chloroplast FBPase is a key enzyme of the Calvin cycle and regulated by light as well as by Mg^{2+}, substrate, and pH. The regulation by light is achieved through the ferredoxin/thioredoxin system. The FBPase is activated by reduction of a regulatory disulfide through reduced thioredoxin *f* (Trx *f*), which in turn is reduced by electrons from the photosynthetic electron flow through ferredoxin: thioredoxin reductase (*1, 2*).

Chloroplast FBPases are homotetrameric enzymes of about 160 kDa. The chloroplast enzymes for which sequences are known (pea, rapeseed, soybean and spinach) possess only seven cysteine residues per subunit, all of which are conserved and located in the mature form of the polypeptide that results from cleavage of the transit peptide. Amino acid sequence alignments of known chloroplastic and cytosolic FBPases show that the light-regulated forms have an insertion of about 15 amino acids, containing three of the conserved cysteines. Two of them, separated by only four residues, were first proposed to be involved in regulatory processes (*3*). The crystal structure determined for the spinach chloroplast FBPase (*4*) provided no evidence for a disulfide bridge, but clearly showed that the amino acid insertion is located on a flexible loop exposed on the surface of the protein (170s loop). Analysis of pea chloroplast FBPase by site-directed mutagenesis, where the three cysteines were replaced one by one by serine, assigned a central role to Cys153 in regulation. Replacement of this cysteine led to a constitutively active FBPase, whereas mutation of Cys173 or Cys178 produced a partially active enzyme, which still required reduction for full activity. In addition, the properties of the C49S and C190S mutants clearly showed that these two residues are not involved in redox regulation (*5*). Similar

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[1] Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FBPase, fructose 1,6-bisphosphatase; mBBr, monobromobimane; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; TEA, triethanolamine; TNB⁻, 5-thio-2-nitrobenzoate anion; Tricine, *N*-tris(hydroxymethyl)methylglycine; Trx *f*, thioredoxin *f*; Trx *m*, thioredoxin *m*.

experiments performed with rapeseed chloroplast FBPase (*6*) confirmed the importance of the Cys in the redox regulatory loop that is closest to the N-terminus. These experiments also demonstrated that the mutations significantly decreased the $S_{0.5}$ of both the oxidized and reduced forms of the enzyme for Mg^{2+}. However, the results obtained with the mutants of the pea and rapeseed enzymes did not allow the unequivocal identification of a specific second cysteine involved in the regulatory disulfide bond. Instead, the experiments suggested the involvement of all three cysteines in regulation.

More recent crystallographic data (*7*), obtained with oxidized pea chloroplast FBPase, provide evidence for a disulfide bond between Cys153 and Cys173 (corresponding to Cys155 and Cys174 in spinach). The authors propose that this bond represents the regulatory disulfide. It is thought to stabilize the inactive form of the enzyme by displacing the glutamate coordinating the Mg^{2+} ion involved in catalysis from its correct position. It has been proposed that this occurs through the movement of the N-terminal β-strands toward the active site about 20 Å away. Upon reduction by Trx *f*, the regulatory loop is probably relaxed, allowing the active site to adopt a competent conformation (*2*). However, structural analysis of the constitutively active FBPase mutant C153S led to the proposal that the reduced FBPase can retain the inactive conformation found in the oxidized protein and that it is the presence of substrate and/or of divalent cations such as Mg^{2+}, which initiates the conformational change at the catalytic site (*7*).

The function of the third conserved cysteine of the 170s loop (i.e., Cys179 in spinach FBPase) remains unclear. It is located at the beginning of an α-helix and oriented toward the interior of the protein structure. On the basis of the structural results, it was proposed that in a C173S mutant of FBPase this Cys might be able to form an artifactual disulfide bond with Cys153 (*7*), explaining the necessity of reduction to achieve full activation.

We have previously reported the oxidation−reduction potentials of WT pea and spinach FBPases and of Trx *f*, the specific regulatory protein for the enzyme (*8*). The potential of spinach FBPase ($E_{m,7.0} = -330$ mV) was significantly more negative than that of Trx *f* ($E_{m,7.0} = -290$ mV) and also more negative than the value obtained for the pea enzyme ($E_{m,7.0} = -315$ mV). Different results were also obtained for the pH dependency of E_m for the two chloroplast FBPases. For the spinach enzyme, the apparent presence of two components with different E_m vs pH profiles was explained by different bonding partners to Cys155 depending on the pH. The oxidation−reduction potential measured for Trx *f* in this study, determined with the mBBr-labeling technique (*8*), was somewhat more negative than a value reported previously for the same protein, which was obtained by measuring the ability of Trx *f* to activate FBPase (*9*).

