

# Chloroplast fructose-1,6-bisphosphatase: modification of non-covalent interactions promote the activation by chimeric *Escherichia coli* thioredoxins

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**Abstract** Although all thioredoxins contain a highly conserved amino acid sequence responsible for thiol/disulfide exchanges, only chloroplast thioredoxin-f is effective in the reductive stimulation of chloroplast fructose-1,6-bisphosphatase. We set out to determine whether *Escherichia coli* thioredoxin becomes functional when selected modulators alter the conformation of the target enzyme. Wild type and chimeric *Escherichia coli* thioredoxins match the chloroplast counterpart when the activation of chloroplast fructose-1,6-bisphosphatase is performed in the presence of fructose 1,6-bisphosphate,  $\text{Ca}^{2+}$ , and either trichloroacetate or 2-propanol. These modulators of enzyme activity do change the conformation of chloroplast fructose-1,6-bisphosphatase whereas bacterial thioredoxins remain unaltered. Given that fructose 1,6-bisphosphate,  $\text{Ca}^{2+}$ , and non-physiological perturbants modify non-covalent interactions of the protein but do not participate in redox reactions, these results strongly suggest that the conformation of the target enzyme regulates the rate of thiol/disulfide exchanges catalyzed by protein disulfide oxidoreductases.

**Key words:** Chloroplast fructose-1,6-bisphosphatase; Chimeric thioredoxin; Thioredoxin-f; Protein perturbant; Protein disulfide oxidoreductase

## 1. Introduction

Thioredoxins comprise a group of ubiquitous proteins whose highly conserved sequence (-W-C-G-P-C-) participates in the reduction of a wide range of proteins via a thiol/disulfide exchange mechanism [1]. In heterotrophic systems, NADPH and NADP-thioredoxin reductase transform the redox active cystine back to sulfhydryl groups whereas in oxygenic photosynthesis photochemically reduced ferredoxin and ferredoxin-thioredoxin reductase carry out this conversion [2]. Two types of thioredoxins found in chloroplasts of higher plants, trx-f and thioredoxin-m, are reduced by the light-actuated mechanism, but only the former seems to activate preferentially enzymes

that regulate photosynthetic  $\text{CO}_2$  assimilation: fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, NADP-glyceraldehyde-3-P dehydrogenase, phosphoribulokinase [3]. Like thioredoxin-m, counterparts from bacterial and animal sources are much less effective in the stimulation of chloroplast enzymes [4]. Some of these thioredoxins have been structurally well characterized but, aside from proving their role in enzyme activation, little is known about the factors involved in their interaction with target proteins. However, knowledge on this issue goes beyond the characterization of trx-f specificity for chloroplast enzymes because it contributes to the understanding of regulatory mechanisms acting on protein disulfide oxidoreductases.

The affinity between two interacting proteins varies to some extent when certain covalent bonds or intramolecular non-covalent interactions on one or both partners are transformed by (bio)chemical or physical means. Thus, alterations of non-functional thioredoxins and target enzymes should ensue variations of the final catalytic activity. Accordingly, Lamotte-Guery et al. found that the mutant D61N trx was more efficient than trx in the stimulation of chloroplast fructose-1,6-bisphosphatase but it did not match trx-f [5]. Conversely, the contribution of the enzyme to the activation process has not been explored in previous studies.

It is well documented that, like trx-f, cosolvents and chaotropic anions in concerted action with reductants, sugar bisphosphates, and bivalent cations accelerate the reduction of chloroplast fructose-1,6-bisphosphatase and, concurrently stimulate its activity [6]. We now report that, hitherto non-functional bacterial thioredoxins are indistinguishable from trx-f in the stimulation of the catalytic activity when the tertiary structure of the enzyme is modified by these protein perturbants. The remarkable outcome of these results is that the rate of thiol/disulfide exchange could be modulated changing not only members of the thioredoxin family but also the conformation of target proteins.

