Cysteine-153 is required for redox regulation of pea chloroplast fructose-1,6-bisphosphatase

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Abstract Chloroplastic fructose-1,6-bisphosphatases are redox regulatory enzymes which are activated by the ferredoxin thioredoxin system via the reduction/isomerization of a critical disulfide bridge. All chloroplastic sequences contain seven cysteine residues, four of which are located in, or close to, an amino acid insertion region of approximately 17 amino acids. In order to gain more information on the nature of the regulatory site, five cysteine residues (Cys^{49}, Cys^{153}, Cys^{173}, Cys^{178} and Cys^{190}) have been modified individually into serine residues by site-directed mutagenesis. While mutations C173S and C178S strongly affected the redox regulatory properties of the enzyme, the most striking effect was observed with the C153S mutant which became permanently active and redox independent. On the other hand, the C190S mutant retained most of the properties of the wild-type enzyme (except that it could now also be partially activated by the NADPH/NTR/thioredoxin *h* system). Finally, the C49S mutant is essentially identical to the wild-type enzyme. These results are discussed in the light of recent crystallographic data obtained on spinach FBPase [Villeret et al. (1995) Biochemistry 34, 4299–4306].

Key words: Chloroplast; Fructose-1,6-bisphosphatase; Redox regulation

1. Introduction

Several chloroplastic enzymes are regulated by light through disulfide/dithiol interconversion reactions [1]. While some of these catalysts like phosphoribulokinase (PRK), fructose-1,6-bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase (GAPD) and sedoheptulose-1,7-bisphosphatase (SBPase) belong to the Calvin cycle responsible for light-dependent CO_2 fixation in plants, others like NADP malate dehydrogenase (NADP-MDH), glucose-6-phosphate dehydrogenase or ATP synthase (CF1) do not [2]. In the dark, Calvin cycle enzymes are essentially in an oxidized and inactive form, but in the light several reports indicate that a critical disulfide has to be reduced to a dithiol in order to get a catalytically active enzyme. The system responsible for the light activation of chloroplast enzymes is now well documented to be constituted of photoreduced ferredoxin, ferredoxin thioredoxin reductase and thioredoxins specific for one or several target enzymes [3]. The structural features of the redox regulatory enzymes have been the subject of much scrutiny lately. One of the first identifications of a regulatory disulfide has been done on PRK, using chemical derivatization and site-directed mutagenesis: in this case, the regulatory site has been demonstrated to be part of the active site [4,5]. Another well-documented case is NADP-MDH. Using a combination of sequence comparison, chemical derivatization and mutagenesis, it has been proposed that this enzyme is actually similar to the non-regulatory NAD malate dehydrogenase and that its redox properties result from two sequence extensions, one N- and one C-terminal, each featuring a thioredoxin-sensitive disulfide bridge, although the exact position of the second disulfide bond is still a matter of debate [6–8]. In the case of FBPase, it has been shown that all chloroplastic enzymes resemble the mammalian gluconeogenic enzyme (pig kidney type), except for the insertion of a region of approximately 17 amino acids, containing four cysteine residues [9–14]. It was proposed earlier that two of these cysteines (namely C173 and C178 in pea) were linked in a regulatory disulfide bridge. Using site-directed mutagenesis, we could indeed show that replacing C173 and C178 together by serines resulted in a deregulated permanently active enzyme [15]. Crystallographic data, however, have been recently published by Villeret et al. [16] which question whether C173 and C178 are actually linked in a disulfide bridge, prompting us to reassess our results by preparing single Cys to Ser mutants of the four individual cysteines of the amino acid regulatory insertion. These experiments are described thereafter.

2. Material and methods

The plasmid used for mutagenesis was the pET-FBPase construction described previously [15]. Site-directed mutagenesis was performed by PCR using N- and C-terminal oligonucleotides as in [15]. The mutagenic oligonucleotides were chosen complementary to one another over a stretch of at least 16 bases so that their immediate adjacent 5′ nucleotide would be an A, as follows (mutagenic bases in bold):