To gain more information on structural and functional roles of the three conserved cysteines of the 170s loop of chloroplast FBPases (i.e., Cys155, 174, and 179 in spinach, Cys153, 173, and 178 in pea), we replaced them individually or in pairs by site-directed mutagenesis and analyzed the redox potentials, the thiol content, the Mg^{2+} dependency, and the activation kinetics of the recombinant mutant proteins. We have also reinvestigated the E_m vs pH profile for wild-type spinach FBPase and the E_m value for spinach Trx *f*.

EXPERIMENTAL PROCEDURES

All proteins used in this study are recombinant proteins expressed in *Escherichia coli*, except for a sample of chloroplast FBPase isolated from spinach leaves (*10*) and used as a control in the oxidation−reduction titrations. Isolation of genes, site-directed mutagenesis, and purification procedures were described previously (*11−15*). Reduced and oxidized forms of dithiothreitol (DTT) were obtained from Calbiochem and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) from Fluka.

Determination of the Molar Absorbency. Recombinant spinach chloroplast FBPase and Trx *f* were extensively dialyzed against 20 mM ammonium formate buffer at pH 5.7 for FBPase and pH 7.5 for Trx *f*. Spectra of the proteins were recorded against dialysis buffer and the protein solutions distributed in desiccated, weighed micro-tubes and lyophilized in a Speed Vac Concentrator (Savant). The mass of the dried samples was measured three times, and the molar absorbency calculated using the molecular weights deduced from the sequences, i.e., 157 300 g/mol for the recombinant FBPase and 12 579 g/mol for a form of Trx *f* truncated at amino acid number nine (*14*). The experimentally determined molar absorbancies, $\epsilon_{279nm} = 144\ 000\ M^{-1}\ cm^{-1}$ for FBPase and $\epsilon_{279nm} = 14\ 200\ M^{-1}\ cm^{-1}$ for truncated Trx *f*, were used to calculate the protein concentrations in all experiments.

Redox Titrations. Oxidation−reduction titrations of WT FBPase, using enzymatic activity to measure the oxidation state of the enzyme, were carried out at 25 °C, under either argon or air atmospheres, essentially as described previously (*8*). FBPase samples were incubated in activation mixtures containing varying amounts of oxidized and reduced DTT, to achieve equilibrium at defined redox potentials. The activation mixtures contained a total DTT concentration of 10 mM in 100 mM buffer and a catalytic amount of Trx *f* (0.05 μM). Incubation was carried out for 2−3 h either under argon or air. Then the activity of the FBPase samples was measured by monitoring the formation of NADPH at 340 nm in a 1 mL reaction medium containing 100 mM Tris-Cl, pH 7.9, 0.1 mM EGTA-Na, 10 mM $MgSO_4$, 1 mM fructose 1,6-bisphosphate, 0.3 mM NADP, 1.75 units of phosphoglucose isomerase, and 0.7 units of glucose-6-phosphate dehydrogenase. For the redox titrations of the C179S FBPase mutant, it was necessary to decrease the Mg^{2+} concentration in the reaction mixture to 1.5 mM to obtain reproducible data. Buffers used were sodium-acetate, pH 5.5, Bis-Tris-Cl pH 6.0, 6.5, 7.0 and tricine-NaOH pH 7.5, 8.0, 8.5. The redox poise of the DTT mixtures was confirmed by titration with DTNB.

Redox titrations of Trx *f* using enzymatic activity were accomplished by incubating thioredoxin in DTT redox mixtures as described above, followed by treatment with *N*-ethylmaleimide to block free cysteinyl residues (*16*). The concentration of unmodified Trx *f* was determined by its capacity to activate FBPase as described by Schürmann et al. (*10*).

The Mg^{2+} dependency of FBPase activity was determined using the assay described above with varying Mg^{2+} concentration in the reaction mixtures and with FBPase either not-activated (i.e., oxidized) or activated (i.e., incubated in 100 mM TEA-Cl, pH 7.0, in the presence of 2 μM Trx *f* and 5 mM reduced DTT for 10 min at 25 °C). An amount of

FBPase corresponding to 0.1 unit was tested for activity in the coupled spectrophotometric assay. Results were evaluated using the simplified Hill equation (*17*) to characterize the sigmoidal curves and to calculate the $S_{0.5}$, the Mg^{2+} concentration at half-maximal activity.

Activation kinetics of FBPase with reduced thioredoxin were measured using the coupled spectrophotometric assay described above. Samples of WT FBPase or of its C179S mutant were mixed with Trx and 5 mM reduced DTT in 100 mM TEA-Cl, pH 7.0. After 0, 5, 10, 20, and 30 min of incubation, samples of activation mixture corresponding to 0.1 unit of FBPase were injected in the reaction mixture. The thioredoxins used to activate the FBPase were Trx *f*, Trx *m*, and C49S Trx *f*, a Trx *f* lacking the buried cysteine of the active disulfide, at concentrations of 0.2 and 2 μM.

