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Rapeseed chloroplast thioredoxin-m Modulation of the affinity for target proteins

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Abstract

The stroma of higher plant chloroplasts contains two thioredoxins (Trx) with different specificity for the reduction of protein disulfide bonds. Based upon electrostatic features of domains that participate in the thiol/disulfide exchange, we prepared mutants of rapeseed Trx-m bearing opposite charges at a single position and subsequently analyzed their action on the activation of rapeseed chloroplast fructose 1,6-phosphate (CFBPase). The replacement of Pro-35 with lysine and glutamic residues enhanced and impaired, respectively, the stimulation of CFBPase relative to the wild-type and the P35A mutant. Furthermore, the shielding of electrostatic interactions with high concentrations of KCl greatly increased and concurrently made indistinguishable the affinity of all variants for CFBPase. The capacity to stimulate the enzyme activity likewise was enhanced concertedly by fructose-1,6-bisphosphate and Ca^{2+} but, at variance with the action of KCl, remained sensitive to charges in the side chain of mutants. These results were consistent with a mechanism in which intermolecular electrostatic interactions and intramolecular non-covalent interactions control the formation of the non-covalent complex between reduced Trx and oxidized CFBPase and, in so doing, modulate the thiol/disulfide exchange.

Keywords: Thioredoxin; Chloroplast fructose 1,6-phosphate; Electrostatic interaction; Redox regulation; Rapeseed

1. Introduction

Thioredoxins (Trx) constitute a family of small (ca. 12 kDa) and ubiquitous proteins whose remarkable feature is the highly conserved amino acid sequence -W-C-G-P-C-K(R)- [1]. In one of the best characterized functions, the cysteine couple enables Trx to reduce a wide array of disulfide-bearing substrates via a thiol/disulfide exchange. Although primary structures vary largely among different species, these protein disulfide reductases sustain two structural features. First, crystallographic data of *Escherichia coli* and human Trx reveal a distinctive folding containing a single domain with a central five stranded β-sheet flanked with four α-helices [2]. Second, the nucleophilic thiolate that initiates the attack on the disulfide bond of the substrate – i.e. Cys-32 – protrudes from the protein surface and locates between the β-2 strand and the α-2 helix [3]. Given that this particular protein family contains members whose primary structure bears a variable number of constituent amino acids, henceforth, the numbering

Abbreviations: DTT, dithiothreitol; CFBPase, chloroplast fructose 1,6-phosphate (EC 3.1.3.11); CMDH, chloroplast malate dehydrogenase (EC 1.1.1.82); Trx, thioredoxin (EC 1.6.4.5)

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indicates the homologous location in *E. coli* Trx while the following parentheses enclose the position in the particular Trx.

In higher plant chloroplasts, Trx-m and Trx-f link the light-triggered generation of reducing power in thylakoid membranes with the regulation of metabolism in the soluble stroma [4]. Photochemically reduced ferredoxin and an iron sulfur protein (ferredoxin-Trx reductase) cleave the unique disulfide bond of Trx which in turn reduces disulfide bonds of chloroplast enzymes. In vitro, chloroplast Trx disclose selectivity for target enzymes. Trx-f is particularly efficient in the stimulation of enzymes involved in the photosynthetic fixation of CO_2 (fructose 1,6-phosphate (CFBPase), sedoheptulose-1,7-bisphosphatase, NADP-glyceraldehyde-3-P dehydrogenase) whereas Trx-m functions in the inhibition of one enzyme related to the catabolism of carbon compounds, glucose-6-P dehydrogenase [4,5]. On the other hand, both chloroplast Trx exhibit an equal capacity for the stimulation of phosphoribulokinase and ATP synthase [6,7]. In vivo, however, the reductive process seems to be more complex than a thiol/disulfide exchange between Trx and target proteins because (i) Trx-m is required for photosynthetic and heterotrophic growth as well as light-dependent regulation of enzymes in cyanobacteria that lack Trx-f [8,9] and (ii) Trx, other than the f type, activate *Chlamydomonas reinhardtii* CFBPase [10]. Given that enzyme modulation in chloroplasts proceeds entirely through a protein to protein transfer of reducing equivalents, attempts to explain large differences in rates of disulfide cleavage require an accurate knowledge of non-covalent interactions that complement the transformation of covalent bonds.

CFBPase (homotetramer, subunit: ca. 40 kDa) is an excellent target for studying chloroplast Trx because the rate of fructose-1,6-bisphosphate hydrolysis is sensitive to the reduction of cystines as well as to chloroplast metabolites not involved in redox reactions (e.g. fructose-1,6-bisphosphate, Ca^{2+}) [11]. Relative to the cytoplasmic counterpart, the primary structure of the chloroplast enzyme contains an insertion that conserves three cysteine residues involved in the reductive activation while the other constituent amino acids vary in number and composition [12,13]. Another important structural feature disclosed by crystal structures is the high density of negative charges distributed on the surface exposed to the solvent because it provides an interesting framework for studying the Trx-mediated modulation [12,14]. We and others invoke the participation of electrostatic components in the intermolecular interaction based on either the adverse or favorable effects elicited by novel charges in side chains of the Trx molecule. For example, K44(58)D, N60(74)D and Q61(75)D spinach Trx-f as well as K69(70)D pea Trx-m exhibit lower capacity for the activation of the homologous CFBPase [15,16] whereas E30K and L94K mutants of *E. coli* Trx are more efficient than the wild-type counterpart in stimulating spinach CFBPase [17]. However, the uncertainty in previous studies was whether opposite charges in point mutations cause diametrically different effects relative to a neutral residue. In conceiving the present study, we searched for amino acids of Trx whose replacement with negatively and positively charged residues does not cause significant structural modifications and hypothesized that these mutations might weaken and enhance, respectively, the Trx-mediated stimulation of CFBPase. We report here that mutants of rapeseed Trx-m at Pro-30(35) not only confirmed this prediction but also were sensitive to the shielding of electrostatic forces with high concentrations of neutral salts. These findings demonstrate that intermolecular electrostatic interactions drive the formation of the noncovalent complex that precedes the thiol/disulfide exchange thereby contributing to the specificity of Trx for CFBPase.

2. Materials and methods

Dithiothreitol (DTT) and other biochemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). For PCR amplifications, oligonucleotides, deoxyribonucleotides and DNA polymerases were from GenSet (La Jolla, CA, USA), Amersham Pharmacia Biotech (Uppsala, Sweden) and Gibco BRL (Gaithersburg, MD, USA), respectively. The Altered Sites II Mutagenesis System was obtained from Promega (Madison, WI, USA). The vector pET22b(+) and the *E. coli* strain JM109 (DE3) were products of Novagen (Madison, WI, USA). Re-

striction enzymes were manipulated according to manufacturer's instructions (New England BioLabs, Beverly, MA, USA), Sambrook et al. [18] or Ausubel et al. [19]. *E. coli* Trx, di-fluoresceinthiocarbamyl-insulin, and recombinant rapeseed CFBPase were obtained as described in Mora-Garcia et al. [17], Heuck and Wolosiuk [20], and Rodriguez-Suarez and Wolosiuk [21], respectively. *Sorghum* chloroplast malate dehydrogenase (CMDH) was kindly provided by Dr. J.P. Jacquot (Université de Nancy I, France) [22].

