Chloroplast thioredoxin mutants without active-site cysteines facilitate the reduction of the regulatory disulphide bridge on the γ-subunit of chloroplast ATP synthase

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The activity of the chloroplast H^+-ATPase (CF_oCF_1) is regulated by the proton electrochemical membrane potential and the reduction or the formation of the disulphide bridge on the γ-subunit mediated by chloroplast thioredoxins (Trx). The latter regulation also applies to the water-soluble portion of CF_oCF_1 (CF_1) and includes two successive steps, namely the binding of Trx to CF_1 and the subsequent reduction or oxidation of CF_1. To study this process thoroughly, a new expression system for spinach Trx-*f* and Trx-*m* was designed. In the presence of dithiothreitol (DTT) both forms of the expressed Trx could reduce the disulphide bridge on the γ-subunit of CF_1 and thus activate the ATPase. Trx mutants deficient in the internal, or both, cysteines of the active site were designed to study the details of the interaction. The Trx mutant proteins could still activate CF_1-ATPase in the presence of DTT and they also increased the apparent affinity of CF_1 for DTT. This implies that the binding of Trx to the CF_1 γ-subunit induces a conformational change facilitating the reduction of the disulphide bridge, and partially explains the high efficiency of Trx as a reductant *in vivo*.

Keywords: ATP hydrolysis, protein–protein interaction, reduction, thiol modulation.

INTRODUCTION

Thioredoxin (Trx) is a small protein found in all living cells [1,2]. It is a protein disulphide oxidoreductase which regulates a number of phenomena in the cell by controlling the activity of other enzymes. The reduced form of Trx reduces a disulphide bridge on a target enzyme, which adopts a new conformation modulating the activity of the enzyme. [3]. This interaction process, formation of a Trx–target protein complex and subsequent reduction of the disulphide bridge on the target enzyme, is widely accepted [4,5]. In contrast, the detailed mode of protein–protein interaction between Trx and the target enzyme, which must determine the specificity between Trx and the target, is not well understood, although there have been some reports on the interaction between Trx mutant forms and their target enzymes [6–8].

Higher plants possess multiple Trx, two of which are found in the chloroplast; *f*-type (Trx-*f*) and m-type (Trx-*m*). They are evolutionarily different from each other and their identity on the amino-acid sequence level is only 27 % in the case of spinach Trx [9]. On the other hand, the active-site sequences (-Trp-Cys-Gly-Pro-Cys-) are conserved, not only in all Trx-*f* and Trx-*m* from plant chloroplasts, but also in almost all of the Trx investigated up to the present time. The three-dimensional structure, solved by X-ray crystallography for *Escherichia coli* Trx [10,11] and by NMR for human and green algae Trx [12,13], suggests that the topology of the secondary structures of all Trx is very similar. The solved crystal structures of both Trx-*f* and Trx-*m* [14] revealed that these two chloroplast Trx have structures similar to those already reported. Therefore the specificity of each Trx must be related to the surface charges on the molecule [15].

Trx-*m* has been found as a Trx which can reduce the $NADP^+$-specific malate dehydrogenase and it is relatively similar to the prokaryotic Trx. Trx-*f* has been designated as the Trx for fructose-1,6-bisphosphatase. This Trx is classified into the eukaryotic group according to amino acid sequence analysis, but it is somewhat different from the cytosolic Trx, Trx-*h*. Following the efficiency of activation by the increase in enzyme activity, several enzymes in the Calvin cycle (glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and phosphoribulokinase) and the ATP synthase on the thylakoid membranes (CF_oCF_1) were assigned as target enzymes of Trx-*f* [9,16]. The preference of the target enzymes for either Trx-*f* or Trx-*m* is determined by the characteristics of the contact region on the target.

The chloroplast target enzyme, CF_oCF_1, is a well-studied membrane protein complex which synthesizes ATP from ADP and P_i at the expense of a proton-motive force across the thylakoid membrane [17,18]. This ATP synthase is a latent enzyme; the activity is regulated by the proton gradient across the thylakoid membrane, which activates the enzyme, and by the reduction or oxidation of a disulphide bridge on the γ-subunit, which modulates the activity. The latter regulation is known as thiol modulation [19,20]. The structural basis for the thiol modulation is assigned to a sequence motif of nine amino acids which include two cysteines in the γ-subunit of CF_1, the water-soluble, membrane-attached moiety of CF_oCF_1. In the γ-subunit of spinach CF_1, these are Cys-199 and Cys-205 [21]. The role of

Abbreviations used: CF_oCF_1, ATP synthase on thylakoid membrane; CF_1, water-soluble portion of CF_oCF_1; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; RT, reverse transcription; TMR, tetra-methylrhodamine; Trx, thioredoxin(s); Trx-f_{C40S}, Trx-*f*-type in which Cys-40 was replaced by serine; Trx-$f_{C37S/C40S}$, Trx-*f*-type in which two cysteine residues at the active site were replaced by serines; Trx-m_{C41S}, Trx-*m*-type in which Cys-41 was replaced by serine; Trx-$m_{C38S/C41S}$, Trx-*m*-type in which two cysteine residues at the active site were replaced by serines.