Thiol Concentrations. Total free thiols were titrated with DTNB in the presence of 2% SDS in 100 mM Tris-Cl, pH 8.0, and surface-exposed cysteines in 100 mM TEA-Cl, pH 7.0, without SDS. Release of TNB^- was monitored at 412 nm and its concentration was calculated with a molar absorbency of 13 600 M^{-1} cm^{-1} (*18*).

Dissociation of the C174S FBPase-TNB Complex. Freshly purified C174S FBPase was treated with DTNB for 20 min at pH 7.0 followed by desalting over a G-25SF column (Pharmacia). The release of TNB^- was followed by recording absorption spectra between 500 and 250 nm every hour during 12 h at 25 °C.

Circular Dichroism Spectroscopy. Samples of WT and mutant FBPase, extensively dialyzed against 10 mM potassium-phosphate buffer, pH 6.0, were diluted to a concentration of 1.5 μM. CD spectra were recorded from 190 to 250 nm in a cell of 0.1 cm path length at 25 °C. The secondary structure composition was predicted using the program "CDdeconvolution" (Dr. G. Boehm, http://bioinformatik-.biochemtech.uni-halle.de/cdnn)

RESULTS

Oxidation−Reduction Titration of Trx f and FBPase. To confirm the redox potential for spinach Trx *f* obtained earlier, in which the fluorescent probe mBBr was used to monitor the extent of reduction (*8*), we have reexamined the protein by an independent method. Using the ability of reduced Trx *f* to activate FBPase the oxidation−reduction midpoint potential at pH 7.0, $E_{m,7.0}$, was found to be −290 ± 10 mV, a value identical to our earlier determination.

The redox potential for spinach FBPase determined earlier was found to be 40 mV more negative (−330 mV, pH 7) than that of Trx *f* and its pH dependency suggested the presence of two components. These results also differed from those observed with the pea enzyme (*8*). For these reasons, and to facilitate comparisons between the WT enzymes and site-specific mutants, we have reexamined the redox properties of WT spinach FBPase. Figure 1 shows a titration of recombinant WT spinach FBPase at pH 7.0. The pH dependency for the E_m values of both recombinant WT FBPase and of the chloroplast enzyme isolated from leaves are shown as an insert in Figure 1. An average redox potential of −305 ± 5 mV at pH 7.0 was determined for both proteins. Identical redox properties were observed regardless of whether redox equilibration was carried out under air or argon. The C179S mutant FBPase, which has an intact
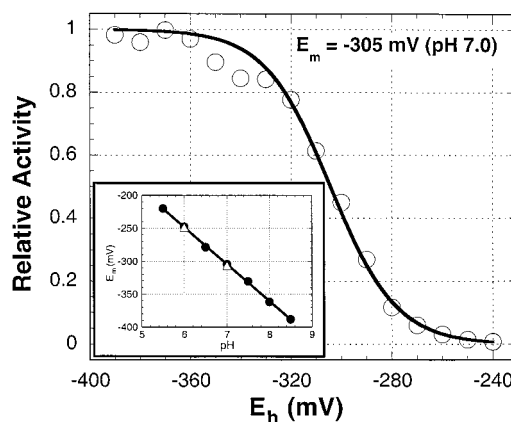

FIGURE 1: Oxidation−reduction titration of recombinant spinach chloroplast FBPase. Average of four titrations at pH 7.0 obtained with the recombinant enzyme. Oxidation−reduction equilibration was carried out for 2 h at ambient temperature under argon using 3.3 units/mL spinach FBPase and a catalytic amount of spinach thioredoxin *f* (50 nM) in 100 mM buffer containing DTT at a total concentration of 10 mM. Aliquots were withdrawn and assayed for FBPase activity in the presence of 10 mM Mg^{2+} as described in the Experimental Procedures. The solid titration curve was calculated by fitting the data to the Nernst equation with the value of n fixed at 2. (Insert) Effect of pH on E_m for recombinant (●) and chloroplast (△) FBPase. The datapoints, with an average deviation of ±5 mV, represent the result from several independent titrations. Buffers used were Na-acetate (pH 5.5), Bis-Tris-Cl (pH 6.0−7.0), and Tricine-NaOH (pH 7.5−8.5).
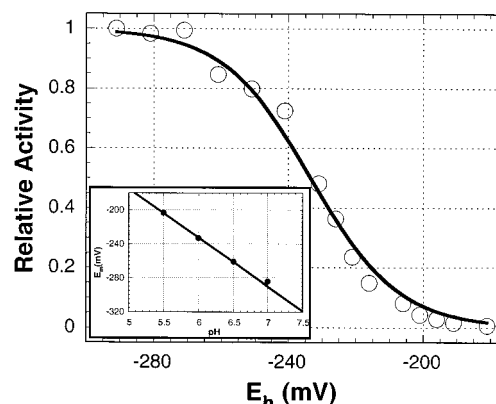