2.1. Site-directed mutagenesis of rapeseed Trx-m

Three successive PCRs were performed for isolating the precursor and the mature form of Trx-m from a cDNA library of rapeseed (*Brassica napus* L. cv Global) leaves prepared by Rodriguez-Suarez and Wolosiuk [23] (details will be given elsewhere) (accession number: AF160870). To generate the P30(35)A, P30(35)K, and P30(35)E variants of rapeseed Trx-m, the DNA fragment encoding the mature form was used in the Altered Sites II Mutagenesis System with the oligonucleotides 5′-TCCACACCATGCTGCCCAAAAGT-3′, 5′-GTCCACACCATTTGCCCAAAAGT-3′, and 5′-GTCCACACCATTCTGCCCAAAAGT-3′, respectively.

2.2. Expression and purification of rapeseed Trx-m variants

The *E. coli* strain JM109 (DE3) was transformed by electroporation with the expression vector pET22b(+) carrying the DNA encoding different variants of rapeseed Trx-m cloned in the *Nde*I/*Bam*HI site. An overnight liquid culture (10 ml) was inoculated into LB medium containing 0.2 mg ampicillin ml^{-1} (2 l) and grown at 37°C to an OD_{600} of 0.6. After the addition of isopropyl-β-D-thiogalactopyranoside (final concentration: 0.5 mM) cells were (i) grown for 3 h, (ii) harvested by centrifugation ($5000 \times g$, 10 min), (iii) washed off from culture medium with 30 mM Tris–HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, (iv) resuspended in the latter buffer devoid of NaCl, and (v) stored frozen at −20°C. Bacteria were subjected to two rounds of freezing and thawing and subsequently lysed by sonication. After removal of cell debris by centrifugation ($15\,000 \times g$, 20 min), proteins of the supernatant fraction were fractionated with ammonium sulfate (20–95% of saturation), dissolved in and dialyzed against 30 mM Tris–HCl (pH 7.9), 1 mM EDTA and 200 mM NaCl. The dialysate was loaded on a Sephadex G-50 column equilibrated and eluted with 30 mM Tris–HCl (pH 7.9), 200 mM NaCl. Fractions containing protein disulfide reductase activity (assayed with di-fluoresceinthiocarbamyl-insulin) were pooled, dialyzed against 30 mM Tris–HCl (pH 7.9) and applied on a DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated beforehand with the latter buffer. A linear gradient of NaCl (100 mM to 600 mM) released fractions containing the protein disulfide reductase activity that were successively concentrated by ultrafiltration, dialyzed against 30 mM Tris–HCl (pH 7.9) and stored at −20°C. This procedure yielded about 20 mg of homogeneous protein whose protein disulfide reductase activity remained for at least 1 year when stored at −20°C in 30 mM Tris–HCl (pH 7.9). Molecular sieve chromatography (BioSil Sec-250 (Bio-Rad, Richmond, CA, USA)) gave no hint for multimerization because the molecular mass (12.5 kDa) was similar to that predicted by the DNA sequence (12 305 Da).

2.3. Denaturation of Trx

Guanidine hydrochloride unfolding of Trx was monitored by following the intrinsic fluorescence in an F-770 Jasco spectrofluorometer at 25°C. After Trx (0.5 μM; $A_{280\,nm} < 0.1$) was incubated for 2 h with varying concentrations of guanidine hydrochloride in 50 mM Tris–HCl (pH 7.0), the fluorescence emission was recorded from 300 to 400 nm (5 nm bandwidth) at the excitation wavelength of 280 nm (10 nm bandwidth) with a scan speed of 50 nm min^{-1}. Variations of the relative fluorescence intensity at 350 nm were fitted to a two-state model – i.e. the native and unfolded states – from which the concentration of denaturant at the midpoint of the transition ($[Gu.HCl]_{0.5}$), the free energy change of unfolding in the absence of denaturant ($\Delta G(H_2O)$), and the dependence of the free energy change of unfolding on denaturant concentration (m) were determined by the linear extrapolation method [24].

2.4. Enzyme assays

2.4.1. Protein disulfide oxidoreductase activity

The analysis of the protein disulfide reductase activity was performed by following turbidimetrically or fluorometrically the cleavage of intercatenary disulfide bonds of native insulin and di-fluoresceinthiocarbamyl-insulin, respectively, as described by Heuck and Wolosiuk [25].

2.4.2. CFBPase activity

The reductive activation of CFBPase was followed at 24°C by the two-stage assay [26]. The rapeseed CFBPase (0.8–1.6 μg) was incubated in a solution containing 100 mM Tris–HCl buffer (pH 7.9), 5 mM DTT and Trx (final volume: 0.05 ml). To measure the rate of activation, aliquots were withdrawn while the specific activity of CFBPase increased with time and injected into the solution for the assay of phosphatase activity (final volume: 1.0 ml): 100 mM Tris–HCl buffer (pH 7.9), 2 mM $MgCl_2$, 1 mM fructose-1,6-bisphosphate, and 0.1 mM EGTA. After 3 min at 24°C, catalysis was halted by adding the reagent for the colorimetric estimation of inorganic phosphate [27].

2.4.3. CMDH activity

The stimulation of CMDH activity was analyzed at 24°C by following essentially the two-stage assay described for CFBPase. The *Sorghum* enzyme (0.16 μg) was incubated in a solution containing 100 mM Tris–HCl buffer (pH 7.9), 5 mM DTT, and Trx (final volume: 0.05 ml). After the preincubation, the catalytic capacity of an aliquot was estimated in 100 mM Tris–HCl buffer (pH 7.9), 0.2 mM NADPH, and 0.75 mM oxaloacetate by following the decrease in $A_{340\,nm}$ (final volume: 0.8 ml).

2.5. Polyacrylamide gel electrophoresis (PAGE) and determination of protein concentration

SDS–PAGE and non-denaturing PAGE were performed on 10% (w/v) and 11% (w/v) acrylamide gels, respectively, using the Mini-Protean Cell (Bio-Rad) as described previously [17]. The protein concentration of rapeseed Trx-m variants was estimated from the molar extinction coefficients calculated according to Gill and von Hippel [28].

2.6. Computational methods

Models of rapeseed CFBPase and variants of rapeseed Trx-m were built with crystal structures deposited in the Protein Data Bank (PDB) by using automatic routines of the SWISS-MODEL program [29–31]. The structures were displayed and analyzed on a Silicon Graphics Iris 4.X computer using the the GRASP program [32]. Electrostatic isopotential contours were obtained with the following parameters: solvent dielectric, 80.0; internal dielectric of proteins, 2.0; ionic strength, 0.15 M; probe radius, 1.4 Å; ionic radius, 2 Å; net charge of all aspartic and glutamic residues, −1; and net charge of all arginine and lysine residues, +1.

