

Heterodimer formation between thioredoxin *f* and fructose 1,6-bisphosphatase from spinach chloroplasts

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Abstract Chloroplast fructose 1,6-bisphosphatase (FBPase) is activated by reduction of a regulatory disulfide through thioredoxin *f* (Trx *f*). In the course of this reduction a transient mixed disulfide is formed linking covalently Trx *f* with FBPase, which possesses three Cys on a loop structure, two of them forming the redox-active disulfide bridge. The goal of this study was to identify the Cys involved in the transient mixed disulfide. To stabilize this reaction intermediate, mutant proteins with modified active sites were used. We identified Cys-155 of the FBPase as the one engaged in the formation of the mixed disulfide intermediate with Cys-46 of Trx *f*. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Spinach; Fructose 1,6-Bisphosphatase; Thioredoxin *f*; Site-directed mutagenesis; Intermolecular disulfide; Heterodimer

1. Introduction

In chloroplasts several enzymes, in particular enzymes involved in CO₂ assimilation like fructose 1,6-bisphosphatase (FBPase), are known to be light-regulated via thioredoxins [1]. Thioredoxins are small ubiquitous proteins with the conserved active site sequence -Trp-Cys-Gly-Pro-Cys- whose disulfide bridge gets reduced, in chloroplasts, by electrons from photosystem I via ferredoxin and ferredoxin:thioredoxin reductase. Chloroplasts contain two types of thioredoxins, the *m*- and the *f*-type. Thioredoxin *f* (Trx *f*) is distinct from the other chloroplast thioredoxin by the presence of a third conserved cysteine of unknown function [2,3]. Reduced Trx *f* modulates the activity of the light-regulated enzymes by reducing their regulatory disulfide. The crystallographic structure of Trx *f* has recently been solved [4]. It confirms that the active site cysteine closer to the N-terminus (Cys-46), which had been shown to be the attacking nucleophile [5], is surface-exposed, whereas the second one, Cys-49, is buried in the protein. The third cysteine, Cys-73, is also surface-exposed.

The regulatory disulfides of several thioredoxin-dependent

enzymes have been located. Phosphoribulokinase was the first where the molecular pathway for the regulation by Trx *f* has been described [6]. Another well studied enzyme is the NADP-dependent malate dehydrogenase, where activation by thioredoxin implies the reduction of at least two disulfide bonds [7,8].

Chloroplastic FBPase is a tetrameric enzyme and resembles the cytosolic counterpart except for the presence, in the chloroplast enzyme, of an insertion of about 20 amino acids containing three conserved cysteines. In the spinach enzyme these cysteines, Cys-155, Cys-174 and Cys-179, are located on a regulatory loop structure [9] and based on early sequencing results the regulatory disulfide was proposed between Cys-174 and Cys-179 [10]. Later site-directed mutagenesis experiments demonstrated the central role of Cys-153 in pea [11,12] and Cys-157 in rapeseed [13] FBPase for regulation. The recently published crystallographic structure of oxidized pea enzyme reveals a disulfide bridge between Cys-153 and Cys-173 [14], corresponding to Cys-155 and Cys-174 in spinach where no such data are available. The model suggests that the sulfur atom of Cys-174 is closer to the surface than the one of Cys-153.

Disulfide reduction by reduced thioredoxins proceeds via the formation of a transition state heterodisulfide between the two reactants [15]. With mutants, where the non-accessible cysteine of one or both participating reactants has been replaced, it is possible to obtain covalently linked, stable heterodimers [6,16]. These provide information on the accessibility of the individual cysteine residues and on the identity of the Cys participating in the intermolecular mixed disulfide. To obtain such information for the reduction of spinach chloroplast FBPase by homologous Trx *f* we replaced on both proteins the cysteine residues potentially involved. With these mutants we obtained evidence that a stable mixed disulfide intermediate is formed between Cys-155 of FBPase and Cys-46 of Trx *f*.

