

Sulfitolysis and thioredoxin-dependent reduction reveal the presence of a structural disulfide bridge in spinach chloroplast fructose-1,6-bisphosphatase

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Abstract A significant difference between cytosolic and chloroplastic fructose-1,6-bisphosphatase (FbPase) is an extra peptide in the middle of chloroplast FbPase which contains three additional cysteine residues. Site-directed mutagenesis experiments have shown that at least two of these cysteine residues are involved in forming the regulatory disulfide bridge [Jacquot, J.-P. et al., FEBS Lett. 401 (1997) 143–147] which is the presupposition for the thioredoxin-dependent control of chloroplast FbPase activity. Here we report that each subunit of the FbPase contains an additional structural disulfide bridge which has been observed by combined application of thioredoxins and sulfitolysis. Observation of the structural disulfide bridges by sulfitolysis was only possible when the FbPase was already specifically reduced by the homologous thioredoxin species TR_m and TR_f from spinach chloroplasts. Interestingly, the accessibility of the structural disulfide bridge for sulfite ions depends on the thioredoxin species engaged in the thioredoxin/FbPase complex.

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Key words: Thioredoxin; Sulfitolysis; Fructose-1,6-bisphosphatase; Protein-protein complex; Disulfide bridge; *Spinacia oleracea*

1. Introduction

It is well established that thioredoxins are versatile redox proteins involved in the regulation of many different processes in all kinds of living cells. The redox activity of thioredoxin is due to reversible disulfide/dithiol interconversions of the active site formed by the tetrapeptide Cys-Gly-Pro-Cys. This enables thioredoxins to receive or to transfer reducing equivalents in biochemical reactions [1]. Higher plants possess multiple thioredoxins which are localized in either cytosol, mitochondria or chloroplasts. Chloroplasts of higher plants usually contain two different thioredoxin species which are essential for the light-dependent regulation of numerous enzymes. Among them are enzymes of the Calvin-Benson cycle (fructose-1,6-bisphosphatase (FbPase), sedoheptulose-1,7-bisphosphatase, phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase), sulfate assimilation (adenylsulfate kinase and 3'-phosphoadenylsulfate reductase) [2], nitrate assimilation (glutamate synthase) [3], ATP synthase (CF1), glucose-6-phosphate dehydrogenase and NADP-ma-

late dehydrogenase (NADP-MDH) [2]. The thioredoxin-dependent regulation of all these enzymes has in common that certain regulatory disulfide bridges are reductively cleaved in a dithiol-disulfide exchange reaction. The disulfide bridge of a target enzyme is initially subjected to nucleophilic attack by one of the thiol groups of reduced thioredoxin, resulting in a transient mixed disulfide bridge between thioredoxin and target enzyme. The adjacent, second thiol group of the active site of thioredoxin cleaves the mixed disulfide, in generating reduced target enzyme and oxidized thioredoxin [1]. This process induces a conformational change of the enzyme which is accompanied by an activity change. Originally, it was assumed that the oxidized thioredoxin is immediately liberated to be regenerated by the ferredoxin thioredoxin reductase. However, ample evidence is available which indicates that the thioredoxins remain complexed with the target enzyme after the dithiol/disulfide exchange reaction [4]. The individual protein-protein interactions between thioredoxin and enzyme are responsible for the fine regulation of the target enzyme activity [5,6]. Moreover, the thioredoxin/enzyme complex protects the enzyme against oxidative inhibition [7].

The redox-active cysteine residues have been identified in some but not all thioredoxin-dependent enzymes [2]. For example, site-directed mutagenesis studies with pea FbPase indicated that cysteine residues Cys¹⁷³ and Cys¹⁷⁸ form the regulatory disulfide bridge in each subunit of the tetrameric enzyme [8]. However, the recently published crystal structure of spinach FbPase revealed that the sulfur atoms of these two cysteine residues probably do not form a disulfide bridge because the distance between the sulphur atoms (Cys¹⁷⁴ and Cys¹⁷⁹ in spinach) is too large. Most recently Jacquot et al. [10] observed that besides Cys¹⁷³ and Cys¹⁷⁸ cysteine residue Cys¹⁵³ is also required for redox regulation of pea chloroplast FbPase. This observation raised the question whether in addition to the regulatory disulfide bridge a structural disulfide bridge is present per FbPase subunit. Here we present evidence for the existence of such a structural disulfide bridge in spinach FbPase derived by combination of thioredoxin-dependent reduction and sulfitolysis.