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these cysteines for thiol modulation was confirmed by the introduction of mutations into this region [22–24] and by the insertion of this region into the γ-subunit of the thiol-insensitive CF_1 from cyanobacteria [25]. Reduction *in vitro* of the disulphide bridge formed by these cysteine residues can be achieved by high concentrations of dithiothreitol (DTT) or other dithiols, but the natural reductant, the reduced form of Trx, is 10^4-fold more reactive [16]. Trx, in turn, is reduced by the photosynthetic electron transport chain via ferredoxin and ferredoxin-Trx reductase *in vivo* [1,26].

There have been few investigations on the interaction between CF_1 and Trx. Dann and McCarty [27] studied the activation of spinach CF_1 by *E. coli* Trx and found that this Trx activated the ATPase of the membrane-bound CF_1 in the presence of a light-induced membrane potential. They also postulated that Trx could form a mixed disulphide complex with CF_1. To investigate why the membrane potential facilitates reduction of CF_1 by Trx, they compared the activation characteristics of the isolated whole CF_1 complex with the ϵ-subunit-deficient complex. However, no differences were found in this experiment [27]. Furthermore, little is known of the difference between Trx-*f* and Trx-*m* for the activation of CF_1 [19,28]. Comparing the effects of recombinant chloroplast Trx-*f* and Trx-*m* on the acceleration kinetics of membrane-bound Cf_oCF_1, Schwarz et al. [16] found that Trx-*f* was a better reductant for this complex. Their report is, to our knowledge, the only one at the present time describing a remarkable difference between Trx-*f* and Trx-*m* in the stimulation of CF_1-ATPase.

In the present study the construction of a new expression system for both the mature *m*- and *f*-type spinach chloroplast Trx in *E. coli* is reported. Mutations at the active-site region were designed in order to investigate the interaction between the chloroplast Trx and CF_1, independently of the subsequent reduction step. The analysis of the activation mode of CF_1 by Trx and Trx mutant forms suggests that the protein–protein interaction between Trx and the target enzyme is not only very important for the recognition of the target but also influences the activation process itself.

MATERIALS AND METHODS

Materials

The *Taq* DNA polymerase was purchased from Takara Inc. (Tokyo, Japan). Restriction endonucleases were obtained from Toyobo Inc. (Tokyo, Japan). The protein assay system was from Bio-Rad Inc. (Hercules, CA, U.S.A.). Urea was purchased from Nacalai Tesque (Kyoto, Japan). DTT and *E. coli* Trx were from Sigma (St. Louis, MO, U.S.A.). Tetra-methylrhodamine (TMR)-maleimide was from Molecular Probes (Eugene, OR, U.S.A.). Other chemicals were of the highest grade commercially available.

Construction and expression of Trx plasmids

Total mRNA from spinach leaves was prepared by the method described in [29]. The full-length DNA of the coding region of Trx-*f*, without transit sequence, was obtained by reverse transcription (RT)-PCR using spinach mRNA. The oligonucleotides CthFR and CthFF (shown below) were designed as primers according to the published sequence for spinach Trx-*f* [30]. The DNA fragment obtained from PCR was cloned into the *Nco*I and *Eco*RI sites of pET-23d(+) (Novagen, Madison, WI, U.S.A.). The full-length DNA of the Trx-*m* without transit sequence was obtained in the same way and cloned into the *Nde*I and *Hin*dIII sites of pET-23a(+). The oligonucleotides CthMR and CthMF (shown below) were designed from the published sequences [31], except that an initial codon ATG was added in front of the coding region of the mature size Trx. The integrity of the coding region of the Trx was confirmed by DNA sequencing (LI-COR, Lincoln, NB, U.S.A.).