FIGURE 2: Oxidation−reduction titration of the spinach FBPase C179S mutant at pH 6.0 and pH dependency of the E_m for the mutant protein. Experimental conditions were as for Figure 1 except that the activated enzyme was tested in the presence of 1.5 mM Mg^{2+}.

regulatory disulfide, showed a slightly more positive potential of −290 ± 10 mV at pH 7.0 (Figure 2), a value that is very similar to that obtained with the corresponding pea FBPase mutant C178S (Figure 3). These titration data all gave excellent fits to the Nernst Equation for a single two-electron transfer. The pH dependency of the E_m value for the WT and C179S mutant spinach FBPases show a slope close to −59 mV/pH unit value expected for a redox reaction that involves the uptake of two protons per two electrons transferred (see inserts in Figures 1 and 2). Titrations of the C179S mutant FBPase at pH values higher than 7.0 showed considerable scatter and gave poor fits to the Nernst equation, probably due to the instability of the mutant at these pHs. The E_m value for the corresponding C178S pea mutant showed the same pH dependency as observed for the spinach enzyme.
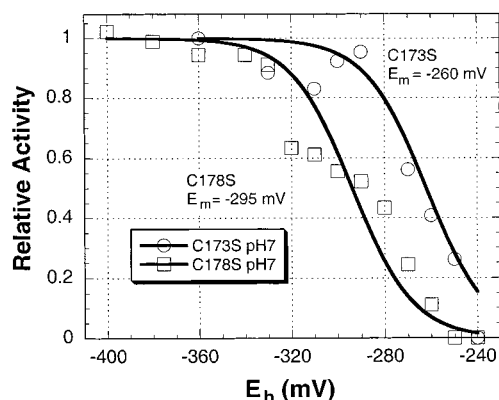
FIGURE 3: Oxidation−reduction titrations of the pea FBPase mutants C178S (□) and C173S (○) at pH 7.0. Oxidation−reduction equilibration was carried out for 3 h at ambient temperature under air using 70 μg/mL pea FBPase and a catalytic amount of pea thioredoxin *f* (0.2 μg/mL) in 100 mM MOPS buffer containing DTT at a total concentration of 10 mM. Aliquots (20−40 μL) were withdrawn and assayed for FBPase activity in 1 mL reaction mixture containing 100 mM Tris-Cl, pH 7.9, 10 mM Mg^{2+}, 1.7 mM EDTA, 2.4 mM fructose 1,6-bisphosphate, 0.3 mM NADP, 1.75 units of phosphoglucose-isomerase and 0.7 units of glucose 6-phosphate dehydrogenase.
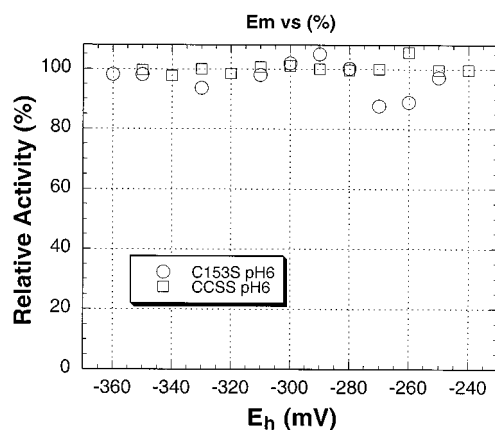

FIGURE 4: Oxidation−reduction titrations of the pea FBPase single mutant C153S (○) and the double mutant C153/173S (□) at pH 6.0 in 100 mM MES buffer. Experimental conditions were as for Figure 3.

When either the Cys closer to the N-terminus or both of the two other cysteines of the regulatory loop are replaced by Ser, a constitutively active enzyme is produced, which no longer shows a response to the redox potential (Figure 4; data for the spinach enzyme not shown). The situation is more complex when the second Cys (Cys174 in spinach and Cys173 in pea) is modified. This enzyme, when freshly isolated, behaves like the Cys155S mutant, i.e., there is no effect of the ambient redox potential on the activity. However, upon aerobic storage, redox control of activity reappears and, after storage for a period of several months, a redox potential has been determined for the pea enzyme (Figure 3). The E_m value found is more positive than for the WT or C178S mutant protein. We have observed that after short-term storage of the spinach enzyme (i.e., for several days) the E_m values are rather variable due to incubation-time dependent changes. The C174S mutant loses activity during incubation with oxidized DTT, suggesting formation of a disulfide bond, whereas during incubation in mixtures

Table 1: Thiol Group Content of WT and Mutant FBPase

FBPase	-SH total	-SH surface
WT	5	0
C155S	6	0.5−1.6[a]
C174S	5.7	0.5−1.6[a]
C179S	4	0

[a] See text for explanation

containing some reduced DTT, the activity increases until it reaches the maximal value.