2. Materials and methods

All chemicals and reagents were of highest purity available. The chloroplast thioredoxin species TR_m and TR_f were generous gifts of Dr. Peter Schürmann, University of Neuchâtel, Switzerland. Purification of spinach chloroplast FbPase followed the procedure by Rother et al. [11]. The incubation mixture for FbPase activation contained in a volume of 0.5 ml, 0.1 M Tris-HCl buffer (pH 7.9); 5 mM MgSO₄; 4 µg FbPase. Incubation of the FbPase occurred in presence of 1 mM DTT plus thioredoxin and different concentrations of Na₂SO₃ as specified below. Reductive activation of the enzyme was completed

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Abbreviations: FbPase, fructose-1,6-bisphosphatase; mBB, monobromobimane; TR_f and TR_m, thioredoxin species from spinach chloroplasts; DTT, dithiothreitol

within 10 min at 30°C. Incubation of the FbPase with sulfite ions was performed at 30°C for 30 min. Enzyme activity was measured by the addition of 0.05 ml of 60 mM fructose-1,6-bisphosphate solution. After 10 min the reaction was stopped with 0.05 ml 72% trichloroacetic acid, 0.2 ml of the assay mixtures were mixed with 0.8 ml of phosphate reagent, and after 10 min the amount of liberated phosphate was determined photometrically at 660 nm [12].

Fluorescence labeling of liberated thiol groups after reduction and/or sulfitolysis (sulfite incubation) of the FbPase was performed with monobromobimane (mBB from Calbiochem, Thiolyte MQ). 13 µg of spinach FbPase was incubated in a total volume of 0.3 ml with different combinations of TR_f (18 µg, 1.4 nmol), TR_m (16 µg, 1.4 nmol), 1 mM DTT and 1 mM Na₂SO₃ as specified below. Reductive incubation was performed at 30°C for 20 min and sulfitolysis at 30°C for 30 min. The final labeling step (60 min; 30°C) was initiated by the addition of 0.05 ml of 60 mM mBB solution, and the labeling reaction was stopped with 0.03 ml of 72% trichloroacetic acid. SDS-polyacrylamide gel electrophoresis (10%) was applied to detect mBB labeling of the FbPase which was observed on a fluorescence screen with an excitation wavelength of 366 nm.

3. Results

Thioredoxins are specific redox regulators capable of differentiating between regulatory and structural disulfide bridges in target enzymes [1]. The specificity is due to protein-protein interactions in the thioredoxin-enzyme complex [5,6]. Numerous studies have shown that the regulatory disulfide bridges of the enzymes can be chemically cleaved with low molecular weight reductants such as dithiothreitol. It is obvious that these reductants are not as specific as the physiological thioredoxin reductant which is a protein of about 120 amino acids. Thus, it cannot be excluded that beside regulatory di-

