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Modification of the reactivity of spinach chloroplast thioredoxin *f* by site-directed mutagenesis

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Abstract

Spinach chloroplast thioredoxin *f* has a third cysteine residue which is surface exposed and close to the active site disulfide. In addition its N-terminus is rather long compared to other thioredoxins. By site-directed mutagenesis the third cysteine has been replaced, the long N-terminal tail has been removed and the properties of the modified proteins have been examined. Truncation of the N-terminus renders the protein more soluble and stable and has little influence on its catalytic capacities. Replacement of the exposed third cysteine clearly impairs its capacity to interact and reduce target enzymes and shows that this cysteine can be involved in homo-dimer formation.

Keywords: Spinach; Thioredoxin *f*; Site-directed mutagenesis; Reactivity; Protein–protein interaction; Dimer; Fructose 1,6-bisphosphatase

1. Introduction

Thioredoxins are ubiquitous, small proteins, with molecular weights of about 12 000, which act as oxido-reductases and fulfill numerous functions in the metabolism [1]. In contrast to bacterial and mammalian cells, where two types of thioredoxins are known, plant cells contain multiple forms of thioredoxins which have been distinguished by their cellular location, function, primary sequence and phylogenetic origin [2]. Two of these thioredoxins, thioredoxin *f* and thioredoxin *m*, are contained in the chloroplasts of higher plants. They are essential members of the ferredoxin/thioredoxin system mediating the light-dependent regulation of enzyme activities in oxygenic CO_2 assimilation. The two chloroplast thioredoxins exhibit strong target enzyme specificity. Reduced thioredoxin *f* is responsible for the activation of fructose 1,6-bisphosphatase (FBPase), sedoheptulose 1,7-bisphosphatase, phosphoribulokinase [3] and H^+-ATPase [4], whereas reduced thioredoxin *m* activates NADP-dependent malate dehydrogenase [5] and deactivates glucose 6-phosphate dehydrogenase [6].

Thioredoxin *f* has been purified from spinach [7–9] and pea [10] chloroplasts and its primary structure determined [11]. It has been cloned [11,12] and overexpressed in *Escherichia coli* [13] and its crystal structure has recently been solved [14]. Thioredoxin *f*, with a molecular mass of 13 500, has beside the active site sequence Trp-Cys-Gly-Pro-Cys, common to most thioredoxins, relatively little resemblance with the second chloroplast thioredoxin sharing only 30% residue identity. It has some properties which make it difficult to purify. It is rather hydrophobic, unstable and it irreversibly precipitates out of solution at higher concentration. The N-terminal end is longer, at least in the spinach protein, than in most thioredoxins and the terminal amino acid is

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blocked. The protein contains in the C-terminal part a third cysteine residue which is surface exposed and close to the active site [14,15]. This third cysteine is conserved in all known thioredoxin *f* structures (Fig. 1). The presence of non-active site thiols is known for the mammalian thioredoxins where they have been shown to form a disulfide bridge between two thioredoxin molecules [16].

The *m*-type thioredoxins are more similar to the thioredoxin from *E. coli* with 46% sequence identity. This similarity enables thioredoxin from *E. coli* to substitute for thioredoxin *m* but not for thioredoxin *f*. Non-active site thiols have also been reported for three *m*-type thioredoxins [17–19]. Thioredoxin *m* from pea has one such thiol in the C-terminal half at position 56 [20], thioredoxin *m* from *Chlamydomonas reinhardtii* has two in the C-terminal half, one of them at a homologous position (Cys53) and thioredoxin *m* from corn has three additional cysteine residues, one on the N-terminal side of the active site and two on the C-terminal side with one of them also at a homologous position. However these additional Cys are no common feature of *m*-type thioredoxins and it is not known whether one of them is surface exposed.