The oligonucleotides used for RT-PCR were as follows (the restriction sites used on the primers are underlined): CthFR, 5′-GCGAATTCAACTACTTCGAGCAGCTTG-3′ (*Eco*RI); CthFF, 5′-AACTGCAGCCATGGAAGCCATTGTAGGGA-AAGTGACTGAAG-3′ (*Nco*I); CthMR, 5′-CCCAAGCTT ATTTTAAGGAGATAAGTATT-3′ (*Hin*dIII); CthMF, 5′-GGGAATTCCATATGAAGGCTAGTGAAGCTGTCAAGG AAG-3′ (*Nde*I). Plasmids for both Trx-*f* and Trx-*m* were transformed into the expression host *E. coli* strain BL21(DE3), and spread on plates of Luria–Bertani medium containing 50 μg/ml ampicillin. A single colony was cultured in 3 ml of liquid medium containing 50 μg/ml ampicillin. An overnight culture in 1 litre of same medium was incubated at 37 °C until a D_{600} 0.8 was attained. After induction with 0.4 mM isopropyl β-D-thiogalactoside, the cells were grown for a further 2–3 h, collected and stored at −20 °C.

Expression of mutant Trx

Oligonucleotide-directed mutagenesis was carried out as described previously [24,32]. The oligonucleotides used to create the mutant Trx are listed in Table 1. The desired plasmids were identified by restriction digestion at the sites introduced into the plasmid DNA by the mutagenesis primers and the entire coding sequences were confirmed by DNA sequencing. Each of the plasmids was transformed into the expression host *E. coli* strain BL21(DE3), and expressed as in case of the wild-type Trx. The mutant Trx forms are designated in the following manner: Trx-f_{C40S} and Trx-m_{C41S} for the single mutants in which one active-site cysteine residue was replaced with serine; Trx-$f_{C37S/C40S}$ and Trx-$m_{C38S/C41S}$ for the double mutants in which both active-site cysteine residues were replaced with serines.

Table 1 Primers used for the Trx mutants

Restriction sites introduced with the primers are underlined. The amino acid sequence of the active site region is shown. The position of the cysteine to serine substitution is reversed.

Mutant	Designed mutagenesis primer in 5′–3′ direction	Restriction enzymes	Amino acids
Trx-f_{C40S}	ATTTTGGAGCCATTGCTTTCGAAGGTCCACACCATTGAG	*Nsp*V	WCGPSK
Trx-$f_{C37S/C40S}$	ATTTTGGAGCCATTGCTTTCGAAGGGCCCGACCATTGAGTAAACATATC	*Nsp*V, *Apa*I	WSGPSK
Trx-m_{C41S}	ACCGGGGCGATGAGCTTCGAAGGTCCACACCATGGG	*Nsp*V	WCGPSK
Trx-$m_{C38S/C41S}$	ACCGGGGCGATGAGCTTCGAAGGTCCTGACCATGGCGCCCAGAAGTCCACCATCA	*Nsp*V, *Nar*I	WSGPSK

Purification of the recombinant Trx

The frozen cells harbouring the recombinant Trx plasmids were suspended in ice-cold lysis buffer (20 mM Tris/HCl, pH 8.0/2.5 mM EDTA) for 10 min on ice. After centrifugation (10 min, 800 ***g***), the supernatant was adjusted to the buffer composition used for hydrophobic-interaction chromatography [50 mM Tris/HCl, pH 7.0, containing 0.5 M $(NH_4)_2SO_4$ for wild-type Trx or 1.0 M $(NH_4)_2SO_4$ for mutant Trx] and loaded on to a Butyl–Toyopearl column (20 mm × 50 mm; Tosoh, Tokyo, Japan) equilibrated with the same buffer. Elution was achieved by 0.5 M $(NH_4)_2SO_4$ (for wild-type Trx) or a linear gradient from 1 M to 0 M $(NH_4)_2SO_4$ (for mutant Trx). Proteins in each fraction were analysed by SDS/PAGE and the fractions containing recombinant Trx were pooled and concentrated using an Ultrafree-15 filter (Biomax-5; Millipore, Bedford, MA, U.S.A.).

Recombinant Trx was further purified by gel-filtration chromatography with a TSK-G2000SW_{XL} column (Tosoh), which was equilibrated with 25 mM Tris/HCl, pH 7.0/150 mM NaCl. The Trx preparations were stored at −20 °C.

The N-terminal sequences of the wild-type Trx were analysed using a protein sequencer (model PPSQ21; Shimadzu, Kyoto, Japan). The protein concentration was calculated from the A_{278} using the published molar absorption coefficient values of 16830 $M^{-1}\cdot cm^{-1}$ for Trx-*f* and 20500 $M^{-1}\cdot cm^{-1}$ for Trx-*m* [33].