3. Results

3.1. Computational study of protein structure

Three-dimensional structures, determined by X-ray diffraction, have been reported for oxidized and reduced pea CFBPase [29] as well as for the reduced spinach counterpart [14]. The modeling of rapeseed CFBPase by the GRASP program, based on either the oxidized pea (accession number: 1D9Q) (Fig. 1) or the reduced spinach enzyme (accession number: 1SPI) (cf. figure 3 in [12]), disclosed significant differences in topology because the insertion bearing the three cysteines necessary for the reductive activation packs against strands β-1 and β-2 in the former template whereas it is flexible in the solvent in the latter. However, as anticipated from more glutamic (21) and aspartic (28) residues than arginine (11) and lysine (22) in the constituent polypeptide, the superficial charge distribution exhibited large patches of negative charges. On the other hand, the recent elucidation of the tertiary structure of spinach Trx-f and Trx-m by X-ray crystallography revealed that both proteins conserved not only the overall three-dimensional structure but also the geometry of the active site region seen in other Trx [30]. However, Trx-f diverged from Trx-m in the disposition of hydrophobic and charged patches around cysteine residues of the active site (Fig. 1), being the extent of positively charged areas in the former larger than in the latter [30,33]. Using the SWISS-MODEL program, the

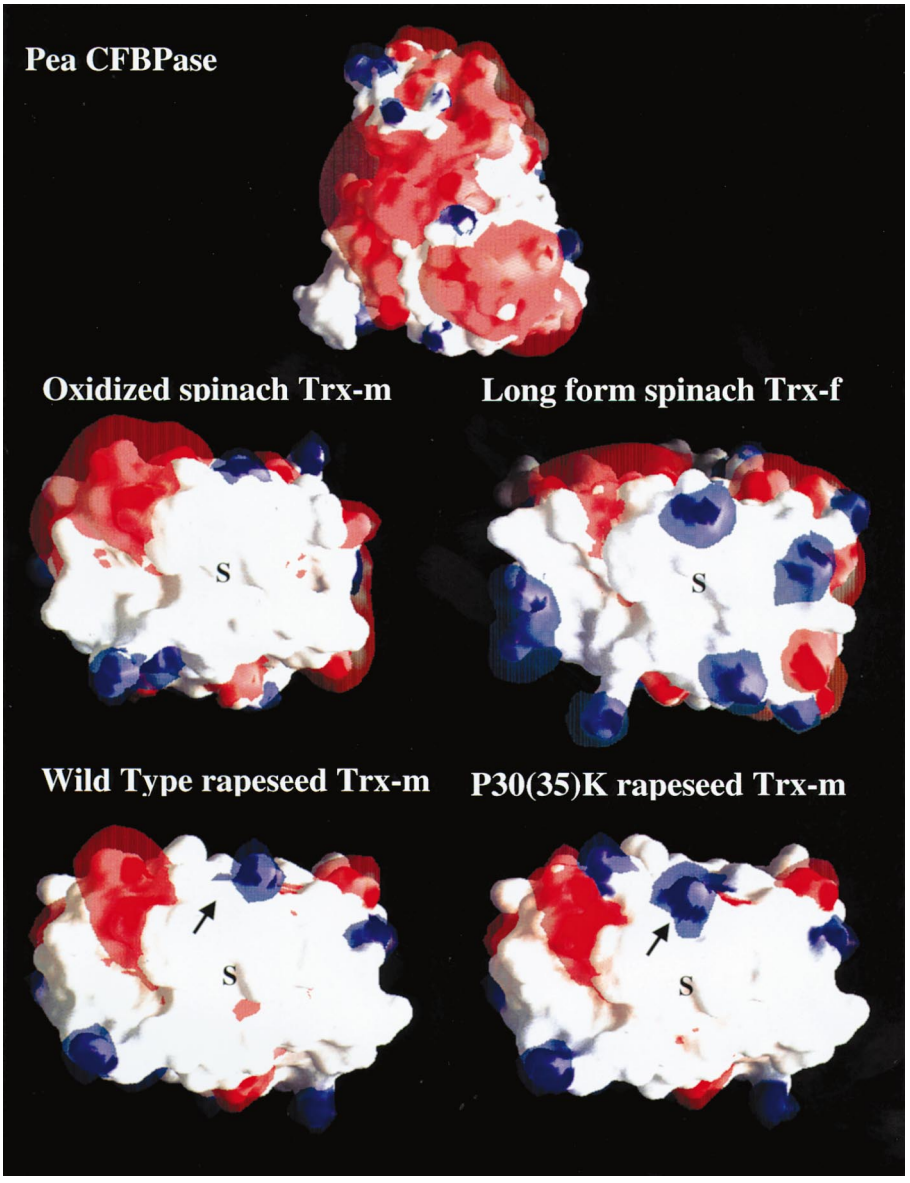


Fig. 1. Electrostatic potentials at the solvent-accessible surface. The pictures of reduced spinach Trx-m and the long form of spinach Trx-f were drawn with PDB files 1FB0 and 1FAA, respectively, while rapeseed CFBPase, wild-type rapeseed Trx-m and P30(35)K rapeseed Trx-m were modeled with the SWISS-MODEL program [29–31]. Charge distributions on surfaces were calculated with the GRASP program at ±2 kT; red, white and blue colors represent the isopotential shell for negative, null and positive potentials, respectively. **S** illustrates the position of the nucleophilic sulfur of Cys-32(37) in spinach and variants of rapeseed Trx-m as well as Cys-32(58) in long form spinach Trx-f. The arrow points out the localization of the proline residue that was modified by site-directed mutagenesis.

structure of rapeseed Trx-m was almost similar to both chloroplast Trx but the superficial topology and charge distribution resembled those of the spinach counterpart while departed from those of spinach Trx-f.

If chloroplast Trx had similar overall three-dimensional structure and redox chemistry but differed in the affinity for target proteins then variations of superficial charges might modulate the accessibility of the nucleophilic sulfur to the negatively charged environment of the CFBPase in which resides the disulfide bond. To uphold this prediction, mutated residues should not alter α-helices and β-strands in order to avoid disturbances of the Trx fold. We

found that Pro-30(35), which is flanked by the highly conserved amino acids Ala-29(34) and Trp-31(36), is conserved not only in all hitherto sequenced Trx-m but also in Trx from cyanobacteria, red algae as well as the Trx motif of eukaryotic protein disulfide isomerases [5,34]. Moreover, a limited variety of residues occupy this position in Trx from other species: acidic (*E. coli*, photosynthetic bacteria), serine (*Chlorobium*, isoforms of *Arabidopsis thaliana*, most of higher plants Trx-h), threonine (animals, *Chlamydomonas* Trx-h, *A. thaliana* Trx-3), glutamine (spinach, rapeseed and *A. thaliana* Trx-f) and lysine (pea Trx-f). Located in the distorted region that links the β-2 strand with the α-2 helix, Pro-30(35) constituted a good target for mutational studies because it is (i) in close contact with the solvent, (ii) outside of secondary elements and (iii) near the active site. As expected from inherent charges, the introduction of lysine and glutamic enlarged the positive and negative, respectively, superficial areas of rapeseed Trx-m mutants near the nucleophilic sulfur.