2. Materials and methods

The cloning of spinach FBPase and the plasmids used for mutagenesis of FBPase (pET 3d-FBPase) and Trx *f* (pET 3d-TFDe1 and TFDe1C73S) have been described previously [17]. All sequences and mutations were checked by automatic sequencing using a T7 primer and the sequencing kit from Amersham-Pharmacia on a MWG LI-COR sequencer.

Expression of recombinant proteins was done in the *E. coli* strain BL21 DE3 pLysS grown in Luria broth containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37°C. When the culture reached an OD_{600 nm} of 0.8, expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM.

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Abbreviations: Mes, 2-morpholinoethanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane; Trx *f*, thioredoxin *f*; FBPase, fructose 1,6-bisphosphatase; EDTA, ethylenediaminetetraacetic acid; 2-MET, 2-mercaptoethanol; DTT, dithiothreitol

After 4 h, cells were harvested by centrifugation ($5000\times g$, 6 min, 4°C).

Extraction and purification of Trx *f* included an osmotic shock [18,19], where cells were resuspended in sucrose containing buffer (50 mM 2-morpholinoethanesulfonic acid (Mes)-NaOH pH 6.1, 2.5 mM ethylenediaminetetraacetic acid (EDTA) and 20% sucrose) and, after 10 min under low agitation, were sedimented by centrifugation ($5000\times g$, 10 min, 4°C). This treatment was repeated with buffer containing no sucrose (50 mM Mes-NaOH pH 6.1, 2.5 mM EDTA and 14 mM 2-mercaptoethanol (2-MET)). After centrifugation ($10\,000\times g$, 10 min, 4°C) the supernatant containing Trx *f* was applied on an SP-Sephacrose column (5×5.5 cm, Amersham-Pharmacia) equilibrated in 50 mM malonate-Cl pH 6.0, 14 mM 2-MET. After elution with a 2 l gradient of 0–200 mM NaCl in this buffer the Trx *f* fractions were concentrated on a YM10 membrane (Amicon) and chromatographed on a Sephadex G-50 column (2.6×95 cm) equilibrated with 50 mM Mes-NaOH pH 6.1, 50 mM NaCl and 14 mM 2-MET. Trx *f* was finally concentrated on a YM10 membrane and diafiltered with 50 mM Mes-NaOH pH 6.1, 14 mM 2-MET.

For the extraction of recombinant FBPase the bacteria were resuspended in 30 mM Tris-Cl pH 8.0, 1 mM EDTA. After freezing and thawing to disrupt cell membranes 25 U/10 ml of benzonase (Merck) was added to digest nucleic acids. The resulting solution was clarified ($48\,000\times g$, 15 min, 4°C) and its pH lowered with formic acid to 5.2. Precipitated proteins were removed by centrifugation and the pH of the supernatant adjusted to 5.5 with 0.1 M NaOH. The protein extract was loaded on a Q-Sepharose column (5×11.5 cm, Amersham-Pharmacia) equilibrated in 50 mM Na-acetate pH 5.5 and eluted with a 2 l gradient of 0–1 M NaCl in buffer. Active fractions were combined and concentrated by ammonium sulfate precipitation (90% saturation). The pellets, redissolved in 20 ml of 50 mM Na-acetate pH

5.5, were chromatographed on a Sephacryl S-200 column (4×98 cm). The peak containing the enzyme was collected and the FBPase concentrated and diafiltered on a XM50 membrane (Amicon) with 50 mM Na-acetate pH 5.5. If needed a final clean up was accomplished on a 1.5×14.5 cm hydroxyapatite column (Bio-Rad, Ceramic Macro-Prep, $40\ \mu\text{m}$) with a 1 l gradient of 20–250 mM K-phosphate buffer pH 7.0. FBPase fractions were concentrated and diafiltered as before and stored frozen.