The presence of two thioredoxins in chloroplasts is puzzling and has prompted experiments to explain the specificity. Both thioredoxins have essentially the same redox potential [21] and mediate identical thiol-disulfide interchange reactions, but have different target protein specificities. Several reports have addressed this question using site-directed mutagenesis. Based on sequence comparisons and modeling various mainly charged residues were replaced either on thioredoxin from *E. coli* [22,23] or thioredoxin *f* from spinach [24] and the resulting proteins tested for interaction with chloroplast target enzymes. In the studies reported here we have tried to obtain information on the function of the third cysteine and of the N-terminal tail of spinach chloroplast thioredoxin *f*, two features which distinguish this protein from its companion thioredoxin *m*. In a previous study, it was observed that the modification of the third Cys with N-ethylmaleimide significantly reduces the capacity of thioredoxin *f* to activate the chloroplast FBPase [15]. This lets one suspect that this residue is at a position which is crucial for the thioredoxin *f*–FBPase interaction. In order to examine the importance of the third cysteine in the activation of FBPase we replaced it by other amino acids using site-directed mutagenesis and

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SPINACIA              1 -MALHLSLSHQSWTSPAHPITSSDPTRSSVPGTGLSRRVDFLGSCKINGVFVVKRKDRRRMRGGE
MESEMBRYANTHEMUM      1 MAMQLSLSHQSWAKSLASPITSFDPARSPPKRVELGPNCLNGGATAGKLMREKVGERMRMSGRSC
BRASSICA              1 -------------MPLSLRLAPSPTALSPTTGGFSPAKKQCRIPSYSGVATTTRRIGLCSLDYVK
ARABIDOPSIS F1        1 -------------MPLSLRLSPSPTALSPTTGGFGPSRKQCRIPYSG---VPTTKIGFCSLDSRK
ARABIDOPSIS F2        1 ------MPLSLRLAPSPTSFRYSPITSTGAGGFSPVKQHCRIPNSGVATKIGFCSGGGGVLDSGR
PISUM                 1 ---------MALNLCTSPKWIGTTVFDSASSSKPSLASSFSTTSFSSSILCSKRVGLQRLSLRRS
ORYZA                 1 ----MALRLSVSSSHGPASSPAISTCRPAACGRFPALLGGGVASQKRSLTVVSGPETRAVIPVRS
consensus             1                    .  .    .   .                .          ..

RECOMBINANT SPINACIA    -MYYLELALGTQEM...
                            1        10        20        30        40        50        60
SPINACIA             65 VRASMEQALGTQEMEAIVGKVTEVNKDTFWPIVKAAGDKPVVLDMFTQWCGPCKAMAPKYEKLAE
MESEMBRYANTHEMUM     66 CVKASLETAVGAESETLVGKVTEVDKDTFWPIANGAGDKPVVLDMYTQWCGPCKVMAPKYQELAE
BRASSICA             53 RGDSSVVRCSLQTVNVSVGQVTEVDKDTFWPIVKAAGEKIVVLDMYTQWCGPCKVIAPKYKALSE
ARABIDOPSIS F1       50 RGDSSVVRCSLETVNVSVGQVTEVDKDTFWPIVKAAGEKLVVLDMYTQWCGPCKVIAPKYKALSE
ARABIDOPSIS F2       60 RIGSCVVRCSLETVNVTVGQVTEVDKDTFWPIVKAAGDKIVVLDMYTQWCGPCKVIAPKYKELSE
PISUM                57 ISVSVRSSLETAGPTVTVGKVTEVNKDTFWPIVNAAGDKTVVLDMFTKWCGPCKVIAPLYEELSQ
ORYZA                62 SGSDTATVGAKAEAVAVTGQVTEVTKDTFWPIVKSAGPKVVLLNMYTQWCGPCKVMAPKFQEMSE
consensus            66    ...       . ...*.****.*******...**.*.*.*.*.*.******..**.. ....

