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Effects of mutations of the cysteines in the regulatory loop on structure and activity of chloroplast fructose-1,6-bisphosphatase (FBPase)

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Introduction

Chloroplast FBPases are homotetrameric enzymes of about 160 kDa. In vivo, activity is regulated by light through the ferredoxin/thioredoxin system (Schürmann and Buchanan 2001) and factors like pH, Mg^{2+} and substrate concentration, which are also modified in a light-dependent manner. Amino acid sequence alignments of chloroplastic and cytosolic FBPases show that the light–regulated forms have an insertion of about fifteen amino acids, the 170's loop, containing three strictly conserved cysteines. In the less active, oxidized enzyme two of them form a disulfide bond which is reduced by thioredoxin *f* in the light. Analysis of the functions of the three cysteines of pea chloroplast FBPase by site-directed mutagenesis revealed the central role of Cys153 in regulation. Replacement of this Cys lead to a constitutively active FBPase, whereas mutation of Cys173 or Cys178 produced a partially active enzyme that still requires reduction for full activity (Jacquot *et al.* 1997). Similar experiments with rapeseed chloroplast FBPase (Rodriguez-Suárez *et al.* 1997) confirmed the central role of the corresponding Cys in regulation and demonstrated that the mutations significantly decreased the $S_{0.5}$ for Mg^{2+} of oxidized and reduced enzyme. However, these results did not provide an unequivocal identification of the second Cys of the disulfide bond, but instead suggested the involvement of all three cysteines in regulation.

Recent crystallographic analysis of oxidized pea chloroplast FBPase (Chiadmi *et al.* 1999) provides evidence for a disulfide bond between Cys153 and Cys173 (corresponding to Cys155 and Cys174 in spinach FBPase) and suggests that this bond represents the regulatory disulfide. It is thought to stabilize the inactive form of the enzyme by displacing a glutamate involved in coordinating the catalytically–essential Mg^{2+} ion from its correct position at the active site. This occurs through a shift of the N–terminal β–strands towards the active site about 20 Å away. Upon reduction by thioredoxin *f*, the regulatory loop probably relaxes, allowing the active site to adopt a competent conformation (Dai *et al.* 2000).

The function of the third conserved cysteine in the 170's loop (i.e., Cys179 in spinach) remains unclear. It is located at the beginning of an α–helix and oriented towards the interior of the protein structure. Structural analysis indicates that in the C173S mutant of pea FBPase

this Cys might be able to form an artifactual disulfide bond with Cys153 (Chiadmi *et al.* 1999). This could explain why this mutant still needs reduction to achieve full activity. To obtain more information on the structural and functional roles of the cysteines in the regulatory loop of spinach and pea chloroplast FBPase we replaced them individually or in pairs and compared redox potentials, Mg^{2+} dependency and thiol content of the recombinant WT and mutant proteins.

Materials and methods

Isolation of genes, site-directed mutagenesis and purification procedures were described previously (Hodges *et al.* 1994; Jacquot *et al.* 1995; Balmer and Schürmann 1998; del Val *et al.* 1999; Balmer and Schürmann 2001). Oxidation–reduction titrations of FBPase using enzymatic activity were carried out at 25°C, essentially as described previously (Hirasawa *et al.* 1999). DTNB titrations were done according to (Habeeb 1972). To determine the Mg^{2+} dependency of reduced FBPases the enzymes were incubated in 100 mM triethanoleamine-Cl buffer pH 7.0 in presence of 2 µM thioredoxin *f* and 5 mM reduced DTT for 10 min at 25°C. Results were evaluated using a simplified Hill equation to characterize the sigmoidal curves and to calculate the $S_{0.5}$, the Mg^{2+} concentration at half maximal activity.

Results

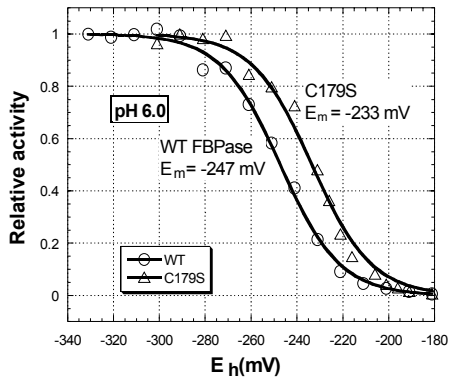

Fig.1. Redox-titration of WT and C179S mutant spinach FBPase at pH 6.0

Figure 1 shows a redox titration of the Cys155/Cys174 disulfide in WT spinach and in its C179S variant at pH 6.0. Mutation of Cys179, a cysteine that is not likely to be involved in regulation, shifts the redox potential of the regulatory disulfide by 14 mV towards more positive values. This change in redox potential is independent of pH. The pH dependencies of E_m for WT and the C179S mutant FBPase are both linear with a slope close to –59 mV/pH unit, as expected for a redox reaction that involves the uptake of two protons per two electrons transferred. An average redox potential of –305 ± 5 mV at pH 7.0 was determined for the recombinant WT spinach FBPase. Similar redox potentials were recorded for the closely related pea chloroplast enzyme. An identical $E_{m,7.0}$ and pH dependency were observed for the enzyme isolated from spinach leaves. Mutation of both regulatory cysteines or only of the one closer to the N–terminus abolishes the effect of ambient redox potential on activity and produces a constitutively fully–active enzyme. A more complex situation results when the second Cys (Cys174 in spinach or Cys173 in pea) is modified. This mutant enzyme, when freshly isolated, behaves like the C155S mutant, exhibiting no effect of the ambient redox potential on activity. During aerobic storage, redox control of activity reappears. A redox potential can be determined for samples of the pea enzyme that have been stored for several months (Fig. 2), suggesting the formation of a new disulfide bridge.