Matrix-assisted laser desorption–time-of-flight (MALDI-TOF) mass spectroscopy

The molecular mass of the wild-type Trx was measured by MALDI-TOF mass spectroscopy (LASERMAT2000, Finnigan Mat, San Jose, CA, U.S.A.). Sinapic acid was used as the matrix. Substance P ($M_r = 1347.7$) and α-lactalbumin ($M_r = 14178$) served as standards for the calibration.

Fluorescence labelling

The Trx sample buffer was changed to 25 mM Hepes/NaOH, pH 7.9/150 mM NaCl by gel filtration. After reduction with 20 μM DTT (1 h at room temperature), the cysteine residues in the protein molecule were labelled with 5 μM TMR-maleimide for 1 h at room temperature, followed by SDS/PAGE to separate the free label from the protein.

Preparation of CF_1 from spinach leaves

CF_1 was extracted from spinach by a modification of the method of Andreo et al. [34]. For further purification, a 5 ml t-butyl hydrophobic-interaction chromatography Econo-Pac Cartridge (Bio-Rad, Hercules, CA, U.S.A.) equilibrated with 50 mM Tris/HCl, pH 8.0/1 mM ATP/0.5 mM EDTA/0.5 M $(NH_4)_2SO_4$ was used. The crude extract, dissolved in the same buffer, was loaded on to the column and the proteins were eluted by a gradient of 0.5–0 M $(NH_4)_2SO_4$. The five-subunit CF_1 was eluted as the third protein peak at approximately 0.15 M $(NH_4)_2SO_4$. The CF_1 preparations were stored as $(NH_4)_2SO_4$ precipitates at 4 °C. Before use, the preparation was desalted by passage through a Sephadex G-25 column (15 mm × 100 mm) equilibrated with 50 mM Tricine/KOH (pH 8.0).

Activation of CF_1 and the measurement of ATPase activity

To reduce CF_1, the enzyme solution (10–50 nM) was incubated in the presence of Trx (0.2–4 μM and 1 mM DTT (if not stated differently) for 15 min at 30 °C. The steady-state ATPase activity was measured as follows: to initiate the reaction 10 μl of the enzyme solution was added to 90 μl of a reaction mixture containing 50 mM Tricine/KOH, pH 8.0/4 mM ATP/1 mM $MgCl_2$/50 mM Na_2SO_3. The reaction was continued for 10 min at 37 °C and terminated by the addition of 100 μl of ice-cold 2.4% (w/v) perchloric acid. The amount of liberated P_i was quantified colorimetrically [35] and is presented as the average of two independent measurements.

Other methods

The protein concentrations were determined by the method described in [36]. SDS/PAGE was carried out as in [37].

RESULTS

Expression and purification of recombinant chloroplast wild-type and mutant Trx in soluble form

A new expression system for mature Trx-*f* and Trx-*m* is described in this section. Starting from total spinach mRNA, the DNA coding for the mature size of Trx-*f* and Trx-*m* was obtained by RT-PCR as described in the Materials and methods section. Direct cloning into pET-23 vectors resulted in the desired expression plasmids as confirmed by DNA sequencing. In the case of Trx-*m*, an N-terminal methionine residue was introduced for the initiation of the translation.

Isopropyl β-D-thiogalactoside induction for the expression of Trx-*f* and Trx-*m* gave proteins of the expected size and in soluble form. N-terminal sequencing confirmed the first ten Trx-*f* and the first 15 Trx-*m* amino acids. Wild-type Trx was purified by hydrophobic-interaction chromatography and gel-filtration chromatography as described in the Materials and methods section. For the four spinach Trx mutants (Table 1) obtained by the Kunkel mutagenesis method [32], the purification was slightly adjusted. A linear-gradient elution was used for the hydrophobic-interaction chromatography, and the purity of the proteins was confirmed by SDS/PAGE (Figure 1). As Trx appear to be smaller on SDS/PAGE than expected, their molecular mass was

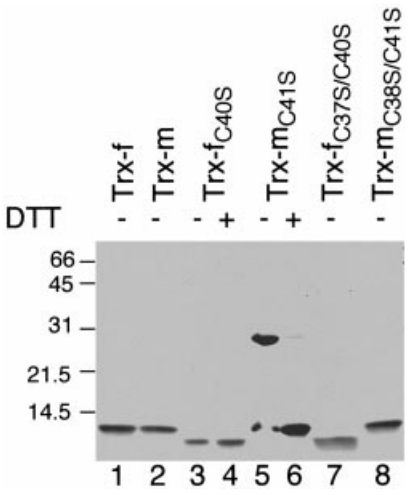


Figure 1 SDS/PAGE analysis of the purified recombinant mature spinach chloroplast Trx and Trx mutants

A 15% (w/v) polyacrylamide gel was used. Each lane contained 3–5 μg purified Trx. Trx-f_{C40S} and Trx-m_{C41S} were incubated for 15 min at room temperature with 10 mM DTT to reduce the dimer (lanes 4 and 6). The Trx-*f* mutants display a smaller apparent size in comparison with wild-type Trx-*f*. The positions of molecular-mass markers are shown on the left.