3.2. Variants of rapeseed Trx-m

The cDNA obtained from rapeseed leaves was used for the isolation of the DNA fragment that encoded the precursor of Trx-m. After cloning the latter in the pET22b(+) vector, the gene that encoded the mature form was prepared by PCR amplification, cloned into the same vector and expressed by induction with isopropyl-β-D-thiogalactoside. The preparation of Trx-m mutants followed essentially similar procedures for cloning and expressing the DNA modified by site-directed mutagenesis. All variants of rapeseed Trx-m were subsequently purified by ammonium sulfate fractionation, gel filtration and anion exchange chromatography as described in the Section 2. As illustrated by non-denaturing PAGE and SDS–PAGE, that were intentionally overloaded (Fig. 2), purified wild-type, P30(35)K, P30(35)A and P30(35)E Trx-m not only were pure at greater than 95% but also migrated according to the absence or the presence of an additional electric charge in the respective polypeptide. The catalytic capacity of these variants was estimated by assaying the protein disulfide oxidoreductase activity towards intercatenary disulfide bonds of insulin. At similar concentrations (10 μM), wild-type and P30(35)K Trx-m reduced insulin at 0.0170 ΔOD_{650} min^{-1} after a lag phase of 33 min whereas P30(35)A and P30(35)E variants did at 0.0127 ΔOD_{650} min^{-1} after a lag of 40 min.

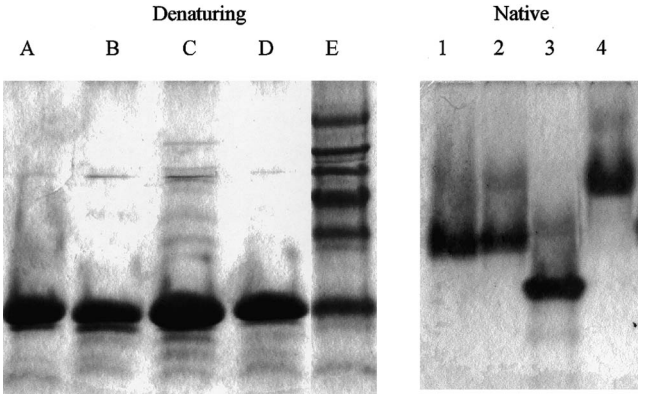


Fig. 2. Electrophoretic analysis of rapeseed Trx-m variants. Preparations of recombinant proteins (20 μg) were loaded onto 10% Tricine/SDS–PAGE (denaturing) and 11% PAGE (native) to analyze the purification of wild-type (lanes A and 1), P30(35)A (lanes B and 2), P30(35)E (lanes C and 3), and P30(35)K (lanes D and 4). Lane E contained the molecular mass markers.

Although mutations at position 30(35) clearly did not impair the capacity to cleave disulfide bonds in target proteins, functional results are difficult to interpret, and may even be misleading, in absence of structural data. The primary structure of rapeseed Trx-m contains three tryptophans: two close to the active site (Trp-28(33) and Trp-31(36)) and another at the start of the α-1 helix (Trp-12(17)). The fluorescence intensities at the λ_{max} of oxidized P30(35)K, P30(35)E and P30(35)A Trx-m were 1.1-, 1.1- and 1.3-fold, respectively, higher than that of the wild-type protein. To further prove that mutations did not cause large structural alterations, the unfolding of recombinant proteins was investigated by monitoring changes of the intrinsic fluorescence elicited by guanidine hydrochloride. The data followed a two-stage model in which the midpoints of single transitions were at 1.8 M and ca. 1.5 M guanidine hydrochloride for the wild-type and mutants, respectively, while the total conversion from the folded to the unfolded species was obtained at 2.5 M guanidine hydrochloride (Table 1). Moreover, slight variations in the free energy change of unfolding in absence of denaturant ($\Delta G(H_2O)$) [24], like fluorescence

Table 1
Unfolding of rapeseed Trx-m variants induced by guanidine hydrochloride

Variant of rapeseed Trx-m	$[Gu.HCl]^{a}_{0.5}$ (M)	$\Delta G(H_2O)^{b}$ (kcal mol^{-1})	m^{c} (kcal mol^{-1} M^{-1})
Wild-type	1.8	5.93 ± 1.09	3.37 ± 0.58
P30(35)K	1.5	7.10 ± 0.88	4.68 ± 0.55
P30(35)A	1.4	5.08 ± 0.70	3.67 ± 0.42
P30(35)E	1.5	5.40 ± 1.40	3.49 ± 0.80

Wild-type and mutants of rapeseed Trx-m (0.5 μM) were treated with guanidine hydrochloride (Gu.HCl) as described under Section 2.
[a]The concentration of denaturant at the midpoint of the transition.
[b]The free energy change of unfolding in the absence of denaturant.
[c]The dependence of the free energy change of unfolding on denaturant concentration.

emission spectra, were in line with a lack of gross structural differences among the variants of rapeseed Trx-m used in this study.

3.3. Modulation of CFBPase activity

To evaluate the action of rapeseed Trx-m variants on rapeseed CFBPase, we relied upon the two-stage assay because it separates clearly the hydrolysis of fructose-1,6-bisphosphate (catalysis) from the conversion of an inactive enzyme to a more active form mediated by reduced Trx (modulation) [4,26]. In these studies, the concentration of DTT exceeded at least 50-fold that of Trx which ensured that the latter is in the reduced form during the activation of the enzyme and the reductive process approached to a pseudo-first-order kinetics because the molar ratio [Trx]/[CFBPase] was at least higher than 10 [25]. Under these conditions, the enhancement of CFBPase specific activity proceeded steadily without bending downwards for at least 70 min. Values of activity thus represented the process of reductive activation rather than the attainment of an equilibrium between the inactive and the active form. Accordingly, at varying concentrations of Trx, the upper limit of the activation (V_{max}) estimated the maximum capacity for the formation of the active enzyme whereas the concentration at half of the V_{max} ($A_{0.5}$) constituted a reliable measurement of the affinity for the target enzyme. Fig. 3A shows that, relative to our

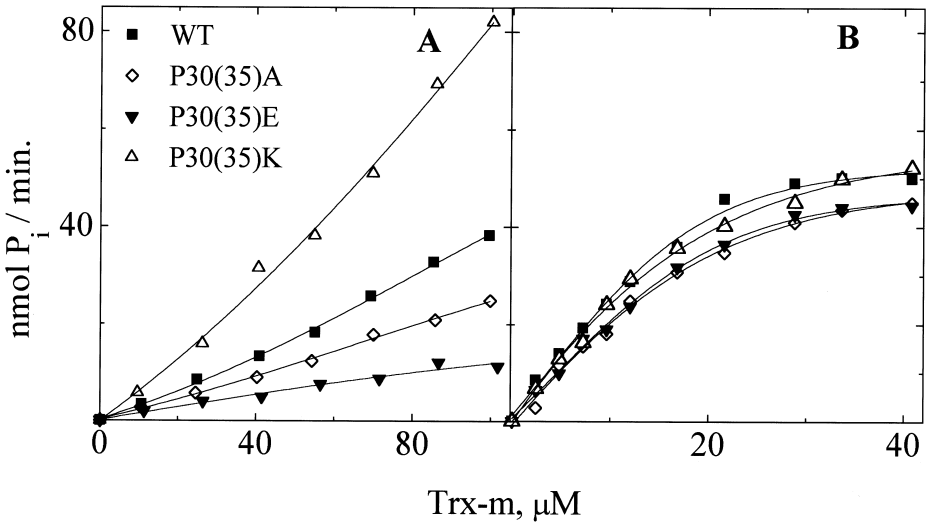


Fig. 3. Activation of rapeseed CFBPase by variants of rapeseed Trx-m. The enzyme (1.6 μg) was incubated at 24°C in 0.05 ml of 100 mM Tris–HCl buffer (pH 7.9), 5 mM DTT, and the wild-type, P30(35)A, P30(35)E, and P30(35)K rapeseed Trx-m, as indicated, in the absence (A) and in the presence of 0.5 M KCl (B). While the specific activity of CFBPase was increasing (30 min), an aliquot was withdrawn and injected into the solution for assaying CFBPase activity and the hydrolysis of fructose-1,6-bisphosphate was estimated as described under Section 2.