The concentration of proteins was calculated based on their absorbance at 280 nm. For recombinant FBPase we determined an extinction coefficient of $\epsilon_{280\text{ nm}} = 144\,570\ \text{M}^{-1}\ \text{cm}^{-1}$ and for Trx *f* we used $\epsilon_{280\text{ nm}} = 14\,230\ \text{M}^{-1}\ \text{cm}^{-1}$ [20]. FBPase and Trx *f* were mixed in a ratio of 1:4 (FBPase:Trx *f*) in 50 mM TEA-Cl pH 7.0 and incubated for 30 min at 25°C . When oxidant was added, it was at a final concentration of 3 mM for cupric ions (CuSO_4) or 10 mM for diamide (N,N,N',N' -tetramethylazodicarboxamide) [21].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [22] using a mini-PROTEAN II system (Bio-Rad). Gels were stained with Coomassie blue.

3. Results and discussion

To obtain stable protein–protein mixed disulfide intermediates between Trx *f* and FBPase we replaced on both proteins cysteines potentially involved in intermolecular disulfides by serines. After incubation of all possible combinations of the mutant proteins under oxidizing conditions the reaction mixtures were analyzed by SDS–PAGE under non-reducing conditions.

In Trx *f*, which contains three strictly conserved cysteines, two are accessible and capable of forming disulfide bonds. Cys-46, the accessible cysteine of the active site, has been shown to be the primary nucleophile in the activation of FBPase [5] and Cys-73, which is close to the active site [4], was found to be able to form disulfide bonds [2]. To distinguish between the two cysteines and detect possible interactions with Cys-73 we produced two Trx *f* mutants. We replaced in both Cys-49 leaving in the first mutant two accessible cysteinyl residues, Cys-46 and Cys-73, while in a second mutant we also replaced Cys-73.

In the spinach FBPase all three cysteines of the regulatory loop, Cys-155, Cys-174 and Cys-179, were individually replaced. In addition we constructed the double mutants C155/174S, C155/179S and C174/179S. Wild-type (WT) and mutant proteins were expressed and purified to homogeneity.

Samples of FBPase WT or mutant (C155S, C174S, C179S, C155/174S, C155/179S, C174/179S) were mixed and incubated at a ratio of 1:4 with samples of WT Trx *f*, mutant C49S or double mutant C49/73S in air-saturated buffer at pH 7.0. Fig. 1A,B shows the analyses of these mixtures by SDS–PAGE where we observe essentially three bands corresponding to Trx *f* (12.5 kDa), FBPase monomer (44 kDa) and a covalently linked complex of Trx *f* with FBPase monomer (~ 60 kDa).

Mixtures containing WT Trx *f* (Fig. 1, lanes a) form no heterodimeric complex since in oxidized thioredoxin its active site cysteines are linked by the internal disulfide bond. Furthermore, the absence of a heterodimer indicates that the third cysteine, Cys-73, does not form a disulfide bond with FBPase. Similarly, in virtually all reaction mixtures containing FBPase mutants lacking Cys-155 there is no complex formation. However, the 60 kDa heterodimer band clearly appears when either Cys-174 or Cys-179, or both are replaced by serine. WT FBPase forms also a small amount of heterodimer with Trx *f* mutants.