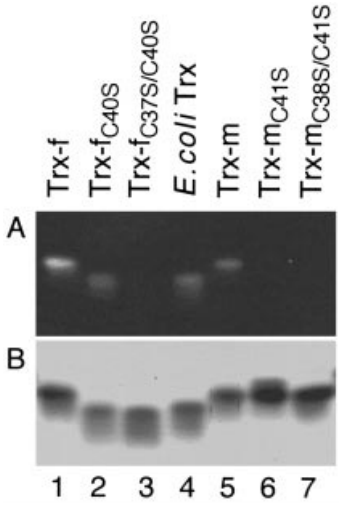


Figure 2 TMR-maleimide labelling of cysteine residues

(**A**) The cysteines of the Trx were reduced with DTT and labelled with TMR-maleimide. Separation from free label was achieved by SDS/PAGE and the labelled proteins were revealed with UV-light. Wild-type Trx-*f* has three cysteine residues, whereas Trx-*m* and *E. coli* Trx have two. (**B**) Coomassie Brilliant Blue R-250 stain of the same gel. Each lane contained 3–5 μg Trx.

verified by MALDI-TOF mass spectroscopy. Values for the wild-type Trx-*f* and Trx-*m* were 12899 Da (calculated mass, 12579 Da) and 12791 Da (calculated mass, 12811 Da) respectively, confirming the integrity of the samples.

Trx-f_{C40S} and Trx-m_{C41S} are the mutants which possess only one exposed cysteine in the active site and could, therefore, possibly form a dimer. During gel filtration chromatography only Trx-m_{C41S} was obtained as a monomer and a dimer in separate fractions. However, the reduction with 10 mM DTT for 15 min at room temperature led to the monomer seen on SDS/PAGE (Figure 1, lanes 5 and 6).

Purity and integrity of the mutant Trx preparations

To assess possible contamination by *E. coli* Trx, the following experiment was designed because no *E. coli* strain with a deletion of all Trx genes was available. Purified Trx-$m_{C38S/C41S}$ was incubated with 10 mM DTT, which should reduce potentially contaminating Trx from *E. coli*. DTT was removed by gel filtration (Sephadex G-25; 15 mm $\times$ 100 mm column) and the Trx fraction was checked for activation of CF_1. *E. coli* Trx was used as a control. Whereas 75 nM *E. coli* Trx activated CF_1 1.5-fold, no activation was observed with 200 nM Trx-$m_{C38S/C41S}$ (results not shown). Therefore the amounts of potentially contaminating Trx from *E. coli* in the recombinant Trx preparations were negligible.

Furthermore, to check the substitution of the thiol group in each of the mutant Trx and to confirm the absence of contaminating *E. coli* Trx in mutant preparations by an independent method, the thiol groups of purified Trx were labelled with TMR-maleimide. Comparable amounts of Trx (stained by Coomassie Brilliant Blue R-250) result in clearly different fluorescence reflecting the loss of cysteines (Figure 2). As Trx-*f* and its mutants contain an additional cysteine residue (Cys-64), their fluorescence intensity is greater than that of the respective Trx-*m*. No contaminating *E. coli* Trx was detected.

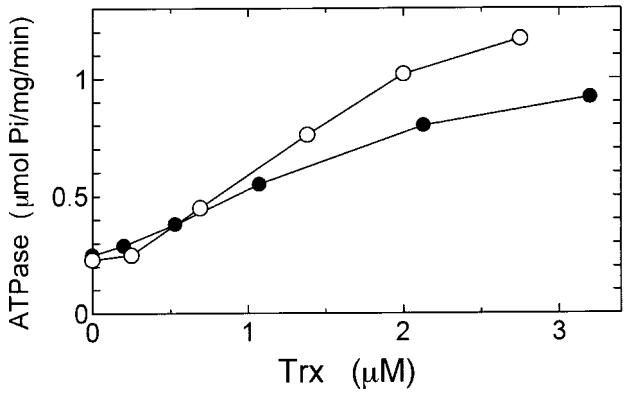


Figure 3 Activation of CF_1 by recombinant Trx-*f* and Trx-*m*

CF_1 (31 nM) was incubated with the indicated concentration of Trx in the presence of 1 mM DTT for 15 min at 30 °C. The ATPase activity was measured as described in the Materials and methods section. The resulting ATPase activity is plotted against the concentration of Trx-*m* (○) and Trx-*f* (●).