C49S up: 5′-GTATTTCTATGGCAT**C**TAAACAAATTGCTTCT-3′
C49S down: 5′-GAAGCAATTTGTTTA**G**ATGCCATAGAAATACT-3′
C153S up: 5′-TGACGAGAGT**C**TTCCTGA-3′
C153S down: 5′-ATCAGGAA**G**ACTCTCGTC-3′
C173S up: 5′-CAAAGG**A**GCATTGTGAATG-3′
C173S down: 5′-CATTCACAATGC**T**CCTTTG-3′
C178S up: 5′-ATGTG**A**GTCAACCAGGAAG-3′
C178S down: 5′-GGTTGCTTCCTGGTTGAC**T**CACATTC-3′
C190S up: 5′-GCTGGCTAC**A**GCATGTATTC-3′
C190S down: 5′-GAATACATGC**T**GTAGCCAGC-3′
FBP-N: 5′-GATCCATGGCTGTGAAGGAA-3′
FBP-C: 5′-TAGGATCCTTAAGCTAAGTACTT-3′

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Abbreviations: FBPase, fructose-1,6-bisphosphatase; DTT, dithiothreitol; NTR, NADPH thioredoxin reductase

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PCR reactions were performed as in [15] and the mutated fragments digested by *Nco*I and *Bam*HI and ligated into plasmid pET-3d. The resulting constructions were used to transform *Escherichia coli* strain BL21 (DE3) [17]. Recombinant enzymes were produced in 5 l cultures induced with 100 μM IPTG for 3 h in the exponential phase. The mutant proteins were released and purified to homogeneity by a combination of French press treatment, heat treatment (3 min at 60°C except for the double C173S/C178S mutant), ammonium sulfate fractionation (30–70% saturation), ACA 44 gel filtration and DEAE Sephacel chromatography. All operations were carried out in the presence of 50 mM sodium acetate buffer, pH 5.5. Generally, the yield was about 100 mg homogeneous enzyme per culture (i.e. ≈20 mg/l). The effect of reduced thioredoxin *f* (either chemically by DTT or by light and the ferredoxin thioredoxin system) on FBPase activation was tested as in [15]. After activation, the FBPase activity was measured in the presence of either 0.8 or 1.6 mM Mg^{2+}, using a coupled spectrophotometric assay [15]. This assay has been used routinely by many researchers in the field because it allows the continuous recording of activity, something that cannot be easily achieved using the direct Pi determination method. The effect of the NADPH/NADPH thioredoxin reductase/thioredoxin *h* system on wild-type FBPase and its mutants was tested as in [18], using recombinant *Arabidopsis thaliana* NADPH thioredoxin reductase (NTR) and *Chlamydomonas reinhardtii* thioredoxin *h* [19,20].

3. Results

Chloroplast redox-regulated FBPase sequences all show seven cysteine residues, most of which are not conserved in the cytosolic plant enzymes or in the mammalian, bacterial and cyanobacterial types. In addition, in the chloroplastic enzymes, there is an amino acid insertion region containing a putative regulatory sequence with four cysteine residues as shown below for the pea sequence, the cysteine residues being in bold characters:

150-NDE**C**LPDFGDDSDDNTLGTEEQR**C**IVNV**C**QPG-SNLLAAGY**C**MY-192

Previous crystallographic structures [16] predict that two of the seven cysteines cannot be involved in the redox regulation process (Cys^{92} and Cys^{306} in pea). Hence, in this study each of the four cysteine residues of the insertion region and Cys^{49} have been individually mutated into a serine residue by PCR as described in Section 2 and all clones were resequenced at the nucleotide level to check whether they contained the appropriate mutation and no other mutation. This mutagenesis method proved to be very efficient since all of them were as expected, except for the C153S mutant which also contained a D156G additional mutation. A second round of mutation was thus effected in order to obtain the C153S mutant devoid of the additional mutation (the two mutants later proved to be identical in their redox-regulatory properties). All mutant proteins were produced in *E. coli* cells and purified to homogeneity as described in Section 2.