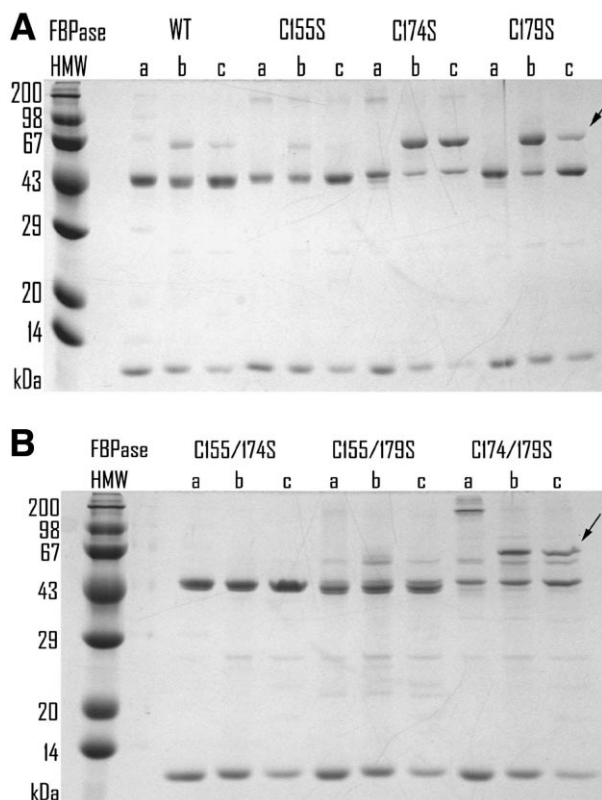


Fig. 1. A and B: SDS–PAGE analysis of reaction mixtures containing Trx *f* and FBPase. The two proteins were mixed in a ratio of 1:4 FBPase:Trx *f* in 50 mM TEA-Cl pH 7.0 buffer and incubated for 30 min at 25°C under aerobic conditions. Aliquots of the incubation mixtures were then separated on non-reducing 15% gels. Protein bands were stained with Coomassie blue. The arrow indicates the Trx *f*/FBPase complex. a: Trx *f* WT, b: Trx *f* C49S, c: Trx *f* C49/73S, HMW: prestained protein molecular weight standards (Gibco BRL).

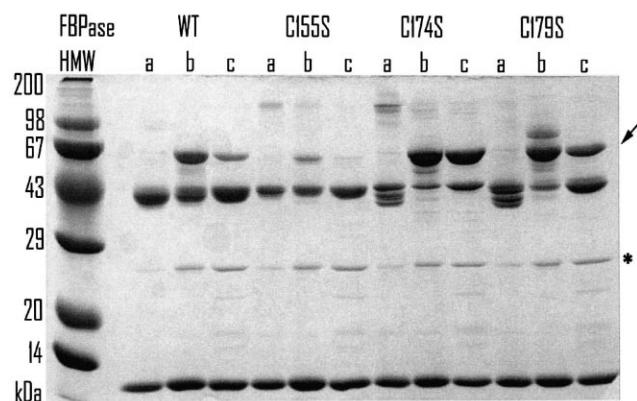


Fig. 2. SDS-PAGE analysis of reaction mixtures containing Trx *f* and FBPase incubated in presence of an oxidant. Incubation conditions were as in Fig. 1 except that 10 mM diamide was added. The arrow indicates the Trx *f*/FBPase complex and the asterisk the homodimer of Trx *f*. a: Trx *f* WT, b: Trx *f* C49S, c: Trx *f* C49/73S, HMW: prestained protein molecular weight standards (Gibco BRL).

The most relevant observation is that the formation of heterodimers is linked to the presence of Cys-155 indicating that this residue is clearly part of the disulfide bridge between FBPase and Trx *f*, although the pea enzyme structure positions the sulfur atom corresponding to Cys-174 closer to the surface [14]. The fact that Cys-155 and not Cys-174 is attacked by Cys-46 of Trx *f* suggests that as a result of the interaction between FBPase and Trx *f*, which is of electrostatic nature [23], there is some change in the conformation of the flexible regulatory loop favoring Cys-155 as the target residue. The weak band of heterodimer seen with Trx *f* C49S and FBPase C155S is probably due to an artefactual disulfide bridge with either Cys-174 or Cys-179 since it is absent from the incubations with the double mutants C155/174S and C155/179S. It could also be formed with Cys-73 of Trx *f* since it is not seen with Trx *f* mutant C73S. Mutation of Cys-155 might render the residues Cys-174 and Cys-179 more accessible due to increased flexibility of the loop in absence of the regulatory disulfide [14].

Surprisingly, the analyses of reaction mixtures containing the FBPase mutant Cys-174 or Cys-179 produce essentially identical patterns for both, favoring heterodimer formation although Cys-179 is, based on the pea enzyme structure, not implicated in a regulatory disulfide bridge. The C179S mutation probably induces some structural change in the regulatory loop enhancing the accessibility of Cys-155 for Trx *f*. However, the large amounts of complex formed between FBPase C174S and both thioredoxin mutants let us conclude that in both proteins only these active site Cys are remaining,

which are part of the intermolecular bond, i.e. Cys-155 in FBPase and Cys-46 in Trx *f* [5] (Table 1).