## 2. Materials and methods

### 2.1. Materials

Chloroplast fructose-1,6-bisphosphatase and trx-f were obtained from frozen spinach leaves as described in [7,8]. trx was purified from *Escherichia coli* cells by the procedure of Holmgren and Reichard [9]. Experimental details on the purification and the characterization of 3aa-trx and GT-trx were recently published [10]. In all preparations, the quality was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis according to Schagger and von Jagow [11] and, the concentration was estimated by the method of Lowry et al [12]. Furthermore, the concentration of trx was determined by using an absorptivity of  $13,700 \text{ M}^{-1} \cdot \text{cm}^{-1}$  [13].

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**Abbreviations:**  $A_{0.5}$ , the concentration of modulator that yields half of the maximum specific activity; trx-f, chloroplast thioredoxin-f; trx, *Escherichia coli* thioredoxin; 3aa-trx, *Escherichia coli* thioredoxin containing G-S-R-bound to the N-terminus; GT-trx, *Escherichia coli* thioredoxin fused to the C-terminus of glutathione-S-transferase of *Schistosoma japonicum*.

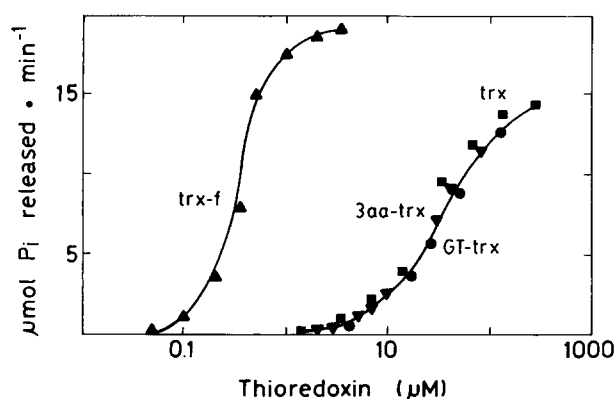


Fig. 1. Activation of chloroplast fructose-1,6-bisphosphatase by *trx-f*, *trx*, *3aa-trx*, and *GT-trx*. Chloroplast fructose-1,6-bisphosphatase (1.0  $\mu$ g) was incubated at 24°C in 0.05 ml of a solution containing (in  $\mu$ mol): Tris-HCl buffer (pH 7.9) 5.0, dithiothreitol 0.25, and the indicated thioredoxin (*trx-f* ( $\blacktriangle$ ); *trx* ( $\blacksquare$ ); *3aa-trx* ( $\blacktriangledown$ ); *GT-trx* ( $\bullet$ )). After 20 min, an aliquot was injected into 0.5 ml of the solution for the assay of fructose-1,6-bisphosphatase activity described in section 2.

## 2.2. Chloroplast fructose-1,6-bisphosphatase assay

The enzyme was incubated at 24°C in a solution of 100 mM Tris-HCl buffer (pH 7.9) containing dithiothreitol, fructose 1,6-bisphosphate,  $\text{CaCl}_2$ , trichloroacetate (or 2-propanol), and the respective thioredoxin. An aliquot was withdrawn and injected into a solution containing (in  $\mu$ mol): Tris-HCl buffer (pH 7.9) 25.0, fructose 1,6-bisphosphate 0.5,  $\text{MgCl}_2$  1.0, and EGTA 0.025 (final volume: 0.5 ml). After 10 min at 24°C, the catalytic reaction was stopped by adding the reagent for the estimation of inorganic phosphate [14].