The effect of dithiothreitol (DTT) or DTT-reduced thiore-

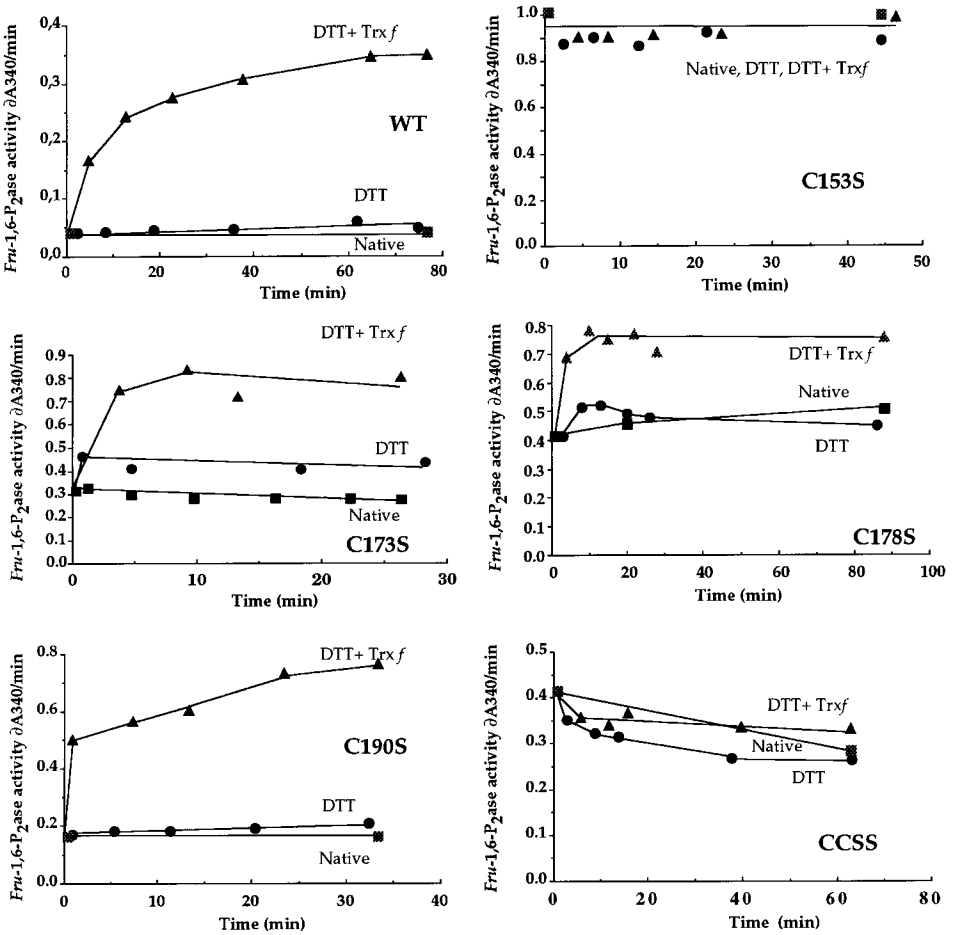

Fig. 1. Effect of DTT and DTT-reduced thioredoxin *f* on FBPase and its mutants. 15 μg enzyme was incubated in the presence of 100 mM Tris-HCl (pH 8.0) and when indicated DTT (2.5 mM) and thioredoxin *f* (3 μg) in a total volume of 150 μl for the indicated time. Aliquots representing 1 μg FBPase were used to determine the activity in the presence of 1.6 mM Mg^{2+} as described in [15]. In these conditions, the increase of 1 unit absorbancy corresponds to a specific activity of 200 units/mg enzyme.

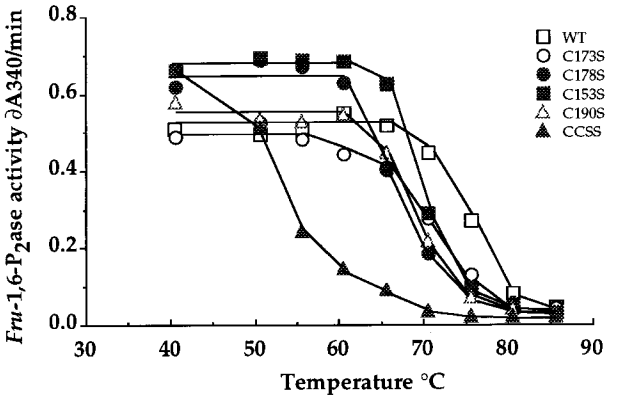

Fig. 2. Thermostability of FBPase and its mutants. Heat denaturation tests of the various enzymes were effected by submitting aliquots of proteins at a concentration of 0.1 mg/ml in 50 mM sodium acetate buffer, pH 5.5, to a 3 min treatment at the indicated temperature. After brief centrifugation to eliminate any denatured material, the remaining FBPase activity was tested by using aliquots containing 1.5 μg enzyme in the presence of 16 mM Mg^{2+} as described in [15]. In these conditions, the increase of 1 unit absorbancy corresponds to a specific activity of 130 units/mg enzyme.