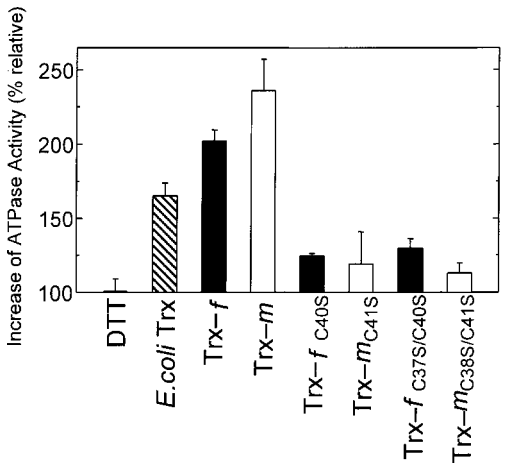


Figure 4 Activation of CF_1 induced by different Trx incubated with DTT

The ATPase activity of 38 nM CF_1 after incubation with different Trx (640 nM) is compared. The activation conditions were as described in the legend of Figure 3. The intrinsic Mg^{2+}-ATPase activity of CF_1, measured without DTT, was used as reference. The relative increase from the control activity by incubation with DTT or various Trx was calculated. Error bars indicate the S.D. calculated from three independent experiments.

Activation of spinach CF_1-ATPase by Trx

Isolated CF_1 is a latent ATPase and can be activated by the reduction of the disulphide bridge on the γ-subunit. The ability of Trx to regulate CF_1 was investigated and the results are shown in Figure 3. Both Trx-*f* and Trx-*m* activated CF_1-ATPase in the same range of Trx concentrations as those reported for the combination of *E. coli* Trx and isolated CF_1 [27]. However, no remarkable differences in the effects of the two spinach chloroplast Trx were found under the chosen conditions.

Effect of the mutant Trx on the activation of CF_1

To separate the interaction between Trx and CF_1 from the subsequent reduction process, we investigated the effect of the mutant Trx lacking active-site cysteines on the activation of soluble CF_1 (Figure 4). All the Trx mutants caused a slight but

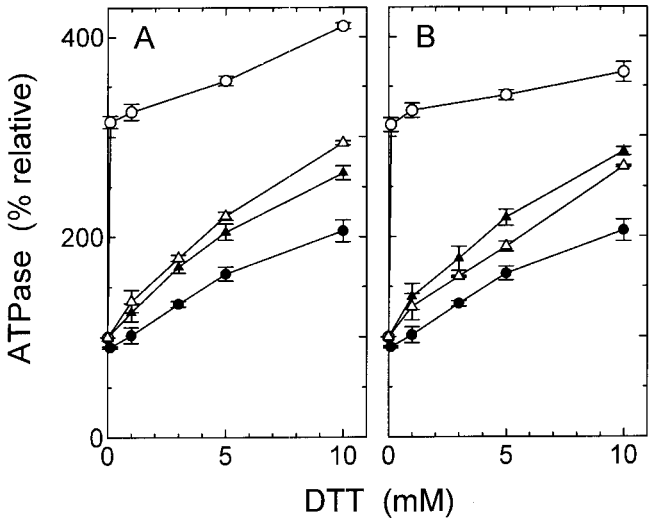


Figure 5 Activation of CF_1 assisted by Trx in the presence of DTT

CF_1 (36–50 nM) was incubated (30 °C for 15 min) with the indicated DTT concentration and the ATPase activity was measured as described in the Materials and methods section. The ATPase activity (0.20 μmol P_i/min per mg of protein) in the absence of DTT was set to 100%. (**A**) Without (●) or with 1 μM Trx-*f* (○), Trx-f_{C40S} (△) or Trx-$f_{C37S/C40S}$ (▲). (**B**) As in (**A**) but with Trx-*m* (○), Trx-m_{C41S} (△), Trx-$m_{C38S/C41S}$ (▲) or without Trx (●). Error bars indicate S.D. from three independent experiments.

detectable increase in the ATPase activity of CF_1 in the presence of DTT. This activation depended on the Trx concentration (results not shown), suggesting that the effect was not directly promoted by DTT in the reaction mixture. CF_1 alone had a relatively low intrinsic Mg^{2+}-ATPase activity (0.20–0.25 μmol P_i/min per mg of protein after incubation with 1 mM DTT), but this could be activated at least 6-fold by wild-type Trx.