## 2.3. Determination of fourth-derivative spectra

The protein (360  $\mu$ g) and modulators dissolved in a solution of 100 mM Tris-HCl buffer (pH 7.9) were incubated at 23°C in a spectrophotometric cuvette (1.0  $\times$  1.0 cm). Final volume: 1.0 ml. After 5 min, wavelengths were scanned from 270 nm to 300 nm in a Gilford Response spectrophotometer (bandwidth: 1.0 nm; scan rate: 60 nm/min). During scanning, digitalized data (interval: 0.1 nm) were acquired by an IBM computer through a RS-232 interface. Data of three successive

spectra were averaged and the fourth-derivative spectra was obtained (interval of derivation: 1.0 nm) by a computer program written in Pascal language (M.A. Ballicora, unpublished). Curves were smoothed by the convolution (Savitzky-Golay) method.

## 3. Results and discussion

### 3.1. Activation of chloroplast fructose-1,6-bisphosphatase by *trx*

It is well established that two successive reactions are required for chloroplast fructose-1,6-bisphosphatase activity [2]. Modulators convert slowly the enzyme to an active form (modulation) which catalyzes the rapid transformation of fructose 1,6-bisphosphate to fructose 6-phosphate and  $\text{P}_i$  (catalysis). Because *trx-f* participates in the former event but it is apparently ineffective in the latter, the  $A_{0.5}$  provides a quantitative estimation of the interaction with the target enzyme during the activation process. Accordingly, we incubated homogeneous chloroplast fructose-1,6-bisphosphatase with variable concentrations of thioredoxins in the presence of 5 mM dithiothreitol prior to assay the activity at 1 mM fructose 1,6-bisphosphate and 1 mM  $\text{Mg}^{2+}$ . As expected, *trx-f* not only was 100-fold more efficient than *trx* in the stimulation of chloroplast fructose-1,6-bisphosphatase – i.e. the  $A_{0.5}$  were 0.33  $\mu$ M and 30  $\mu$ M, respectively – but also increased the maximum specific activity 1.4-fold over that elicited by the bacterial counterpart (Fig. 1).

Given that data reported on the regulation of chloroplast fructose-1,6-bisphosphatase primarily concern the interaction with a variety of bacterial and chloroplast thioredoxins [15–17], we considered it worthwhile to make a systematic analysis of the structure-function relationship by using the *trx* molecule as ‘building block’ for the preparation of chimeric protein disulfide oxidoreductases. When a 354 Da peptide (*3aa-trx*) and a 26.6 kDa polypeptide (*GT-trx*) were linked to the N-terminus of *trx*, the former reduced insulin faster than the latter [10]. At variance, both fusion proteins were similar to *trx* in improving quite markedly the activity of chloroplast fructose-1,6-bisphosphatase.