doxin *f* on the wild-type enzyme and various mutants (including the previously engineered CCSS, i.e. C173S/C178S) is shown in Fig. 1. All these experiments are time course assays, and all samples were used to assay for activity in the presence of 1.6 mM Mg^{2+}. First, none of the mutations resulted in a loss of catalytic activity but, on the contrary, all of them increased the specific activity of the oxidized enzyme (3–18-fold when assayed at low Mg^{2+} concentration). Thus it can be concluded that none of these cysteines is required for catalysis. In addition, two mutations completely abolished the redox-dependent regulation of the enzyme (i.e. C153S and CCSS). C173S and C178S were partially deregulated (each was activated by a factor of ≈ 2 after addition of the reductants compared to 5 for the C190S mutant and 7 for the wild-type enzyme). Chemically reduced thioredoxin was required for activation of all these mutants, DTT alone being ineffective (see Fig. 1). Another important feature is that the spontaneous activity of C173S and C178S was approximately 7-fold higher than the spontaneous activity of the wild-type enzyme, indicating a shift toward a catalytically more efficient conformation in these mutants. These results were fully confirmed when using light and the ferredoxin–thioredoxin system as an activator instead of DTT (data not shown). FBPase is a complex enzyme which can be activated both by reduction or by incubating it in the presence of high Mg^{2+} concentrations and we wanted to make sure that the results obtained were not due to an effect of Mg^{2+} in the subsequent catalytic phase. The experiments were thus repeated at a different, lower Mg^{2+} concentration (0.8 vs. 1.6 mM) with similar results (Table 1). This table also shows that the C49S mutant behaves as the wild-type protein and is not deregulated. Since FBPase is also regulated by the Mg^{2+} concentration, an index showing the relative activities of the oxidized proteins at two different Mg^{2+} concentrations (low, 2.5 mM; high, 10 mM) is also included in Table 1. Mg^{2+} ions could not be replaced by Ca^{2+} ions for any of the mutants, the latter strongly inhibiting catalysis (data not shown). It can be seen that the mutations that induce a deregulation of the redox properties also strongly influence the ratio of activity, the deregulated proteins being relatively more active at lower Mg^{2+} concentrations. In this test also, C49S gave results identical to the wild-type protein and C190S classified between wild-type and C173S or C178S.

We have earlier shown [15] that the CCSS (C173S/C178S) mutation had the additional effect of destabilizing the protein. In general, the presence of a disulfide bond in a protein is supposed to make it more stable and, in this respect, the CCSS mutant conformed to the prediction. In order to gain more insight into the nature of the mutant fructose-1,6-bisphosphatases, their irreversible thermal denaturation properties have been compared (Fig. 2). Clearly the wild-type enzyme is the most resistant enzyme with a temperature for half denaturation (T_m) of approximately 75°C and the CCSS mutant was the most unstable protein (T_m 55°C). All other mutations moderately decreased the stability of the proteins with T_ms close to 70°C, but none had the very strong destabilizing effect observed for CCSS. It is thus difficult to conclude from these data whether the destabilization observed for the single mutants results from the disappearance of a disulfide bond or is the result of more local conformational changes. Another observation can be drawn from Fig. 2: since these assays have been performed in the absence of reductant at a high Mg^{2+} concentration (16 mM), it also appears that the increase in specific activity is still true for the CCSS, C153S and C178S mutants but with a very much reduced margin (40% at most). Wild-type FBPase and the C173S and C190S mutants exhibit similar catalytic rates in these conditions.

When using chemically or light-reduced thioredoxin *f*, the C153S, C173S and C178S clearly appear to be very different from the wild-type protein, but the C190S mutation appears to have a much weaker effect. We could, however, detect one additional difference between the C190S mutant and the wild-type FBPase when using thioredoxin *h* and the NTR system

Table 1
Effect of Mg^{2+} and DTT or DTT-reduced thioredoxin *f* on pea fructose-1,6-bisphosphatases

Treatment	FBPase						
	WT	C49S	C153S	C173S	C178S	C190S	CCSS
DTT	0.002	0.003	0.021	0.06	0.014	0.011	0.005
DTT+Trx *f*	0.115	0.258	0.034	0.19	0.026	0.106	0.006
Redox stimulation %	60	81	1.6	3.1	1.8	9.6	1.2
Ratio of activity high/low Mg^{2+}	6	7	1.9	1.2	1.7	3.5	1.6