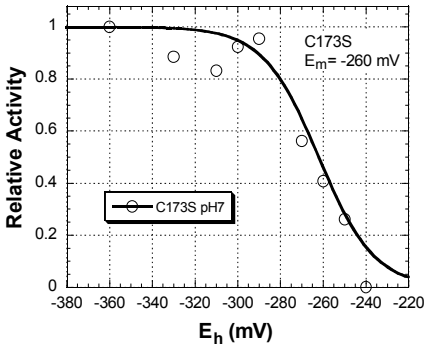

Fig.2. Redox-Titration of pea C173S mutant FBPase at pH 7.0

This has been verified by titration of the thiols in WT and mutant spinach FBPase. The primary structure indicates the presence of seven cysteines per subunit in the WT protein, two of them involved in the regulatory disulfide. Titration of WT FBPase under denaturing conditions yields the five thiols expected per subunit. Titrations of freshly purified mutant proteins also agree with the theoretical numbers. We obtained a total of six thiols for the C155S and C174S mutants, and four thiols for the C179S mutant. WT FBPase and the C179S mutant show no surface–exposed thiols. In contrast, the number of exposed thiols observed for the C155S and C174S mutants increases with increasing time in the reaction mixture until a final number of 0.5 to 1.6 thiols/subunit is reached.

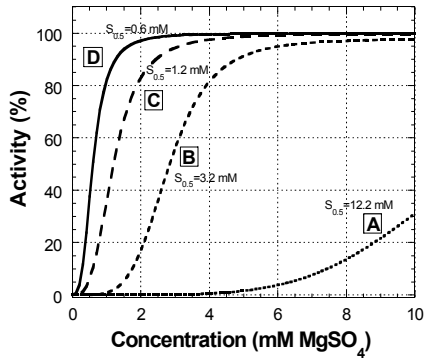

Fig.3. Mg^{2+} dependency of spinach WT and mutant FBPase

This suggests that internal cysteines become slowly exposed to the solvent. After aerobic storage of both regulatory–site spinach C155S and C174S mutants, we observed a decrease in the total free thiol content from six to about four thiols per subunit. This change was confirmed by the observation that during incubation at 25°C TNB^- is released from DTNB–modified C174S mutant protein. This implies that the Cys155–TNB bond is attacked by some other Cys, since the presence of air, which leads to an oxidizing environment, prevents the liberation of the TNB^- unless there is formation of a new disulfide bridge.

Replacements of Cys in the regulatory loop promote significant changes in the Mg^{2+} requirement of FBPase. Oxidized WT spinach FBPase is active only at very high Mg^{2+} concentrations with a half maximal saturation concentration of $S_{0.5}$ =12.2 mM (Fig. 3, curve A). By contrast, activated, reduced WT FBPase has a $S_{0.5}$ of 0.6 mM (curve D). All mutations involving only the regulatory disulfide provide an enzyme with an identical requirement for Mg^{2+} with $S_{0.5}$ = 0.6 mM (curve D). The oxidized C179S mutant (curve B) has a Mg^{2+} requirement almost four times lower ($S_{0.5}$ = 3.2 mM) than the oxidized WT enzyme. However, reducing either the C179S mutant or the double mutants containing the C179S

mutation produces a Mg^{2+} requirement intermediate between the one for the oxidized C179S form and the reduced WT protein (curve C). The C179S mutation also changes the activation kinetics. Whereas an activation by reduced thioredoxin *f* is still needed, it occurs almost instantaneous upon addition of thioredoxin.

Discussion

Our results provide independent evidence that the regulatory disulfide in spinach chloroplast FBPase is formed between Cys155 and Cys174. Whereas the WT spinach enzyme has an $E_{m,7.0}$ of –305 mV mutation of either of these two cysteines produces a protein which is constitutively active and no longer exhibits redox response. When Cys179 is replaced by serine, redox response is maintained, however the $E_{m,7.0}$ is somewhat more positive. This change in E_m, which makes reduction by thioredoxin thermodynamically more favorable, may explain the much more rapid activation by thioredoxin *f*. This indicates that structural changes occur in the regulatory loop facilitating the interaction with thioredoxin *f* and the reduction of the regulatory disulfide. The flexibility of the loop and perturbations due to the mutations of the regulatory cysteines also seem to allow the formation of an artifactual disulfide bond. During aerobic storage, the number of total free thiols in the C155S mutant decreased from 6 to around 4 per subunit and also the amount of α–helical structure decreased. For the C174S mutant, the appearance of a partial dependence on reduction was accompanied by a similar decrease of the number of total free thiols per subunit. One of the essential elements for FBPase activity is the Mg^{2+} ion. At physiological Mg^{2+} concentrations oxidized FBPase is practically inactive. Replacement of a regulatory Cys by Ser decreases the Mg^{2+} requirement to the same level as observed with the reduced WT enzyme, whereas mutation of the third Cys significantly decreases the Mg^{2+} requirement but not to the same low levels observed with the regulatory–site mutants.
In conclusion, these results clearly show that the third Cys is unlikely to have a physiological role in redox activation and that the main effect of reductive activation of chloroplast FBPase is a large decrease of the Mg^{2+} concentration needed for catalytic activity.

Acknowledgments

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