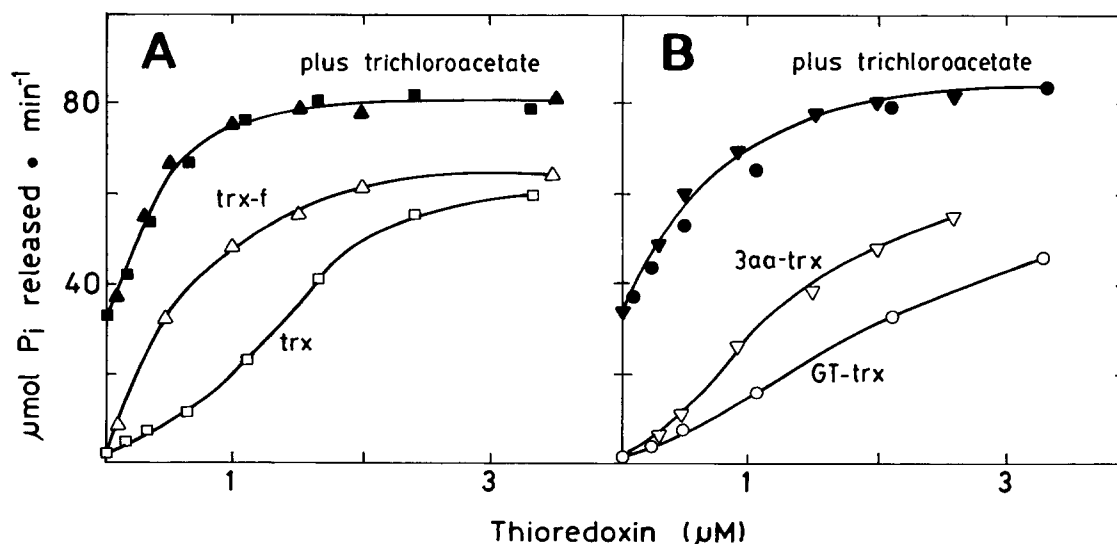


Fig. 2. Effect of trichloroacetate on the activation of chloroplast fructose-1,6-bisphosphatase by thioredoxins. Chloroplast fructose-1,6-bisphosphatase (1.0  $\mu$ g) was incubated at 24°C in 0.05 ml of a solution containing (in micromoles): Tris-HCl buffer (pH 7.9) 5.0, dithiothreitol 0.125, fructose 1,6-bisphosphate 0.075,  $\text{CaCl}_2$  0.0025 (open), and, as indicated, sodium trichloroacetate 0.45 (closed). *trx-f* ( $\blacktriangle$ ); *trx* ( $\blacksquare$ ) in A and, *3aa-trx* ( $\blacktriangledown$ ); *GT-trx* ( $\bullet$ ) in B. After 5 min, an aliquot was injected into the solution for the assay of activity described in section 2. Given that trichloroacetate accompanies the enzyme in the injection, its concentration was kept below 10 mM during catalysis.

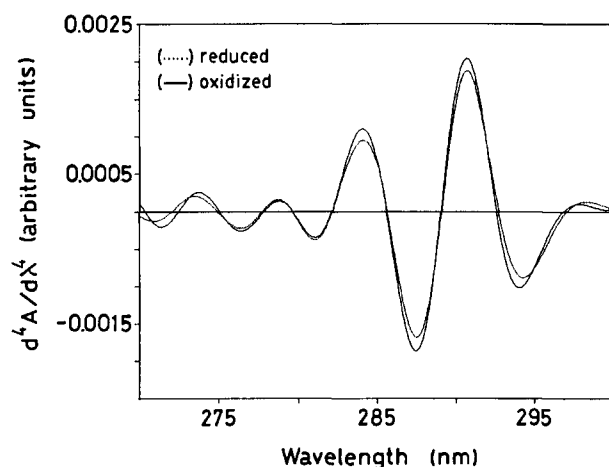


Fig. 3. Fourth-derivative spectra of *trx*. In a solution of 100 mM Tris-HCl buffer (pH 7.9), *trx* (30  $\mu$ M) was incubated at 23°C in the presence and in absence of 1 mM fructose 1,6-bisphosphate, 0.1 mM  $\text{CaCl}_2$  and, 90 mM sodium trichloroacetate. After 5 min, spectra recording, data acquisition and data handling were carried out as described in section 2. The reduced form of *trx* contained additionally 5 mM dithiothreitol during the preincubation and the subsequent scanning of the spectrum.

Of special interest was the effect of fructose 1,6-bisphosphate and  $\text{Ca}^{2+}$  because their concerted action enhances the catalytic activity and prevents the irreversible inactivation triggered by alkaline pH or protein denaturants [18,19]. When the activation of chloroplast fructose-1,6-bisphosphatase was carried out at 2.5 mM dithiothreitol, 1.5 mM fructose 1,6-bisphosphate and 0.05 mM  $\text{Ca}^{2+}$ , the  $A_{0.5}$  of *trx*, 3aa-*trx*, and GT-*trx* abruptly fell (1.3  $\mu$ M, 1.1  $\mu$ M and 1.8  $\mu$ M, respectively) whereas the  $A_{0.5}$  of *trx-f* was only slightly modified (Fig. 2). It was clear, however, that non-photosynthetic thioredoxins still did not match the ability of *trx-f* to stimulate the enzyme activity. At this stage, the incorporation of trichloroacetate (90 mM) further lowered the  $A_{0.5}$  of *trx*, 3aa-*trx*, and GT-*trx* (0.4  $\mu$ M, 0.4  $\mu$ M, and 0.7  $\mu$ M, respectively) and increased the specific activity. These results clearly demonstrated that the concerted action of fructose-1,6-bisphosphate,  $\text{Ca}^{2+}$ , and trichloroacetate makes the activation of chloroplast fructose-1,6-bisphosphatase by wild type and chimeric *Escherichia coli* thioredoxins indistinguishable from that by *trx-f*.