Because the mutant Trx used for these experiments did not possess active site cysteines, and all mutants had a similar effect, the activation observed must relate to protein–protein interaction between the mutant Trx and the γ-subunit of CF_1 itself. However, as no activation by the mutants was observed without DTT (results not shown) a synergistic effect was conceivable. Therefore the DTT-concentration-dependence of CF_1-ATPase activation was investigated in combination with different Trx preparations (Figure 5). DTT alone was used as a control. The addition of wild-type Trx substantially increased the CF_1-ATPase activity at concentrations as low as 0.1 mM DTT. As shown in Figure 5, the stimulatory effect of each of the Trx mutants was DTT-concentration-dependent, and they were unable to activate the ATPase in the absence of DTT. These results strongly suggest that Trx is not only a reductant for the target disulphide bridge but also an important factor which specifically interacts with the cysteine region of the γ-subunit, changing its conformation and facilitating reduction by DTT.

DISCUSSION

Higher plants have at least five Trx in the cytosol [38] in addition to the two classical chloroplast Trx [39]. The physiological importance of multiple forms of Trx in the plant cell is far from being understood. Moreover, the reason for the specificity of the Trx types remains enigmatic and justifies further study of the reaction mechanism. In the case of CF_oCF_1, Trx-*f* was more efficient than Trx-*m* [16], even though both the active site and overall structure are conserved. Therefore the interaction between Trx and the target CF_1-γ must determine this specificity. The efficiency of the interaction should relate to the surface topology of each protein molecule and to the relative positions of their complementary amino acid. One recent study focused on the effect of amino acid substitutions to make *E. coli* Trx more similar to Trx-*f* [15]. The authors concluded that surface charges determined the affinity of Trx for its target enzyme.

The amino acid sequence identity among spinach Trx-*f*, Trx-*m* and *E. coli* Trx is relatively low. Therefore only studies carried out with proteins obtained from the same organism can be considered to be close to physiological. This prerequisite prompted us to set up a new expression system for mature spinach chloroplast Trx. One of the advantages of our system, in contrast to earlier methods [33,40], is that no inclusion bodies were formed during expression. As a result, the same purification steps could be used for the various Trx forms, making comparison between the effects of Trx-*f* and Trx-*m* easier.

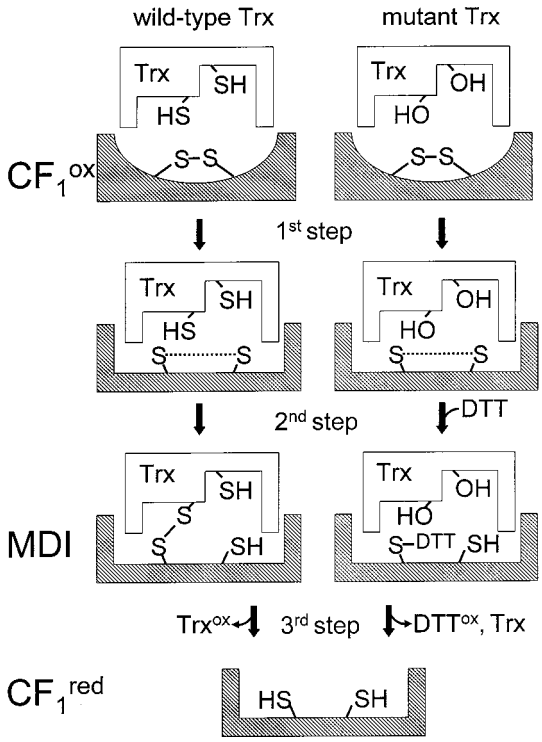
Both Trx from spinach chloroplasts could activate latent CF_oCF_1, but the activation rate by Trx-*f* was approx. six times that with Trx-*m* [16]. This difference was not observed for soluble CF_1 (Figure 3) and is in agreement with an earlier report that *E. coli* Trx and Trx-*m* have the same efficiency [28]. The difference in second-order rate constants observed for Trx-*m* and Trx-*f* activation of CF_oCF_1 is probably due to the fact that Schwarz et al. [16] were comparing Trx effects on the membrane-embedded enzyme in the presence of a proton gradient. This is known to affect the overall structure of the complex [41]. It should be noted that, in the present work, the Mg^{2+}-ATPase activity was always measured, which yields 10-fold lower values compared with the Ca^{2+}-ATPase activity [27]. This difference does not affect the activation characteristics.