previous studies [17,35], rapeseed Trx-m was much less effective than Trx-f and *E. coli* Trx in the activation of CFBPase. Indeed, the actual degree of stimulation was difficult to assess quantitatively because the recombinant protein did not show rate saturation up to concentrations of 100 μM. To overcome this inconvenience an estimate of the effect of mutations on the conversion of inactive CFBPase to an active form was obtained by comparing the extent of the activation process. Thus, the activation by the wild-type Trx-m was consistently higher than that mediated by the P30(35)E variant and, more important, lower than that elicited by the P30(35)K mutant. If negative charges on rapeseed Trx-m impaired the approximation to the negatively charged domain of CFBPase, then screening repulsive forces with high concentrations of neutral salts should minimize the contribution to the intermolecular interaction and, as a consequence, enhance the reductive process [17]. Confirming this prediction (Fig. 3B), the modulation of rapeseed CFBPase in the presence of 0.5 M KCl yielded roughly similar $A_{0.5}$ and V_{max} for all variants; i.e. the catalytic efficiency ($V_{max}/A_{0.5}$) at high ionic strength is identical for all rapeseed Trx-m variants. But more important, the convergence of the $A_{0.5}$ to a common value (ca. 11 μM) resembled our previous finding in which not only the $A_{0.5}$ of the inefficient *E. coli* Trx (33 μM) but also the $A_{0.5}$ of the highly efficient Trx-f (0.9 μM) approached to limiting value (9 μM) when the modulation of spinach CFBPase was performed at concentrations of KCl higher than 0.5 M [17]. Thus, the high ionic strength shielded electrostatic forces between interacting proteins thereby restricting the activation (reduction) of CFBPase to the thiol/disulfide exchange reaction.

In the reduction of CFBPase, Trx-f is highly efficient whereas Trx-m and heterologous Trx are inefficient when the modulation is performed in absence of modulators. Because the concerted action of chloroplast metabolites elicits conformational changes of CFBPase which in turn cause large variations in the activation process [11,16,35–37], we tested the regulatory activity in the presence of both 0.75 mM fructose-1,6-bisphosphate and 0.05 mM Ca^{2+}. Fig. 4 shows that the stimulation of CFBPase was saturable by all variants of rapeseed Trx-m and concurrently brought the $A_{0.5}$ of all variants to the common value of 14–15 μM but with different V_{max}: 74, 58, 49 and

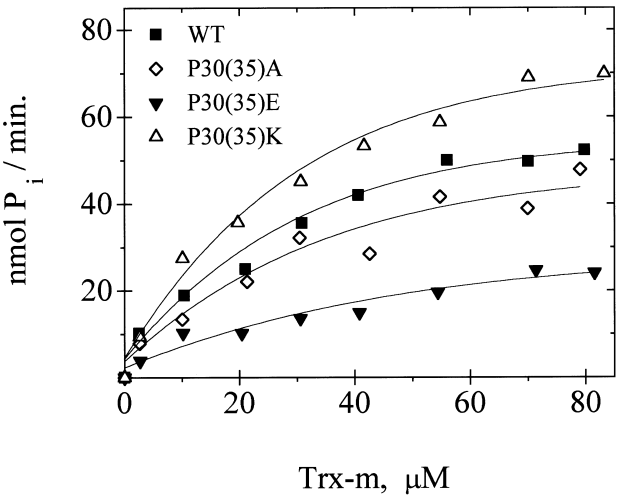


Fig. 4. Effect of fructose-1,6-bisphosphate and $CaCl_2$ on the activation of rapeseed CFBPase. The enzyme (0.8 μg) was incubated in the presence of 0.75 mM fructose-1,6-bisphosphate and 0.05 mM $CaCl_2$ with the wild-type, P30(35)A, P30(35)E, and P30(35)K rapeseed Trx-m, as described in Fig. 3A. After 10 min, CFBPase activity was assayed as indicated under Section 2.

30 μmol Pi min^{-1} for the P30(35)K, wild-type, P30(35)A and P30(35)E rapeseed Trx-m, respectively. Thus, at variance with the action of KCl, the stimulation of CFBPase in the presence of modulators showed the highest and the lowest catalytic efficiency, with P30(35)K and P30(35)E Trx-m, respectively. Clearly, the concerted action of fructose-1,6-bisphosphate and Ca^{2+} improved the reductive activation of CFBPase, perhaps by shifting the enzyme to a conformation that was more adequate for interaction with Trx-m, without changing the sensitivity to charges at position 30(35) [11]. Although not shown, a similar trend was observed when native spinach CFBPase replaced the recombinant rapeseed enzyme.

3.4. Modulation of CMDH activity

Given that Trx-m is an efficient modulator in the stimulation of CMDH, it was of interest to examine the action of rapeseed variants on this particular activity. At variance with the activation of CFBPase in absence of modulators, all variants of rapeseed Trx-m exhibited rate saturation in the enhancement of *Sorghum* CMDH (Fig. 5). The P30(35)K mutant ($A_{0.5}$ = 14 μM; V_{max} = 37 nmol NADPH oxidized min^{-1}) was virtually similar to the wild-type Trx-m ($A_{0.5}$ = 13 μM; V_{max} = 41 nmol NADPH oxidized

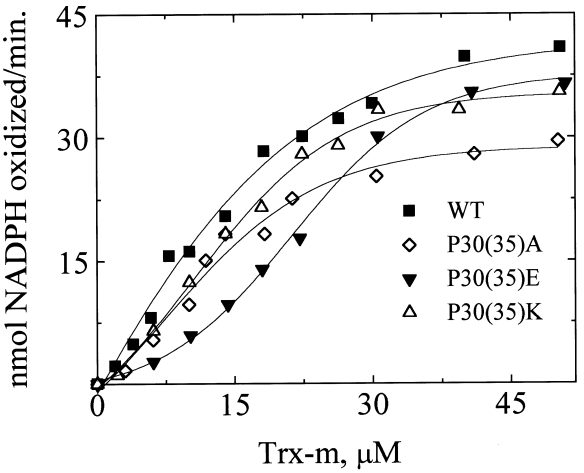


Fig. 5. Activation of CMDH by variants of rapeseed Trx-m. *Sorghum* chloroplast CMDH (0.16 μg) was incubated at 24°C in 0.05 ml of 100 mM Tris–HCl buffer (pH 7.9), 5 mM DTT, and wild-type, P30(35)A, P30(35)E, and P30(35)K rapeseed Trx-m, as indicated. After 2.5 min, an aliquot was injected into the solution for assaying CMDH activity as described under Section 2.

min^{-1}). However, the P30(35)E mutant had the lowest affinity for CMDH ($A_{0.5} = 22$ μM) but retained the maximum efficiency for the conversion of the enzyme to a more active form ($V_{max} = 38$ nmol NADPH oxidized min^{-1}) whereas the P30(35)A mutant did the opposite ($A_{0.5} = 14$ μM; $V_{max} = 29$ nmol NADPH oxidized min^{-1}). The number of variants was limited but sufficient for showing clearly that the reduction of CMDH by Trx-m proceeded by a mechanism different from that involved in the activation of CFBPase.