Cosolvents can substitute for chaotropic anions in the enhancement of chloroplast fructose-1,6-bisphosphatase [7,20]. When 15% (v/v) 2-propanol was used in place of 90 mM trichloroacetate, the  $A_{0.5}$  of wild-type and chimeric *trx* were identical to *trx-f* (not shown). However, these two protein perturbants activate chloroplast fructose-1,6-bisphosphatase by somehow different mechanisms because *trx* lowered the concentration of trichloroacetate needed for maximal stimulation from 150 mM to 90 mM but it did not alter that of 2-propanol (15% (v/v)).

Comment is in order concerning the biphasic effect of neutral salts and cosolvents on chloroplast fructose-1,6-bisphosphatase. As the concentration of trichloroacetate or 2-propanol rises, the specific activity increases steadily up to a point beyond which it decays only when the structure of chloroplast fructose-1,6-bisphosphatase is stabilized previously by both fructose 1,6-bisphosphate and  $\text{Ca}^{2+}$  [7,20]. In contrast, the

posterior addition of sugar bisphosphate and the bivalent cation does not revert the inactivation of the enzyme caused by these protein perturbants [19].

### 3.2. Fourth derivative spectra of *trx*

The analysis of the absorption spectra by means of fourth-derivative spectrophotometry resolves the contribution of tyrosine and tryptophan residues, thus allowing the study of their environments [21,22]. In a previous publication [19], we detailed four different spectra of chloroplast fructose-1,6-bisphosphatase obtained by this technique: (i) the native form, and the species incubated with (ii) fructose 1,6-bisphosphate and  $\text{Ca}^{2+}$ , (iii) fructose 1,6-bisphosphate,  $\text{Ca}^{2+}$ , and trichloroacetate, and (iv) trichloroacetate. Of these, present data show that the first three states of the enzyme were selectively activated by *trx-f* and bacterial thioredoxins whereas the latter form was totally inactive (not shown). Recent experiments on the denaturation of chloroplast fructose-1,6-bisphosphatase by urea were consistent with these states of chloroplast fructose-1,6-bisphosphatase (Hagelin, K., unpublished results).

These findings raise the question whether protein perturbants modify also the conformation of thioredoxin. As illustrated in Fig. 3, the fourth-derivative spectrum of the oxidized form shows two maxima – at 284.5 nm and 291 nm – and two troughs – 287.6 nm and 294.4 nm – whose changes in intensity were marginal upon reduction with dithiothreitol. Congruent with other studies [23], the transition from the oxidized to the reduced state did not cause large variations around two tryptophan and two tyrosine residues placed in different locations of *trx*. Subsequently, the ultraviolet spectra of the oxidized and the reduced species were scanned in the presence of 1 mM fructose 1,6-bisphosphate, 0.1 mM  $\text{Ca}^{2+}$  and 90 mM trichloroacetate. Although included in Fig. 3, these data are not perceptible