                                70        80        90       100       110
SPINACIA            130 EYLDVIFLKLDCNQENKTLAKELGIRVVPTFKILKENSVVGEVTGAKYDKLLEAIQAARSS--------
MESEMBRYANTHEMUM    131 KLLDVVFLKLDCNQENKPLAKELGIRVVPTFKILKGGKIVDEVTGAKFDKLVAAIEAARSS--------
BRASSICA            118 KYEDVVFLKLDCNPENRPLAKELGIRVVPTFKILKDNQVVKEVTGAKYDDLVAAIETARSASSSG----
ARABIDOPSIS F1      115 KYDDVVFLKLDCNPDNRPLPKELGIRVVPTFKILKDNKVVKEVTGPKYDDLVAAIETARSAASG-----
ARABIDOPSIS F2      125 KYQDMVFLKLDCNQDNKPLAKELGIRVVPTFKILKDNKVVKEVTGAKYEDLLAAIEAARSG--------
PISUM               122 KYLDVVFLKLDCNQDNKSLAKELGIKVVPTFKILKDNKIVKEVTGAKFDDLVAAIDTVRSS--------
ORYZA               127 KDQDVVFFKLDCNQQNNFFAKEPGTKVVPTFKIFKDGKFFKAVTGPQTDELIQAIETAKSSCGIPRKFA
consensus           131 .. *..*.*****..*....**.*..*******.*.......***.....*..**....*.
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Fig. 1. ClustalX alignment [33] of all known thioredoxin *f* sequences. The transit peptide of spinach thioredoxin *f* is given in *italics* and the mature protein numbered above the sequence. The N-terminal sequence of the recombinant spinach thioredoxin *f* is also given. Database accession numbers: Spinach (X14959), Mesembryanthemum (AF069314), Brassica (AF018174), Arabidopsis F1 and F2 (Y. Meyer, personal communication), Pisum (X63537), Oryza (AF022741).

Table 1
Oligonucleotides used to introduce site-directed mutations into recombinant thioredoxin *f*[a]

Mutation	Mutagenic primer sequence
C 73 A	pCCTGGTT**AGA**ATCGAGC
C 73 S	pTTCCTGGTT**AGC**ATCGAGCTTG
C 73 G	pCCTGGTT**ACC**ATCGAGC

[a] The mutations introduced in the cysteine codon (**ACA**) are underlined.

studied the properties of the modified proteins. In addition an N-terminal truncated thioredoxin *f* was prepared and its properties were compared with those of the recombinant protein described earlier [13].

2. Materials and methods

2.1. Material

Restriction endonucleases were from Boehringer and DNA modifying enzymes from Appligene, Biolabs, GIBCO-BRL and Promega. Radioisotopes, Pharmalytes and chromatography supports (Phenyl-, Q-Sepharose, Resource-isopropyl, Superdex-75, HiTrap) and FPLC equipment were from Amersham Pharmacia. All chemicals were of analytical grades.

2.2. Bacterial strains and plasmids

E. coli strain XL1-Blue was used to produce high levels of plasmids, M13 mp18 single strand and replicative forms. *E. coli* strain RZ1032 dut^- ung^- served to produce dU-substituted DNA templates for oligonucleotide-directed mutagenesis [25]. The expression vector pKK-MTTL was kindly provided by Dr I. Kopetzky, Boehringer Penzberg, and the *E. coli* strain BN103 used as host to overexpress recombinant thioredoxin *f* was a gift from Dr N. Müller, University of Berne [13].

Bacteria were grown at 37°C in a modified Luria broth, containing 10 g of Bacto-Tryptone, 5 g of yeast extract and 5 g of NaCl/l. Ampicillin at 50 μg/ml and tetracycline at 12 μg/ml were included as selective markers.

2.3. Subcloning and mutagenesis of Cys73

A subfragment (364 nucleotides) containing the complete coding part of a thioredoxin *f* cDNA was introduced in the *Eco* RI site of pKK-MTTL as described by [13]. This construction was called pKK-TF.

A *Kpn* I–*Hin*d III fragment containing an excized thioredoxin *f* coding sequence was ligated into the M13 mp18 phagemid polylinker. The resulting construct, named M13-TF was used to provide single-stranded DNA for site directed mutagenesis. All point-mutations were introduced by production of dU-substituted DNA templates by the method of Kunkel [25]. The mutagenic primers, listed in Table 1, were purchased from Microsynth (Balgach, Switzerland). A mutant screening was performed by preparing replicative forms from a few plaque isolates and sequencing them through the mutagenesis site by PCR (fmol DNA Sequencing System kit, Promega). Positive mutants were then subcloned again in the *Kpn* I–*Hin*d III sites of the pKK-TF vector.

2.4. Expression and purification of recombinant and mutant thioredoxin f

The bacteria were grown in 1 l culture medium in a labor fermentor (Multi-Gen, New Brunswick Scientific) as described earlier [26]. Since the mutant thioredoxins, like the recombinant thioredoxin *f*, were expressed as inclusion bodies the same solubilization and purification methods could be applied [13,26].