4. Discussion

The formation of a non-covalent complex functional in the thiol/disulfide exchange requires a collision between the protein disulfide oxidoreductase and the target protein followed by an adequate movement of one molecule relative to the other for the subsequent transformation of covalent bonds. In these successive intermolecular interactions, long range electrostatic forces would approach interacting proteins whereas short range interplays between complementary residues dock correctly the nucleophilic thiolate for cleaving the disulfide bond. Most Trx have similar overall three-dimensional structure but significant differences in both the surface topology and the distribution of charges that surround the active site thereby non-covalent interactions at the exposed residues might contribute to the specificity of chloroplast Trx for target proteins [30]. Previous research on this issue showed either the impairment or the enhancement of CFBPase activity in response to novel negatively or positively charged residues, respectively, but lacked the contrasting action of amino acids bearing opposite charges at a single fixed position [15–17,38]. Although the latter approach is convincingly acceptable, it further requires that those replacements are not accompanied by structural changes, such as imperfect packing, which in turn might alter protein–protein interactions [15]. Satisfyingly, the spectra of intrinsic fluorescence emission and the response to chemical denaturants indicate that Pro-30(35) of rapeseed Trx-m easily accommodates large charged amino acids as well as small hydrophobic residues without substantial changes in the tertiary structure.

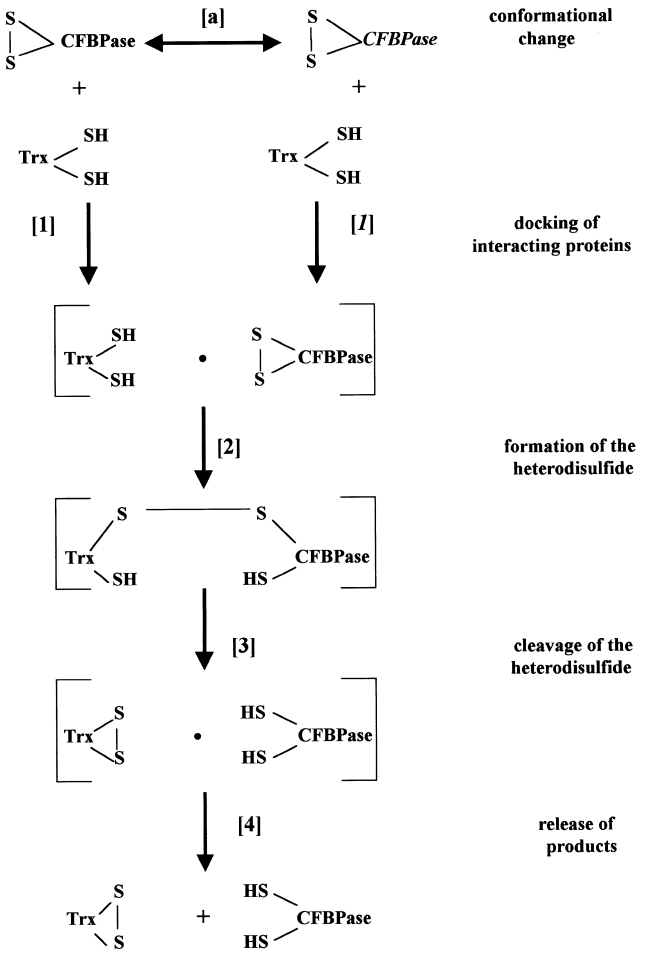
In the present paper we have shown that the replacement of Pro-30(35) with a lysyl residue enhances the capacity of rapeseed Trx-m for the stimulation of rapeseed CFBPase whereas a glutamyl residue causes a detrimental response. Interestingly, the former and the latter amino acids occupy the homologous position in an efficient (pea Trx-f) and an inefficient (*E. coli* Trx) modulator, respectively [5,17,39]. Opposite charges in single replacements are convenient for establishing whether intermolecular electrostatic interactions prevail in the formation of the non-covalent heterocomplex but the rigorous demonstration requires that the binary association must also be sensitive to shielding by high concentrations of neutral salts [40]. In line with this requisite, all variants of rapeseed Trx-m have much higher affinity for rapeseed CFBPase when the stimulation is performed at concentrations of KCl beyond 0.5 M. Accordingly, we have shown previously that high concentrations of neutral salts improve the affinity of *E. coli* Trx for spinach CFBPase while they impair that of Trx-f [17]. Two independent studies further support the idea that intermolecular electrostatic interactions play an important role in the modulation of CFBPase. First, negatively charged dithiols and Tris(carboxyethyl)phosphine do not enhance the catalytic capacity whereas positively charged mono-

thiols and the neutral tributylphosphine do so very efficiently [41,42]. Second, the incorporation of polyamines to the modulation of CFBPase impairs the action of Trx-f, perhaps by competing for the negatively charged surface of CFBPase, but the subsequent removal restores the activation process [43]. Although the role of intermolecular electrostatics, itself, is clear, other Trx residues may participate in complementary interactions. This possibility was first suggested by the high-salt-mediated convergence of the $A_{0.5}$ for either *E. coli* Trx or rapeseed Trx-m towards a value that was quantitatively higher than that of Trx-f. Perhaps, as was recently proposed [30], additional structural factors that contribute to the 'specificity' of spinach Trx-f may comprise the unstructured region that links the β-3 strand with the α-3 helix whose amino acid composition clearly differs from Trx-m

	(66)	
-L N T D	D S P A-	rapeseed Trx-m
-L N T D	E A P G-	spinach Trx-m
-L D C N Q	E N K T-	spinach Trx-f
	(74)	

Early studies on CFBPase provided evidence for a synergistic enhancement of the Trx action by both fructose-1,6-bisphosphate and Ca^{2+} [16,26,35,37]. Our kinetic and structural studies revealed that the concerted action changes the tertiary structure of CFBPase while the conformation of Trx remains invariant [11,26]. Moreover, the removal of fructose-1,6-bisphosphate and Ca^{2+} impairs the reductive stimulation of the inactive CFBPase but does not alter the specific activity of the activated form; i.e. both metabolites drive the enzyme to an adequate conformation for the formation of the initial non-covalent heterocomplex rather than stabilize the activated enzyme [11,26,39]. Present data show that these chloroplast metabolites increase abruptly the affinity of all variants of rapeseed Trx-m for CFBPase but, at variance with KCl, the extent of the reductive stimulation responds to charges in side chains of position 30(35). Thus, the modulation of the CFBPase activity by reduced Trx seems to be more complex than the reduction of cysteines and involves also the modification of intramolecular non-covalent interactions by compounds that do not participate directly in redox reactions.