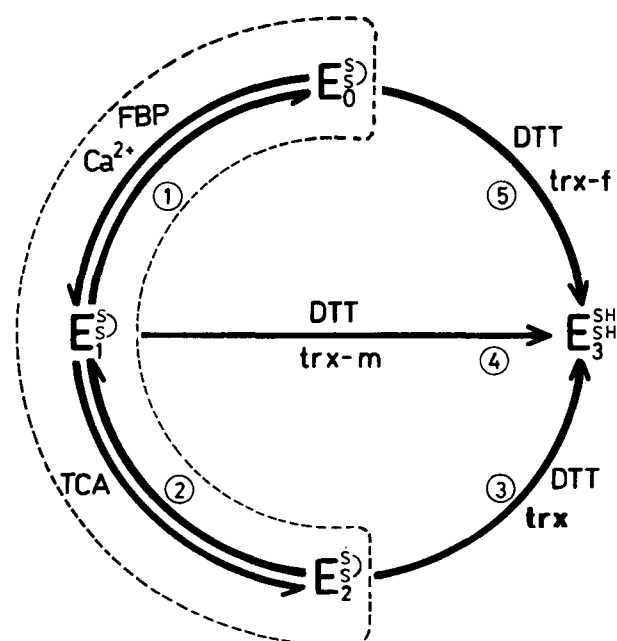


Fig. 4. The effect of thioredoxins on chloroplast fructose-1,6-bisphosphatase. E: chloroplast fructose-1,6-bisphosphatase; DTT: dithiothreitol; FBP: fructose 1,6-bisphosphate; TCA: trichloroacetate; *trx-m*: chloroplast thioredoxin-m.

because they coincided totally with those obtained in absence of modulators. Interestingly, results from parallel studies evinced similar spectra when 3aa-trx was used in place of trx. Taking together present experiments provide evidence that modulators functional in the activation and the modification of chloroplast fructose-1,6-bisphosphatase did not alter the tertiary structure of trx and 3aa-trx.

### 3.3. Activation of chloroplast fructose-1,6-bisphosphatase by thioredoxins

In principle, the low capacity of trx for the activation of chloroplast fructose-1,6-bisphosphatase could be enhanced by changing either the protein disulfide oxidoreductase or the target enzyme. The use of heterologous thioredoxins and, more recently, site-directed mutagenesis were the basis for the former approach. When we examined the latter approach, the model depicted in Fig. 4 comes to the fore (cf. Scheme II in ref. [19]). trx-f activates preferentially native chloroplast fructose-1,6-bisphosphatase (Eo) (reaction 5). Although less efficiently than trx-f, three bacterial thioredoxins that are dissimilar in the reduction of insulin have similar  $A_{0.5}$  in the activation of the native enzyme. However, the concerted action of fructose 1,6-bisphosphate and  $\text{Ca}^{2+}$  elicited the reversible appearance of a new form (E1) (reaction 1) whose activity was enhanced more efficiently by bacterial thioredoxins. At this stage, trichloroacetate or 2-propanol lead chloroplast fructose-1,6-bisphosphatase to productive folding (E2) (reaction 2) rather than commitment to denaturation and, once this partitioning occurs, the activation of the enzyme by wild type and chimeric trx are indistinguishable from trx-f (reaction 3). Although it remains to be established whether the catalytically active state (E3) constitutes a unique entity or represents various forms of the enzyme with similar kinetic properties, the important feature is that chloroplast fructose-1,6-bisphosphatase reaches the functional state through multiple paths.

It is immediately noticeable that the above model leads to consider two different aspects of thiol/disulfide exchanges in proteins. On the one hand, we know that chloroplast thioredoxin-m becomes a functional modulator of chloroplast fructose-1,6-bisphosphatase in the presence of fructose 1,6-bisphosphate and  $\text{Ca}^{2+}$  (reaction 4) [24]. Given that trx-f and thioredoxin-m coexist with sugar bisphosphates and bivalent cations in the stroma of chloroplasts, current findings are not congruent with the former as the specific activator of chloroplast enzymes. On the other hand, we can surmise that modulators partition target proteins among different conformational states, and in so doing they could regulate the action of protein folding catalysts that apparently lack (protein) substrate specificity [25].

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