Thiol Content of the WT and Mutant FBPases. To verify whether the site-specific mutations that replace Cys by Ser affect the number of accessible thiol groups, we have titrated the WT and mutated FBPases with the thiol-specific reagent DTNB. Table 1 summarizes the contents of accessible and total free thiols found per subunit of enzyme for WT and mutant spinach FBPases. The primary structure indicates that seven cysteines are present per subunit in the WT protein, two of which are involved in the regulatory disulfide. Titration of WT spinach FBPase with DTNB under denaturing conditions yields the five thiols expected per subunit. Titrations of the mutants were more variable, especially when the enzymes had been stored under air. However, measurements performed on freshly purified proteins gave good agreement with the theoretical value. We obtained a total of 6 thiols for the C155S and C174S mutants and 4 thiols for C179S. WT FBPase and mutant C179S show no surface-exposed thiols, even after prolonged incubation times. In contrast, the number of exposed thiols observed for the C155S and C174S mutants increases with increasing time in the reaction mixture (data not shown), suggesting that internal cysteines become slowly exposed to the solvent. The final values obtained for both these mutants are between 0.5 and 1.6 thiols/subunit. It is important to point out that, after aerobic storage, we observed a decrease in the total free thiol content from 6 to about 4 thiols/subunit for both the C155S and C174S active-site spinach mutants. To analyze this change, the C174S mutant was treated with DTNB to obtain TNB-labeled FBPase. This complex has an absorbance spectrum with the protein peak at 280 nm and a peak at 330 nm due to the complex of the enzyme with TNB (*19*). The labeled enzyme was incubated at 25 °C and spectra recorded at intervals (Figure 5). These spectra exhibit an increase in absorbance at 412 nm, due to liberation of TNB^-, whereas the absorbance at 330 nm decreases. This observation implies that the Cys155−TNB bond is attacked by some other cysteine, since the presence of air, which leads to an oxidizing medium, prevents the liberation of the TNB^- unless there is formation of a new disulfide bridge.

Mg^{2+} Dependency of WT and Mutant FBPase. Mg^{2+} is an absolute requirement for FBPase activity, and it is known that reduction of the enzyme decreases the level of this divalent cation required for full activity. We compared the Mg^{2+} requirement of the regulatory site mutants with that observed for the WT spinach FBPase. Figure 6 shows the results obtained with oxidized and reduced WT enzyme and for the FBPase Cys/Ser mutants. The results clearly fall into four groups. Oxidized WT FBPase is active only at very high Mg^{2+} concentrations with a half-maximal saturation concentration $S_{0.5}$ of 12.2 mM (Table 2). Mutating Cys179, a residue that is not supposed to be involved in regulation (*7*),
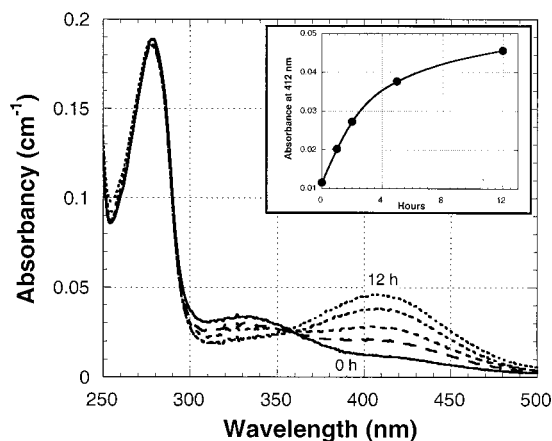
FIGURE 5: Release of TNB$^-$ from DTNB-modified FBPase mutant C174S. The labeled protein was incubated at 25 °C and spectra recorded at intervals. The spectra represented were recorded after 0, 1, 2, 5, 12 h incubation. The insert shows the time course of absorbency increase at 412 nm due to the release of TNB$^-$.
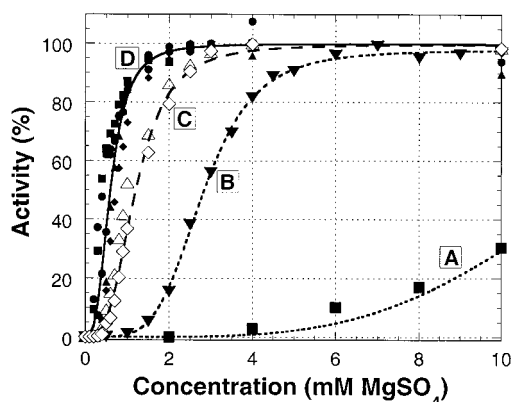

FIGURE 6: Effect of Mg^{2+} concentration on the activity of oxidized and reduced WT spinach FBPase and its mutants. The solid curves represent average values from several independent experiments. (A) Oxidized WT FBPase; (B) oxidized mutant C179S; (C) reduced mutant C179S and oxidized or reduced double mutants C155/179S and C174/179S; (D) reduced WT FBPase and oxidized or reduced C155S and C174S single or double mutants.