Using the information reported here, we developed a more detailed picture for the activation of CFBPase which is consistent with existing data (Scheme 1). The reductive process necessarily entails the non-covalent docking of reduced Trx to oxidized CFBPase (reaction [**1**]) for the generation of an intermolecular disulfide (reaction [**2**]) [44–47]. Due to the intramolecular nature, the subsequent formation of the disulfide bond in Trx is very rapid (reaction [**3**]) and drives the reaction to the release of products (reaction [**4**]). Given that most chloroplast Trx have close midpoint redox potentials for performing the thiol/disulfide exchange, non-covalent interactions appear as responsible for differences in their action on target proteins [48]. Accordingly, the present activation of CFBPase correlates with perturbations in the electrostatic charge distribution at the interface that separates interacting proteins. On the other



Scheme1. Mechanism of the thiol/disulfide exchange between Trx and CFBPase.

hand, the kinetic analysis on the reduction of chloroplast H^+-ATPase by Trx further demonstrates that the non-covalent docking of the latter to the former is the rate-limiting step relative to the irreversible cleavage of disulfide bonds in the coupling factor [49]. An examination of the mechanism depicted in Scheme 1 also reveals the importance of intramolecular interactions in the reductive process (reaction [**a**]). The conformation of target proteins constitutes an issue that is not generally addressed in the analysis of protein disulfide oxidoreductases but the sharp increase in the affinity of Trx when CFBPase is incubated with fructose-1,6-bisphosphate and Ca^{2+} provides a compelling evidence for conformations of the enzyme that are more effective in the reductive process than the native form [11,17,35]. Therefore, our results collectively make evident that the stimulation of CFBPase is a mechanism in which intermolecular electrostatic interactions as well as intramolecular non-covalent interactions provide effective controls for the action of different Trx that perform the same redox chemistry within the same cellular compartment. In this context, a challenge for the future will be to identify the contribution of other non-covalent interactions to the reductive process, e.g. reaction [**4**] [7,50].

Current data do not allow to extend the present mechanism to the formation of non-covalent heterocomplexes with other target proteins. Trx accepts disulfide bonds from an enormous diversity of substrates ranging from low molecular weight compounds to large proteins whose accessible surfaces do not exhibit the extremely high density of negative charges observed in CFBPase. As a matter of fact, we found that P30(35)K rapeseed Trx-m is superior to the wild-type counterpart in the activation of CFBPase while both variants are indistinguishable in the cleavage of insulin intercatenary bonds and the stimulation of CMDH. Thus, molecular characteristics of interacting partners prompt to different alternatives for the non-covalent docking that precedes the thiol/disulfide exchange.

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References

[1] H. Eklund, F.K. Gleason, A. Holmgren, Structural and functional relations among thioredoxins of different species, Proteins Struct. Funct. Genet. 11 (1991) 13–28.
[2] J. Martin, Thioredoxin, a fold for all reasons, Structure 3 (1995) 245–250.
[3] S.K. Katti, D.M. LeMaster, H. Eklund, Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution, J. Mol. Biol. 212 (1990) 167–184.
[4] R.A. Wolosiuk, M.A. Ballicora, K. Hagelin, The reductive pentose phosphate cycle for photosynthetic CO_2 assimilation: enzyme modulation, FASEB J. 7 (1993) 622–637.
[5] J.P. Jacquot, J.M. Lancelin, Y. Meyer, Thioredoxins: structure and function in plant cells, New Phytol. 136 (1997) 543–570.
[6] M.K. Geck, F.C. Hartman, Kinetic and mutational analysis on the regulation of phosphoribulokinase by thioredoxins, J. Biol. Chem. 275 (2000) 18034–18039.
[7] M.T. Stumpp, K. Motohashi, T. Hisabori, Chloroplast thioredoxin mutants without active-site cysteines facilitate the reduction of the regulatory disulfide bridge on the γ-subunit of chloroplast ATP synthase, Biochem. J. 341 (1999) 157–163.
[8] E.D.G. Muller, B.B. Buchanan, Thioredoxin is essential for photosynthetic growth, J. Biol. Chem. 264 (1989) 4008–4014.
[9] F. Navarro, J. Florencio, The cyanobacterial thioredoxin gene is required for both photoautotrophic and heterotrophic growth, Plant Physiol. 111 (1996) 1067–1075.
[10] H.C. Huppe, B.B. Buchanan, Activation of a chloroplast type fructose bisphosphatase from *Chlamydomonas reinhardtii* by light-mediated agents, Z. Naturforsch. 44c (1989) 487–494.
[11] M.A. Ballicora, R.A. Wolosiuk, Enhancement of the reductive activation of chloroplast fructose 1,6-phosphate by modulators and protein perturbants, Eur. J. Biochem. 222 (1994) 467–474.
[12] R.J. Rodriguez-Suarez, S. Mora-Garcia, R.A. Wolosiuk, Characterization of cysteine residues involved in the reductive activation and the structural stability of rapeseed (*Brassica napus*) chloroplast fructose 1,6-phosphate, Biochem. Biophys. Res. Commun. 232 (1997) 388–393.
[13] J.P. Jacquot, J. Lopez-Jaramillo, M. Miginiac-Maslow, S. Lemaire, J. Cherfils, A. Chueca, J. Lopez-Gorge, Cysteine-