10 μg enzyme was activated in the presence of 100 mM Tris-HCl (pH 8.0) and 2.5 mM DTT and thioredoxin *f* (3 μg) when indicated, in a total volume of 100 μl for 2 min. Aliquots representing 2 μg FBPase were then used to determine the activity in the presence of 0.8 mM Mg^{2+} as described in [15]. The results are expressed as ΔA at 340 nm/min. A ΔA of 0.2 corresponds to a specific activity of 40 units/mg enzyme.
The ratio of activity in the presence of high and low Mg^{2+} was estimated using oxidized FBPase and measuring its activity in the presence of either 10 or 2.5 mM Mg^{2+}. The variations in the ratios are the result of increased basal activity at the lower Mg^{2+} concentration.

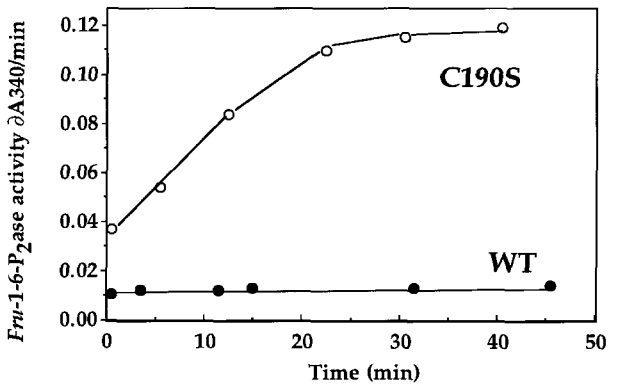

Fig. 3. Effect of the NTR system on wild-type and C190S FBPase. 8 μg enzyme was incubated together with 7 μg *Chlamydomonas reinhardtii* thioredoxin *h*, 1.5 μg *Arabidopsis thaliana* NTR, and 20 μg NADPH in the presence of 100 mM phosphate buffer (pH 6.0, a pH compatible with optimal NTR activity), in a total volume of 100 μl. After the indicated times, 15 μl aliquots were used for activity determination. FBPase activity was tested in the presence of 0.8 mM Mg^{2+}. In these conditions, the increase of 1 unit absorbancy corresponds to a specific activity of 20 units/mg enzyme.

as a reductant (Fig. 3). While the wild-type enzyme cannot be activated by reduced thioredoxin *h* in the experimental conditions of Fig. 3, the C190S mutant became activatable by thioredoxin *h* although not to the same extent as by reduced thioredoxin *f*.