Table 2: $S_{0.5}$ of Magnesium for Oxidized and Reduced WT and Mutant Spinach FBPase

FBPase	oxidized $S_{0.5}$ (mM MgSO$_4$)	reduced $S_{0.5}$ (mM MgSO$_4$)
WT	12.2	0.6
C155S	0.6	0.6
C174S	0.6 (1.2)[a]	0.6
C179S	3.2	1.2
C155/174S	0.6	ND
C155/179S	1.2	ND
C174/179S	1.2	ND

[a] After storage under aerobic conditions.

significantly decreases the Mg^{2+} requirement of the oxidized enzyme to $S_{0.5}$ = 3.2 mM. Upon reduction of this mutant, its Mg^{2+} requirement further decreases to $S_{0.5}$ = 1.2 mM, but does not reach the level characteristic of the reduced WT protein. The two double mutants, containing the C179S mutation and an open regulatory site disulfide, displayed the same $S_{0.5}$ values. When the regulatory disulfide is either opened by reduction of the WT enzyme or by mutation of Cys155, Cys174, or both, we observe the lowest $S_{0.5}$ values for Mg^{2+} of about 0.6 mM. Table 2 summarizes the $S_{0.5}$
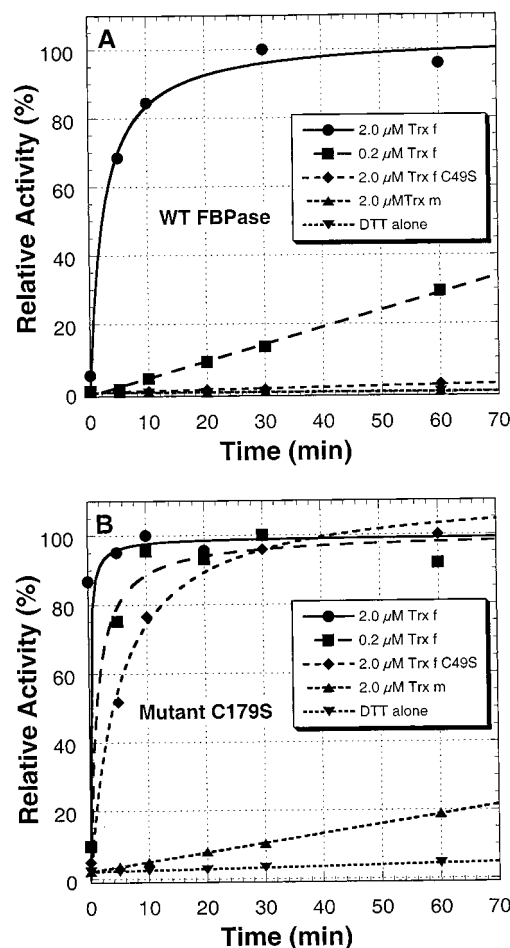

FIGURE 7: Activation kinetics of WT FBPase (A) and its C174S mutant (B). The enzyme was activated by chemically reduced thioredoxins. After the indicated times a sample, corresponding to 0.1 units of FBPase was withdrawn and its activity measured.

values for magnesium obtained for the different oxidized and reduced enzymes at pH 7.9. It also should be mentioned that during aerobic storage we observe an increase of the $S_{0.5}$ for the oxidized C174S mutant.

Activation Kinetics of WT and Mutant C179S. Figure 7 shows the activation kinetics of the WT spinach FBPase (Figure 7A) and its C179S mutant (Figure 7B). Under the experimental conditions used for these experiments, only Trx *f* (at 2 μM) is able to significantly activate the WT enzyme. Full activity is reached after an incubation time of approximately 20 min. At a 10 times lower Trx *f* concentration, 0.2 μM, FBPase is very slowly activated. However, C49S Trx *f* and Trx *m* at 2 μM, or DTT alone have virtually no effect on the WT enzyme. In contrast, the C179S mutant of FBPase is fully activated almost immediately after addition of 2 μM Trx *f* and also easily reduced by 0.2 μM Trx *f* and a 2 μM concentration of the C49S Trx *f* mutant. Even Trx *m*, at a concentration of 2 μM, is able to slowly activate this mutant, while DTT alone is not.