153 is required for regulation of pea chloroplast fructose 1,6-phosphate, FEBS Lett. 401 (1997) 143–146.
[14] V. Villeret, S. Huang, Y. Zhang, Y. Xue, W.N. Lipscomb, Crystal structure of chloroplast fructose 1,6-phosphate at 2.8 Å resolution, Biochemistry 34 (1995) 4299–4306.
[15] M.K. Geck, F.W. Larimer, F.C. Hartman, Identification of residues of spinach thioredoxin f that influence interactions with target enzymes, J. Biol. Chem. 271 (1996) 24736–24740.
[16] J. Lopez-Jaramillo, A. Chueca, J.P. Jacquot, R. Hermoso, J.J. Lázaro, M. Sahrawy, J. Lopez-Gorge, High yield expression of pea thioredoxin m and assessment of its efficiency in chloroplast fructose 1,6-phosphate activation, Plant Physiol. 114 (1997) 1169–1175.
[17] S. Mora-Garcia, R.J. Rodriguez-Suarez, R.A. Wolosiuk, Role of electrostatic interactions on the affinity of thioredoxin for target proteins, J. Biol. Chem. 273 (1998) 16273–16280.
[18] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[19] F.M. Ausubel, R. Brent, R. Kingston, E.W. Moore, J.G. Seidman, J.A. Smith, K. Struhl, Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1995.
[20] A.P. Heuck, R.A. Wolosiuk, Fluoresceinthiocarbamyl-insulin: a potential analytical tool for the assay of disulfide bond reduction, J. Biochem. Biophys. Methods 34 (1997) 213–225.
[21] R.J. Rodriguez-Suarez, R.A. Wolosiuk, High level expression in *Escherichia coli*, purification and properties of chloroplast fructose 1,6-phosphate from rapeseed (*Brassica napus*) leaves, Photosynth. Res. 46 (1995) 313–322.
[22] J.P. Jacquot, E. Keryer, E. Issakidis, P. Decottignies, M. Miginiac-Maslow, J.M. Schmitter, C. Cretin, Properties of recombinant NADP-malate dehydrogenases from *Sorghum vulgare* leaves expressed in *Escherichia coli* cells, Eur. J. Biochem. 199 (1991) 47–59.
[23] R.J. Rodriguez-Suarez, R.A. Wolosiuk, Sequence of a cDNA encoding chloroplast fructose 1,6-phosphate from rapeseed, Plant Physiol. 103 (1993) 1453–1454.
[24] C.N. Pace, Determination and analysis of urea and guanidine hydrochloride denaturation curves, Methods Enzymol. 131 (1986) 266–280.
[25] A.P. Heuck, R.A. Wolosiuk, Di-fluoresceinthiocarbamyl-insulin: a fluorescent substrate for the assay of protein disulfide oxidoreductase activity, Anal. Biochem. 248 (1997) 94–101.
[26] C.M. Hertig, R.A. Wolosiuk, Studies on the hysteretic properties of chloroplast fructose 1,6-phosphate, J. Biol. Chem. 258 (1983) 984–989.
[27] P.S. Chen, T.I. Toribara, H. Warner, Microdetermination of phosphorus, Anal. Chem. 239 (1956) 1756–1758.
[28] S.C. Gill, P.H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data, Anal. Biochem. 182 (1989) 319–326.
[29] M. Chiadmi, A. Navaza, M. Miginiac-Maslow, J.P. Jacquot, J. Cherfils, Redox signalling in the chloroplast: structure of oxidized pea fructose-1,6-bisphosphate phosphatase, EMBO J. 18 (1999) 6809–6815.
[30] G. Capitani, Z. Markovic-Housley, G. DelVal, M. Morris, J.N. Jansonius, P. Schurmann, Crystal structures of two functionally different thioredoxins in spinach chloroplasts, J. Mol. Biol. 302 (2000) 135–154.
[31] M.C. Peitsch, Protein modeling by e-mail, Biotechnology 13 (1995) 658–660.
[32] A. Nicholls, K. Sharp, B. Honig, Protein folding and association: insights from the interfacial and thermodynamics properties of hydrocarbons, Proteins Struct. Funct. Genet. 11 (1991) 281–296.
[33] M.F. Jeng, A.P. Campbell, T. Begley, A. Holmgren, D.A. Case, P.A. Wright, H.J. Dyson, High-resolution solution structures of oxidized and reduced *Escherichia coli* thioredoxin, Structure 2 (1994) 853–868.
[34] H. Eklund, F.K. Gleason, A. Holmgren, Structural and functional relations among thioredoxins of different species, Proteins Struct. Funct. Genet. 11 (1991) 13–28.
[35] S. Mora-Garcia, M.A. Ballicora, R.A. Wolosiuk, Chloroplast fructose 1,6-phosphate: modification of non-covalent interactions promote the activation by chimeric *Escherichia coli* thioredoxins, FEBS Lett. 380 (1996) 123–126.
[36] C. Hertig, R.A. Wolosiuk, A dual effect of Ca^{2+} on chloroplast fructose 1,6-phosphate, Biochem. Biophys. Res. Commun. 97 (1980) 325–333.
[37] P. Schurmann, J. Roux, L. Salvi, Modification of thioredoxin specificity of chloroplast fructose 1,6-phosphate by substrate and Ca^{2+}, Physiol. Veg. 23 (1985) 813–818.
[38] F. deLamotte-Guery, M. Miginiac-Maslow, P. Decottignies, M. Stein, P. Minard, J.P. Jacquot, Mutation of a negatively charged amino acid in thioredoxin modifies its reactivity with chloroplastic enzymes, Eur. J. Biochem. 196 (1991) 287–294.
[39] F.E. Prado, J.J. Lázaro, R. Hermoso, A. Chueca, J. Lopez-Gorge, Purification and properties of pea (*Pisum sativum* L.) thioredoxin f, a plant thioredoxin with unique features in the activation of chloroplast fructose 1,6-phosphate, Planta 188 (1992) 345–353.
[40] G. Schreiber, A.R. Fersht, Rapid electrostatically assisted association of proteins, Nat. Struct. Biol. 3 (1996) 427–431.
[41] M. Stein, R.A. Wolosiuk, The effect of chaotropic anions on the activation and the activity of chloroplast fructose 1,6-phosphate, J. Biol. Chem. 262 (1987) 16171–16179.
[42] M.A. Ballicora, A.C. D'Alessio, S. Mora-Garcia, R.J. Rodriguez-Suarez, R.A. Wolosiuk, The reductive modulation of chloroplast fructose 1,6-phosphate by tributylphosphine and sodium borohydride, Cell. Mol. Biol. 42 (1998) 673–682.
[43] E. Corley, R.A. Wolosiuk, C.M. Hertig, Regulation of the activation of chloroplast fructose 1,6-phosphate: inhibition by spermidine and spermine, Biochem. Biophys. Res. Commun. 115 (1983) 707–714.
[44] C.-H. Hu, C.-L. Tsou, Formation of enzyme–substrate disulfide linkage during catalysis by protein disulfide isomerase, FEBS Lett. 290 (1991) 87–89.
[45] J. Park, J.E. Churchich, Interaction of thioredoxin with oxi-

dized aminobutyrate aminotransferase. Evidence for the formation of a covalent intermediate, FEBS Lett. 310 (1992) 1–4.

[46] J. Quin, G.M. Clore, W.M.P. Kennedy, J.R. Huth, A.M. Gronenborn, Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NFκB, Structure 3 (1995) 289–297.

[47] H.K. Brandes, F.W. Larimer, F.C. Hartman, The molecular pathway for the regulation of phosphoribulokinase by thioredoxin *f*, J. Biol. Chem. 271 (1996) 3333–3336.

[48] M. Hirasawa, P. Schurmann, J.P. Jacquot, W. Manieri, P. Jacquot, E. Keryer, F.C. Hartman, D.B. Knaff, Oxidation–reduction properties of chloroplast thioredoxins, ferredoxin:thioredoxin reductase and thioredoxin *f*-regulated enzymes, Biochemistry 38 (1999) 5200–5205.

[49] O. Schwarz, P. Schurmann, H. Strotmann, Kinetics and thioredoxin specificity of thiol modulation of the chloroplast H^+-ATPase, J. Biol. Chem. 272 (1997) 16924–16927.

[50] I. Haberlein, M. Wurfel, H. Follmann, Non-redox protein interactions in the thioredoxin activation of chloroplast enzymes, Biochim. Biophys. Acta 1121 (1992) 293–296.