Complex formation happened very rapidly. Within minutes after mixing the proteins the complexes could be demonstrated and no significant further increase in their amounts was detected even after overnight incubation, which we consider as an indication for the specificity of the interaction.

In order to further enhance formation of covalent complexes we tested the addition of oxidants during incubation. Fig. 2 shows results obtained with diamide. The amount of heterodimer formed between WT FBPase and Trx *f* mutants increases significantly reaching almost the level seen with FBPase C179S, while FBPase C174S is almost completely in the intermediate complex form. By increasing the ratio of thioredoxin C49S/FBPase C174S it has not been possible to completely saturate the tetrameric FBPase with Trx *f* (data not shown). Chromatographic separation of such reaction mixtures yielded four species of heterodimeric complexes containing variable numbers of thioredoxins linked. On the other hand addition of oxidant produced detectable levels of thioredoxin homodimers implicating Cys-73 in the WT, Cys-46 and/or Cys-73 in the C49S mutant and Cys-46 in the C49/73S double mutant. Addition of cupric ions (Cu^{2+}) as oxidant [6] resulted in essentially the same patterns, but with many additional minor bands, which seem to be due to modifications of the FBPase since they are also observed after incubation of FBPase alone.

The dimerizations obtained with diamide are entirely reversible. The covalently linked heterodimers were easily separated by reduction with dithiothreitol (DTT) (Fig. 3), whereas monothiol like glutathione or 2-MET were much less efficient (data not shown).

In conclusion, we have obtained evidence for the formation of covalent heterodimers between spinach chloroplast FBPase and Trx *f* active site mutant C49S. We identified Cys-155 of FBPase as the cysteinyl residue, which forms the protein–protein mixed disulfide bond with the primary nucleophile Cys-46 of Trx *f* during the reduction/activation process. Replacement of Cys-174 and/or Cys-179, the other two cysteines on the

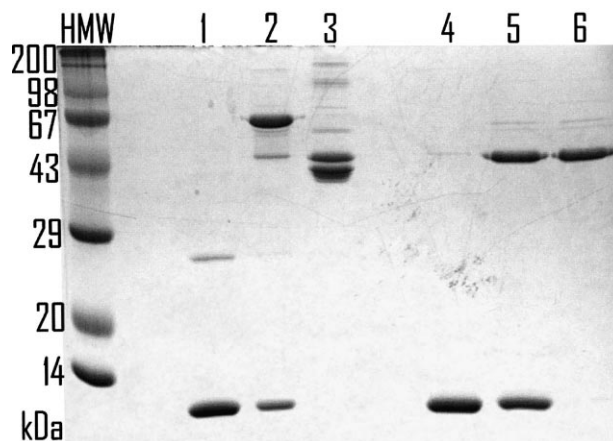


Fig. 3. SDS-PAGE analysis of reaction mixtures containing Trx *f* and FBPase incubation under oxidizing and reducing conditions. FBPase C174S and Trx *f* C49S were incubated in presence of 10 mM diamide (lanes 1–3), followed by reduction with 10 mM DTT (lanes 4–6). Lanes 1 and 4: Trx *f* C49S, lanes 2 and 5: Trx *f* C49S and FBPase C174S, lanes 3 and 6: FBPase C174S. HMW: prestained protein molecular weight standards (Gibco BRL).

Table 1
Heterodimer formations observed with different combinations of WT and mutant FBPase and Trx *f*

	Trx <i>f</i> WT	C49S	C49/73S
FBPase WT	—	++	+
C155S	—	±	—
C174S	—	+++	+++
C179S	—	+++	++
C155/174S	—	—	—
C155/179S	—	—	—
C174/179S	—	+++	++

regulatory loop of FBPase, or addition of oxidizing compounds enhance the formation of the heterodimer whereas replacement of Cys-155 abolishes it (see Table 1). These results might open the way to structural studies of the heterodimeric complex.

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