Circular Dichroism. Freshly purified WT FBPase and single mutants show no differences in their CD spectra. This is not the case for preparations stored for two weeks in the presence of air where the spectrum of mutant C155S clearly differs from the others (Figure 8). Computer analysis of the spectra indicates a loss of about 3% of α-helical structure in the C155S mutant compared to the three other proteins.
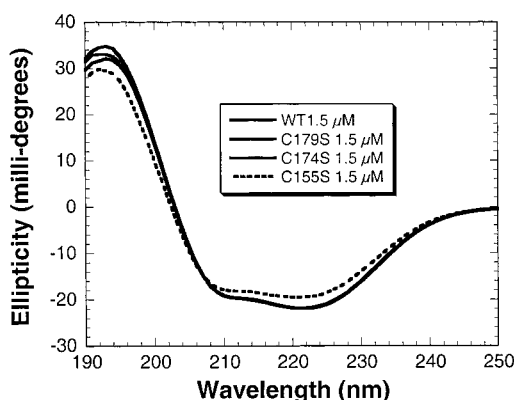
FIGURE 8: Circular dichroism spectra of 1.5 μM WT and mutant FBPases in 10 mM potassium-phosphate, pH 6.0, were recorded in 0.1 cm path-length cuvettes.

DISCUSSION

The activity of the chloroplast FBPase depends on several different factors, such as pH, Mg^{2+} concentration (20, 21) and reduction state of the regulatory disulfide (22). All three of these parameters are light dependent. Illumination of chloroplasts leads to an alkalization of the stroma (23), to an increase in stromal Mg^{2+} concentration (24), and to activation through reduction mediated by the ferredoxin/ thioredoxin system (2, 25). The interaction with reduced Trx *f*, which is highly specific (Figure 7A), results in the reduction of a regulatory disulfide bond, located on a loop structure that also contains a third conserved Cys (Cys 179 in spinach). Analysis of the structure of pea chloroplast FBPase (7) and studies on heterodimer formation between Trx *f* and FBPase (15) indicate that this third Cys is probably not involved in physiological regulation. Of the other two, Cys155 appears to be the residue attacked by Trx *f* forming the transient mixed disulfide whereas Cys174 does not interact with Trx *f* (15). To obtain reduction of FBPase by Trx *f* there has to be, in addition to a specific interaction, a sufficient thermodynamic driving force, which depends on the difference in the E_m values of the two proteins (26). Since the ΔE_m calculated from our earlier measurements (8) was rather unfavorable, we have redetermined the oxidation– reduction potentials for spinach Trx *f* and FBPase. While the earlier $E_{m,7.0} = -290 \pm 10$ mV value for Trx *f* was confirmed using a different determination method, the potential for FBPase was found more positive than the value reported earlier. Thus, as is the case for pea FBPase and Trx *f*, the two spinach proteins do not differ greatly in E_m. This new value for $E_{m,7.0} = -305$ mV has been obtained independently in two other laboratories (9, 27) and with redox mixtures incubated either under argon or in air. In addition, we also obtained identical results, regardless of whether recombinant FBPase, expressed in *E. coli*, or FBPase that had been isolated from leaves was titrated. We also tested the effect of pH on the E_m values for FBPase and obtained data that gave a good fit to a straight line with a slope of about -59 mV/pH unit over the entire pH range from 5.5 to 8.5. This is the slope expected for a process in which two protons are taken up per disulfide reduced (28). These results compare well to those obtained in our laboratories with the pea proteins (Table 3) (8, 27). In the case of the spinach enzyme, the redox potential of FBPase is about 15 mV more

Table 3: Summary of Oxidation–Reduction Midpoint Potentials of Spinach Thioredoxin *f* and Spinach and Pea WT and Mutant Chloroplast FBPase

protein	disulfide titrated	E_m at pH 7.0 (mV)
spinach thioredoxin *f*	C46/C49	-290 ± 10
spinach FBPase WT	C155/C174	-305 ± 5
spinach FBPase C179S	C155/C174	-290 ± 10
pea thioredoxin *f*	C33/C36	-290 ± 10^a
pea FBPase WT	C153/C173	-315 ± 10^a
pea FBPase C178S	C153/C173	-295 ± 10
pea FBPase C173S	C153/C178	-260 ± 10

a From ref *8*.

negative than that of Trx *f*, so that most of the Trx *f* has to be reduced for significant activation of the enzyme to occur.