Studies with reduced wild-type Trx always focus on the reduction step, which is several orders of magnitude faster than the preceding step, complex formation [16]. To study complex formation separately, mutants deficient in the active-site cysteines were designed. This new approach might lead to an understanding of the specificity of Trx for its target, that goes beyond the established reaction mechanism which emphasizes the role of the two active-site cysteines [42]. Up to this time the transfer of reducing equivalents has been the main interest in the field and the preceding recognition step has not been well studied.

In the mutants described in this report, the disulphide bridge between the active site cysteines cannot be formed, and these mutant Trx were necessarily in the 'reduced conformation'. This small change caused a difference in behaviour during hydrophobic-interaction chromatography and SDS/PAGE (Figure 2). Moreover, of the mutants with only one exposed cysteine, Trx-m_{C41S} tended to form dimers as observed by SDS/PAGE (Figure 1) and gel filtration.

All of the mutants were less efficient than wild-type Trx for the activation of CF_1 in the presence of DTT (Figure 4) and did not activate CF_1 in the absence of DTT (results not shown). In the presence of 1 mM DTT, wild-type Trx activated the ATPase activity of CF_1 up to 6-fold, depending on the concentration of added Trx (Figure 3). However, the mutants showed much less activation at 1 mM DTT. Wild-type Trx was reduced by DTT and then could directly activate CF_1 very efficiently. In contrast, the mutants could not be reduced by DTT because of the lack of one or both of the active-site cysteines, which is why an additional role for DTT must be considered to explain this observation.

When the DTT concentration dependence of the activation of CF_1-ATPase was measured in the presence or absence of Trx and their mutants, both mutants of Trx-*f* and Trx-*m* showed a behaviour very different from the wild-type (Figure 5). The



Scheme 1 Model showing the protein–protein interaction of Trx and CF_1 before reduction of the disulphide bridge on the γ-subunit of CF_1

Trx binds to inactive CF_1 (CF_1^{ox}, oxidized) and induces a conformational change affecting the disulphide bridge of the target protein (1st step). The mixed-disulphide intermediate (MDI) can be formed (2nd step) with wild-type Trx (left) or DTT (right). Finally, Trx^{ox} (left) or DTT^{ox} and the Trx mutant (right) is released (3rd step).

observed dependency implied that the affinity of DTT for CF_1 was altered by the addition of the Trx mutants. Mutants Trx-f_{C40S} and Trx-m_{C41S} were equally efficient, indicating that the rate-limiting step was the same for both mutants. On a molecular level, Trx mutants are thought to bind to the target enzyme and concomitantly induce a conformational change so that the disulphide bridge of the target enzyme becomes more susceptible to reduction by DTT. The same should apply to wild-type Trx but this phenomenon could not be observed because the reduction by Trx itself was 10^4-fold more efficient [16]. It can be speculated that the same effect should be observed when using alkylated Trx together with CF_1. In a similar experiment with malate dehydrogenase and fructose-1,6-bisphosphatase, however, no activation could be induced by alkylated *E. coli* Trx in the presence of DTT [6]. Possibly, this difference relates to the oxidation potential of the target enzyme; DTT can slowly activate CF_1 (Fig. 5) but not malate dehydrogenase.

Therefore we postulate a more detailed model for the activation process of CF_1 by Trx (Scheme 1). In the first step, Trx recognizes the disulphide bridge region on the inactive CF_1. For this interaction, the difference in the surface charges between Trx-*f* and Trx-*m* might determine a difference in affinities, which could explain the difference in the initial rates of activation reported previously [16]. As described above, the double-mutant Trx could facilitate the reduction of the disulphide bridge in the presence of DTT. Therefore the binding of Trx to CF_1 must concomitantly induce a conformational change on the γ-subunit that finally causes the formation of the mixed-disulphide bridge between one cysteine on CF_1-γ and the reduced form of Trx or DTT.

Following the first two steps in our model, the mixed-disulphide intermediate is finally reduced by the internal attack of either wild-type Trx or DTT (Scheme 1, 3rd step). Our model is entirely compatible with the reaction mechanism previously described for the activation of fructose-1,6-bisphosphatase [15], but our version adds detail to the preceding steps.

In conclusion, our findings suggest that Trx is not a mere reductant of the disulphide bridge of CF_1, but that it also induces a critical conformational change. This conformational change renders the disulphide bridge more susceptible to DTT, and probably more so to the reduced form of Trx itself, which explains the 10^4-fold higher reactivity of the reduced form of Trx compared with DTT. This quality marks Trx as an excellent class of regulators *in vivo*.

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