sulfide bridges additional structural disulfides will be cleaved by unphysiological chemical reductants. The FbPase from chloroplasts is a tetrameric enzyme, in which each subunit possesses one regulatory disulfide bridge [9,10]. We established an experimental system which combines the specific reduction of FbPase with the homologous thioredoxins TR_m and TR_f from spinach chloroplasts with non-enzymatic cleavage of disulfide bridges by sulfite ions in order to investigate whether the FbPase contains additional structural disulfide bridges. Sulfityolytic cleavage of disulfide bridges into an S-sulfonyl group and an adjacent thiol group is due to nucleophilic attack by the sulfite ions. To observe the reduction and/or the sulfitolysis of disulfide bridges the liberated thiol groups were alkylated with mBB, and the incorporation of mBB was followed by its fluorescence emission. We confirmed an earlier observation of Droux et al. that oxidized spinach FbPase does not possess an accessible thiol group [15], since labeling of the FbPase with mBB did not occur in the oxidized state (Fig. 1, lanes 1 and 3). In contrast, oxidized TR_f is labeled because it contains a third cysteine residue beside the cysteine residues in the active site (Fig. 1, lanes 2 and 3). Addition of 1 mM sodium sulfite to the oxidized FbPase and TR_f caused sulfitolysis of the disulfide bridge in the active site of TR_f, indicated by the increased mBB labeling (Fig. 1, lanes 5 and 6), but sulfitolysis of the oxidized FbPase was not observed (Fig. 1, lanes 4 and 6). To observe the thioredoxin-dependent liberation of thiol groups in FbPase the specific thioredoxin reductase was replaced by 1 mM DTT. This DTT concentration was sufficient for complete reduction of the thioredoxin, but too small to significantly reduce FbPase (Fig. 1, lanes 8 and

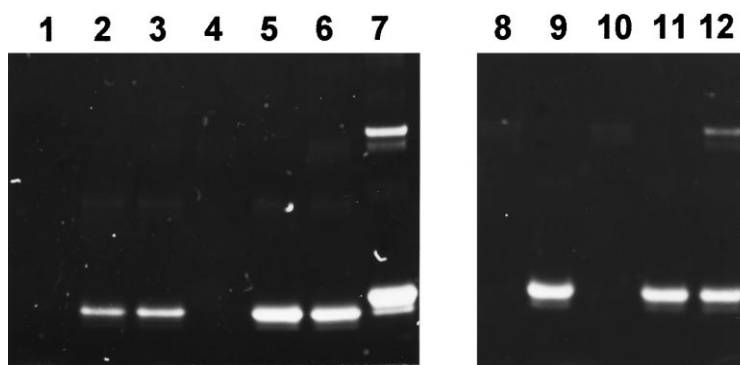


Fig. 1. Liberation of thiol groups in 18 µg TR_f and 13 µg FbPase from spinach chloroplasts, observed in response to reduction with 1 mM DTT and/or sulfitolysis with 1 mM sulfite ions by labeling thiol groups with monobromobimane (mBB). The upper and lower signals correspond to FbPase and TR_f, respectively. The reaction conditions are described in Section 2, and the different reaction mixtures are summarized as follows:

lane	FbPase	TR _f	DTT	sulfite	mBB
1	X				X
2		X			X
3	X	X			X
4	X			X	X
5		X		X	X
6	X	X		X	X
7	X	X	X	X	X
8	X		X		X
9		X	X		X
10	X		X	X	X
11		X	X	X	X
12	X	X	X		X

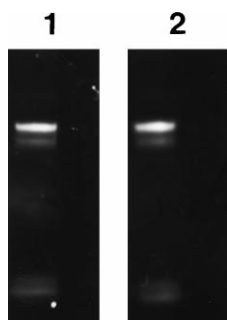


Fig. 2. Liberation of thiol groups in 16 μg TR_m and 13 μg FbPase from spinach chloroplasts, observed in response to reduction with 1 mM DTT and/or to sulfitolysis with 1 mM sulfite ions by labeling thiol groups with monobromobimane (mBB). The upper and lower signals correspond to FbPase and TR_m , respectively. For further details see Fig. 1. The reaction mixtures contained FbPase, TR_m and DTT (lane 1) and FbPase, TR_m , DTT and sulfite (lane 2).

9). Labeling of the FbPase by mBB occurred when the enzyme was reduced in presence of 1 mM DTT and 18 μg TR_f (Fig. 1, lane 12). Addition of 1 mM sodium sulfite to this mixture

produced a significant increase of the FbPase labeling with mBB (Fig. 1, lane 7). A control experiment evidenced that this labeling was not due to the combined action of DTT and sulfite ions (Fig. 1, lane 10). In our previous study of TR_f saturation kinetics it had been measured that 18 μg TR_f (1.4 nmol) saturate the activation process of FbPase [5]. Thus, the additional liberation of thiol groups in the FbPase by sulfitolysis, which is not observed with FbPase in the oxidized state, indicates that the cleavage of the regulatory disulfide bridge by thioredoxin induces a conformational change which makes a buried structural disulfide susceptible for nucleophilic attack by sulfite ions.