The mutation C179S produces a protein with a behavior comparable to the wild-type FBPase, but which is more easily activated by Trx *f*. At a concentration of only 0.2 μM Trx *f*, this protein is rapidly reduced (Figure 7B), and at 2 μM it is virtually instantly activated. This might be due to the fact that E_m for the mutant, $E_{m,7.0} = -290$ mV, is more positive than that of the WT enzyme by 15 mV. The fact that the C179S mutant and WT Trx *f* are isopotential (Table 3) could facilitate the thiol-disulfide interconversion. The specificity of the interaction with the Trx *f* in activating this mutant FBPase is well maintained, as evidenced by the slow activation rate observed with Trx *m*. Surprisingly, the Trx *f* C49S mutant, in which the nonaccessible, active-site Cys49 is replaced by serine, activates the FBPase C179S mutant quite well, despite the fact that this mutant does not activate the WT enzyme. It seems that the C179S mutation affects the structure of the 170s loop, increasing the accessibility of the regulatory disulfide of the FBPase. The ability of the Trx *f* C49S mutant to reduce the disulfide bridge formed between Cys155 and 174 could thus be explained by the attack of the primary nucleophile of Trx *f* (Cys46) on Cys155 followed by the reduction of the heterodisulfide by DTT present in the activation mixture (15, 29). The Mg^{2+} dependency of the C179S mutant enzyme tends to support the structural role of Cys179. The $S_{0.5}$ for Mg^{2+} of the oxidized mutant is almost four times lower than for the oxidized WT enzyme (Table 2). On the other hand, the $S_{0.5}$ of the reduced C179S mutant or of the two double mutants C155/179S and C174/179S are both approximately 1.2 mM, which is twice that observed with the other reduced FBPases. This demonstrates that the disturbance induced by the replacement of Cys179 in the regulatory loop affects the binding of Mg^{2+} at the catalytic site (7).

Mutation of Cys155 leads to a protein with a requirement for Mg^{2+} comparable to that characteristic of the reduced WT FBPase ($S_{0.5} = 0.6$ mM) and which has completely lost the redox-dependent regulation. However, during aerobic storage we observed a decrease in the number of total free thiols from 6 to around 4/subunit, without any detectable effect on the catalytic activity. Analysis of the proteins by circular dichroism spectroscopy supports the hypothesis of the appearance of a new disulfide bridge. CD spectra of freshly purified WT and mutant FBPases are completely identical. However, after aerobic storage the spectrum of the C155S mutant diverges from the others (Figure 8). Computation of the relative contribution by the different structural

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elements shows a loss of 3% of α-helix in the C155S mutant compared to the other proteins. Interestingly, the α-helix formed by the region of the protein between Ala169 and Val178 in the 170s loop corresponds to 3% of the enzyme's helical structure. This correlation suggests that under oxidizing conditions an artifactual disulfide bond between Cys174 and 179 could be formed in the C155S mutant. This would be made possible by the high flexibility of the 170s loop and would disturb the secondary structure of this region.

Mutation of Cys174 yields a protein, which is constitutively fully active, like the C155S mutant, and has a Mg^{2+} dependency like that of the reduced WT FBPase. However, during aerobic storage, a partial dependence of the activity on redox state appears. This oxidized enzyme can be easily reactivated by Trx f and, less efficiently, by Trx m and DTT alone (data not shown). In parallel to the emergence of a redox potential effect on activity, we also observe an increase of the $S_{0.5}$ for Mg^{2+} from 0.6 to 1.2 mM under nonreducing conditions (Table 2) and a decrease of the number of total free thiols from 6 to about 4/subunit. In contrast, freshly purified enzyme shows a $S_{0.5}$ for Mg^{2+} around 0.6 mM under either nonreducing or reducing conditions. These observations can also be explained by the formation of an artifactual disulfide bridge between Cys155 and Cys179. This proposal was made previously for the pea chloroplast enzyme (*7*), where only a small shift in the structure would allow a disulfide bridge to form between Cys153 and Cys178, due to the short 7 Å distance separating these two cysteines. Analysis of the release of TNB⁻ from the TNB-mutant C174S complex also supports this hypothesis. DTNB incubation of a freshly purified sample of C174S FBPase yields a TNB complex, with TNB⁻ associated with Cys155 and/or Cys179. This complex is not stable under aerobic conditions, but disintegrates releasing free TNB⁻ (Figure 5). Under our experimental conditions, this could only occur if a disulfide bond is formed.

Our results provide experimental evidence that only the two most N-terminal cysteines on the regulatory loop of chloroplast FBPase are responsible for redox regulation, whereas the third cysteine does not seem to be involved under normal physiological conditions. The most striking effect of the opening of the regulatory disulfide bridge, either by reduction with Trx f or by mutation, is the change of the Mg^{2+} requirement, which is lowered about 20-fold. This strengthens the proposal, based on the structural analysis, that the reduction of the regulatory disulfide has a positive allosteric effect on the binding of the catalytically essential Mg-ion in the active site.

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