4. Discussion

In an earlier work [15], based on sequence comparisons suggesting that Cys^{173} and Cys^{178} of chloroplastic FBPase could be linked in a disulfide bridge responsible for the redox regulatory properties of the enzyme, we have shown that mutating these two residues together resulted in a redox-independent enzyme with conserved catalytic activity. Taken together with the fact that the mutation strongly decreased the stability of the enzyme, these results were compatible with the disappearance of the redox-sensitive disulfide bridge of FBPase. However, a recent paper dealing with the crystallographic structure of spinach FBPase [16] has brought new information about the nature of the regulatory insertion. Although Villeret et al. could not precisely locate the disulfide bridge(s) in the structure, they observed that, in the spinach enzyme, the sulfur atoms of Cys^{174} and Cys^{179} were separated by distances varying from 5 to 16 A°, depending on the monomers and could thus hardly be bridged together unless there was an important conformational change in the regulatory loop. On the other hand, the sulfur atom of Cys^{155} was generally closer to either Cys^{174} or Cys^{179} with an average distance of approximately 9 A°. They concluded that this distance is still too far for a disulfide bridge to be formed between these residues, unless there was also an important conformational change in the loop. The site-directed mutagenesis data obtained in this paper clearly indicate that Cys^{153} is definitely the most essential cysteine residue for redox regulation. The obtained data are actually very consistent with Cys^{153} being bridged alternately with Cys^{173} or Cys^{178} in the pea enzyme, since the C173S and C178S mutations only partially abolish the redox dependency, while the C153S and C173S/C178S mutants are completely redox inactive. Thus, the regulatory disulfide cannot be located between Cys^{173} and Cys^{178}, but Cys^{173} and Cys^{178} are likely to be the two redox partners of Cys^{153}, which explains why the double mutant C173S/C178S is redox inactive, leading us earlier to an incorrect interpretation of these data [15]. On the other hand, Cys^{190} does not seem to be essential for the redox regulation although its mutation into a serine residue results in an enzyme more promiscuous in terms of thioredoxin requirement, since it can be activated by reduced thioredoxin *h* while the wild-type protein cannot. It is still difficult to understand why three cysteine residues (Cys^{153}, Cys^{173} and Cys^{178}) are required for the full redox properties of FBPase if only a single disulfide bridge is present, unless the reduction of this disulfide involves a transient isomerization as in BPTI [21]. With respect to the latter proposal, it is to be noted that the very specific thioredoxin *f* contains in addition to the disulfide bridge WCGPCK an additional cysteine both in spinach or pea (Cys^{59} in pea) [23,24]. Very interestingly, the mutation of this additional cysteine which seems to be on the surface in spinach thioredoxin *f* decreases the affinity versus FBPase (del Val, G., Morris, M. and Schürmann, P., communication at the 4th International Conference on Thioredoxins and Related Proteins, Kassel, August 1995, and del Val, G., Thesis University of Neuchâtel, 1996). It could thus be that the very high specificity observed in the thioredoxin *f*/FBPase reaction is not only due to specific surface electrostatic charges [25,26], but also to the presence of this third cysteine in thioredoxin *f*. Crystallizing the new mutants obtained or using a new method based on single cysteine mutants of thioredoxin *f* able to trap covalently target enzymes would help elucidating the activation mechanism of FBPase [27]. A mechanism based on a disulfide bridge isomerization would also fit rather well with the notion that the enzyme does not solely require reduction for its activation, but is also active in the oxidized form in the presence of high Mg^{2+} concentrations, independently of the reduction.

Based on the three-dimensional structure of FBPase, Villeret et al. have proposed that Cys^{191} could be linked in a disulfide with Cys^{51} in the spinach enzyme (the two sulfur atoms being separated by about 4 A°), although such a disulfide was not visible on the structure. Similar conclusions have been put forward for the wheat chloroplast FBPase based on molecular modeling studies [22]. In addition, several plant cytosolic sequences of FBPase have now been published which show two cysteine residues in the position of Cys^{49} and Cys^{190} of chloroplastic pea FBPase and it was thus suggested that the cytosolic enzyme could actually also contain such a disulfide bridge [28]. However, all plant cytosolic enzymes consistently show in their N-terminus part the sequence CKFVC, the first of the two cysteines being the one homologous to Cys^{49} of the chloroplastic pea sequence and based on the available 3-dimensional structures these two vicinal cysteines could also very well be linked together in a disulfide bridge.

The C190S mutation in the pea chloroplast enzyme indeed indicates that the resulting enzyme has changed conformation as evidenced by its new reactivity with thioredoxin *h*. In addition, all mutants examined, including C190S, exhibited a decreased thermostability, an observation which could be consistent with the disappearance of a disulfide bridge. However, the fact that the C49S mutant is identical to the wild-type protein in contrast to C190S, is not in favor of a disulfide bond between Cys^{49} and Cys^{190}. Moreover, neither of the

two mutations abolishes the redox regulation of the enzyme, thus demonstrating that the regulatory disulfide is not located between Cys^{49} and Cys^{190}. In order to estimate whether there is an additional structural disulfide bridge on the FBPase monomer, we have performed DTNB titrations on several oxidized and denatured preparations of the various mutants. Although the results were somewhat scattered, they did consistently show an increase of approximately one thiol group per subunit in the C153S mutant, which agrees with the breaking of a disulfide bridge (data not shown). No clear information could, however, be gathered from these experiments concerning the existence of an additional structural disulfide in pea FBPase. New attempts to tackle this problem by using mBBr before and after reduction of the enzyme preparations are under way. Whatever the result, the mutagenesis results obtained in this study indicate that, if there is a second disulfife bridge between Cys^{49} and Cys^{190}, it is certainly not the regulatory one. In conclusion, these mutagenesis results favor a model in which there is only one regulatory disulfide bond on chloroplastic FBPase, this bond being located between Cys^{153} and either Cys^{173} or Cys^{178}.

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