In another set of experiments we studied whether interaction between TR_m , the second thioredoxin in spinach chloroplasts, and FbPase produce a comparable conformational change in response to reduction. After treatment with 1 mM DTT and 16 μg TR_m (1.4 nmol) the FbPase was labeled by mBB (Fig. 2, lane 1). In presence of 1 mM sulfite ions a much smaller increase of the FbPase labeling was observed (Fig. 2; lanes 1 and 2) than in the FbPase/ TR_f combination (Fig. 1, lanes 7 and 12). Although TR_f and TR_m possess identical

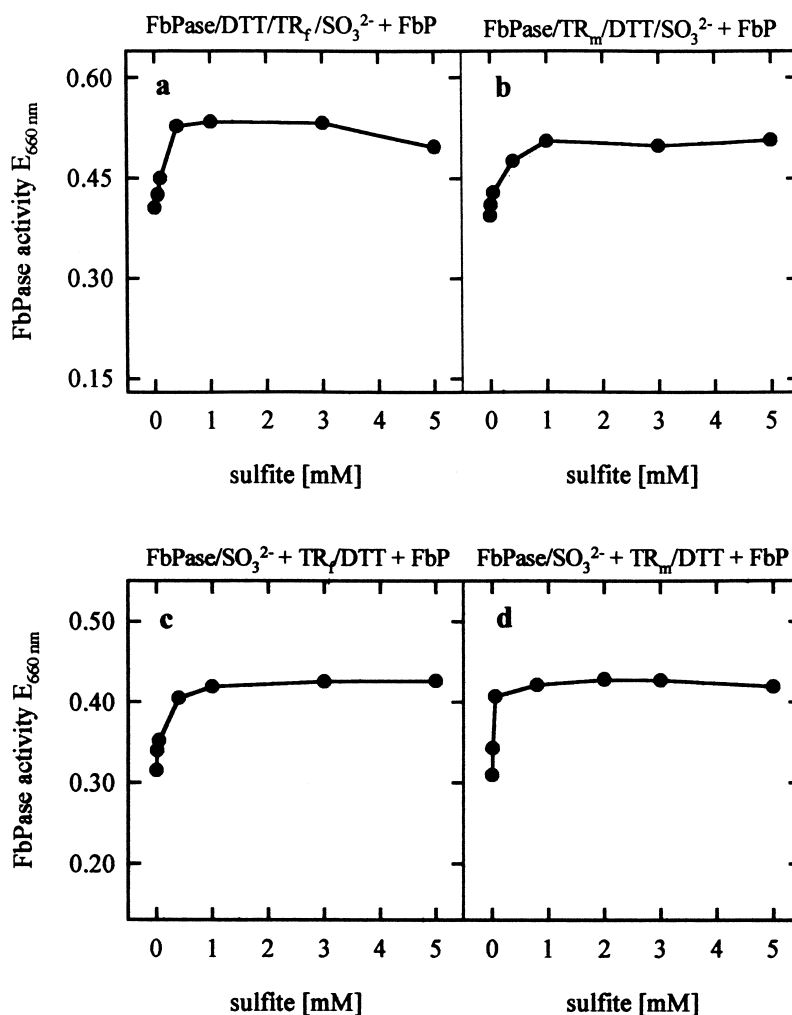


Fig. 3. Influence of increasing concentrations of sulfite ions on the fructose-1,6-bisphosphatase (4 μg) from spinach chloroplasts before and after specific reduction of the regulatory disulfide bridges with either TR_f (15 μg) or TR_m (15 μg). The sequence of incubation steps is indicated by the headline of each graph. a, b: The FbPase was incubated together with thioredoxin/DTT and sulfite ions before the catalytic reaction was initiated by the addition of FbP. c, d: The FbPase was first treated with sulfite ions and then incubated with thioredoxin/DTT before the catalytic reaction was initiated by the addition of FbP. E_{660} of 0.3 is equivalent to a specific FbPase activity of 27.5 $\mu\text{mol Pi}$ liberated/min/mg protein. The FbPase activity measured without the presence of sulfite is given at zero mM sulfite.

active sites they are not equivalent because of major differences in the amino acid sequences. Obviously, the individual protein-protein interactions between FbPase and TR_m or TR_f produce different conformational states of the FbPase. When TR_m is bound to FbPase either the structural disulfide is less accessible for sulfite ions or the liberated thiol group, which must be labeled with mBB to observe sulfitolysis, is more buried in this protein complex.

The addition of sulfite ions to FbPase assays containing 1 mM DTT and either TR_m or TR_f is not inhibitory for FbPase activity (Fig. 3a,b). Moreover, increasing concentrations of sulfite ions induced a small activity increase and it made no difference whether the sulfite ions were present during reductive activation or whether incubation with sulfite ions preceded reductive activation with TR_m or TR_f (Fig. 3c,d). Thus, sulfitolysis of the structural disulfide bridge of the FbPase is not detrimental to its catalytic activity.

4. Discussion

Fructose-1,6-bisphosphatase is a key enzyme of the Calvin-Benson cycle. The nuclear-encoded enzyme is composed of four identical subunits. The amino acid sequences of the chloroplast FbPases from spinach [16], pea [17], *Brassica napus* [18], wheat [19], *Arabidopsis thaliana* [20] and potato [21] have been determined. A significant extra feature of the chloroplast FbPases is an insertion of about 17 amino acid residues in the middle of the protein containing additional three cysteine residues. Site-directed mutagenesis experiments have revealed that the cysteine residues Cys¹⁵³, Cys¹⁷³, and Cys¹⁷⁸ (in pea FbPase numbering) are involved in the thioredoxin-dependent redox regulation, but it was not clarified which pairing of these three cysteine residues forms the regulatory disulfide bridge [10]. The three-dimensional crystal structure of the FbPase from spinach chloroplasts shows that the inserted peptide is localized at the outside corner of each subunit [9]. This exposed position confers the redox regulation to chloroplast FbPase, and mutagenesis experiments have shown that the docking site for thioredoxin precedes the regulatory sequence [22]. It was recently suggested that an additional structural disulfide bridge should be present in each subunit of the FbPase [10]. We applied a combination of specific opening of the regulatory disulfide bridge by thioredoxins and chemical cleavage of other disulfide bridges by sulfite ions. The latter reaction, which is called sulfitolysis, has been recognized to be responsible for the phytotoxic action of SO₂ in higher plants [13,14]. Liberated thiol groups were labeled with monobromobimane, and incorporation of the label was observed by the fluorescence emission of protein-bound mBB. The results demonstrate that oxidized FbPase possesses a buried structural disulfide bridge which becomes accessible for sulfitolysis only when the regulatory disulfide bridge was specifically reduced by thioredoxin (Figs. 1 and 2). Cleavage of this structural disulfide bridge by sulfite ions did not significantly influence the catalytic activity of the FbPase (Fig. 3) which indicates that this disulfide bridge is not involved in the activation process. Interestingly, the accessibility of the structural disulfide bridge was dependent on the thioredoxin species applied in the experiments. Sulfitolysis occurred to a much greater extent in combination with TR_f than with TR_m (Figs. 1 and 2). Obviously, the protein-protein interactions between FbPase and the individual thioredoxins

cause different FbPase conformations. This is further evidence that the formation of a thioredoxin-target enzyme complex is responsible for the fine regulation of enzyme activity [1]. Moreover, the results of protein labeling, e.g. the detection of disulfide bridges, depend on the individual applied thioredoxin species. Thus, only homologous systems comprising thioredoxins and target enzymes from the same plant and cell organelle will completely describe the regulatory action of thioredoxins. The data presented here indicate that the conformation of the FbPase is flexible and that crystal packing forces have probably generated a three-dimensional structure in which the distance between the cysteine residues is too long for a regulatory bridge in spinach chloroplast FbPase [9]. Our finding of a structural disulfide bridge besides the regulatory one complicates the identification of the individual cysteine residues which are involved in the regulatory, or structural disulfide bridges, respectively. It was recently suggested that cysteine residues Cys⁴⁹ and Cys¹⁹⁰ (pea FbPase) may form a disulfide bridge [10], but further experiments are necessary to evaluate this suggestion.

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References

- [1] Follmann, H. and Häberlein, I. (1996) *BioFactors* 5, 147–156.
- [2] Jacquot, J.P., Lancelin, J.-M. and Meyer, Y. (1997) *New Phytol.* 136, 543–570.
- [3] Lichter, A. and Häberlein, I. (1998) *J. Plant Physiol.* (in press).
- [4] Häberlein, I., Würfel, M. and Follmann, H. (1992) *Biochim. Biophys. Acta* 1121, 293–296.
- [5] Häberlein, I. and Vogeler, B. (1995) *Biochim. Biophys. Acta* 1253, 169–174.
- [6] Braun, H., Lichter, A. and Häberlein, I. (1996) *Eur. J. Biochem.* 240, 781–788.
- [7] Morell, S., Follmann, H., De Tullio, M. and Häberlein, I. (1997) *FEBS Lett.* 414, 567–579.
- [8] Jacquot, J.P., Lopez-Jaramillo, J., Chueca, A., Cherfils, J., Lemaire, S., Chedozeau, B., Miginiac-Maslow, M., Decottingnes, P., Wolosiuk, R.A. and Lopez-Gorge, J. (1995) *Eur. J. Biochem.* 229, 675–681.
- [9] Villeret, V., Huang, S., Zhang, Y., Xue, Y. and Lipscomb, W.N. (1995) *Biochemistry* 34, 4299–4306.
- [10] Jacquot, J.P., Lopez-Jaramillo, J., Miginiac-Maslow, M., Lemaire, S., Cherfils, J., Chueca, A. and Lopez-Gorge, J. (1997) *FEBS Lett.* 401, 143–147.
- [11] Rother, Th., Acker, G. and Scheibe, R. (1988) *Bot. Acta* 101, 311–320.
- [12] Taussky, H. and Shorr, H.E. (1963) *J. Biol. Chem.* 202, 675–685.
- [13] Häberlein, I. (1994) *Eur. J. Biochem.* 223, 473–479.
- [14] Häberlein, I. and Follmann, H. (1991) *Naturwissenschaften* 78, 259–265.
- [15] Droux, M., Crawford, N.A. and Buchanan, B.B. (1987) *C.R. Acad. Sci. Paris Sér. III Physiol. Vég.* 305, 335–341.
- [16] Marcus, F., Edelstein, I., Reardon, I. and Henrikson, R.L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7161–7165.
- [17] Carrasco, J.L., Chueca, A., Prado, F.E., Hermoso, R., Lazaro, J.J., Ramos, J.L., Sahrawy, M. and Lopez-Gorge, J. (1994) *Planta* 193, 494–501.
- [18] Rodriguez-Suarez, R.J. and Wolosiuk, R.A. (1993) *Plant Physiol.* 103, 1453–1454.
- [19] Raines, C.A., Lloyd, J.C., Willingham, N.M., Potts, S. and Dyer, T.A. (1992) *Eur. J. Biochem.* 205, 1053–1059.
- [20] Horsnell, P.R. and Raines, C.A. (1991) *Plant Mol. Biol.* 17, 185–186.
- [21] Kossmann, J., Müller-Röber, B., Dyer, T.A., Raines, C.A., Sonnenwald, U. and Willmitzer, L. (1992) *Planta* 188, 7–12.
- [22] Sahrawy, M., Chueca, A., Hermoso, R., Lazaro, J.J. and Lopez-Gorge, J. (1997) *J. Mol. Biol.* 269, 623–630.