2.5. N-terminal deletion

The thioredoxin *f* gene was deleted by polymerase chain reaction (PCR) amplification of the shortened gene with two specific primers. The first (deletion) primer was designed to start inside the gene (at Met10), creating a deletion of 9 residues, while the second primer hybridized at the 3′ gene extremity. Each primer contained a restriction site, *Nco* I and *Bam* HI, to allow further cloning in the pET-3d expression vector.

Nco I
Deletion primer pCC ATG GAA GCC ATT GTA GGG
M_{10} E A I V G_{15}

Bam HI
Second primer pGGATCCTCA ACT ACT TCG AGC AGC
Stop S_{122} S R A A_{118}

Amplification was performed at standard PCR conditions for 30 cycles in a 100 μl mixture containing 10 nM of pKK233-2 MTTL cloning vector recombined with the full length thioredoxin *f* gene [13], 0.5 μM phosphorylated primers, 0.2 mM dNTP and 2.5 U of Taq DNA polymerase in appropriate buffer. The amplified fragment corresponding to deleted thioredoxin *f* was subcloned in pBluescript, blunt end digested with *Eco* RV and sequenced to verify that no other mutations were inserted. The recombined pBluescript was digested with *Nco* I and *Bam* HI and the deleted thioredoxin *f* gene subcloned in the pET-3d expression vector.

2.6. Expression and purification of deleted thioredoxin *f*

For overexpression, freshly transformed BL21 DE3 host bacteria were grown overnight in a preculture that was used to inoculate 10 l of medium in a fermentor (Bioengineering AG) kept at 22°C. Cells were induced with 1 mM IPTG at an OD_{600nm} of 0.6 and harvested at the end of their exponential growth. The bacteria (30–60 g) were gently resuspended at 4°C in 1 l hypertonic solution containing 20 mM Tris–Cl pH 8.0, 2.5 mM EDTA–Na and 20% saccharose (w/v) and stirred for 10 min at 4°C. The bacteria were then pelleted by centrifugation (10 min, 14 500 × *g*) and resuspended in 1 l hypotonic solution containing 20 mM Tris–Cl pH 8.0, 2.5 mM EDTA–Na, 100 μM PMSF, 1 μM E-64 and 1 μM leupeptine. This was followed by stirring and centrifugation as indicated above. The resulting supernatant, containing the thioredoxin, was subjected to a heat shock at 80°C for 3 min and rapidly cooled in ice. Some contaminating proteins were then removed by ammonium sulfate fractionation at 40% saturation and the deleted thioredoxin *f* precipitated by ammonium sulfate at 100% saturation. Precipitated proteins were dissolved in 50 mM phosphate buffer at pH 7.0 containing 0.6 M ammonium sulfate, collected on a 130 ml Phenyl-Sepharose column in the same buffer and eluted with a 600 ml gradient of 0.6–0 M ammonium sulfate in phosphate buffer. Thioredoxin *f* fractions were combined and concentrated by ultrafiltration on a YM-5 membrane (Amicon). Final purification was achieved on a 1 ml Resource-isopropyl column with a 15 ml gradient of 2–0 M ammonium sulfate in 50 mM phosphate buffer at pH 7.0.

2.7. FBPase affinity column

A total of 4.75 nmol (equal to 7 mg) spinach chloroplast FBPase were dialyzed against coupling buffer (0.2 M $NaHCO_3$ pH 8.5, 1.0 M NaCl) and then bound to a 1 ml HiTrap NHS-activated column following the manufacturer's instructions.

Each thioredoxin sample to be chromatographed was dialyzed against 5 mM Tris–acetate pH 7.5 and then diluted to 10 μM with the 5 mM Tris–acetate pH 8.0, 0.1 mM EDTA elution buffer. Two nmol (equal to 34 μg) of thioredoxin were loaded and eluted with a 10 ml gradient of 0–200 mM NaCl in elution buffer at a 1 ml/min flow rate.

2.8. Electrophoretic analyses

Electrophoretic separations under denaturing conditions were done according to [27] with a Mini-Protean System from Biorad. Isoelectric focusing gels, covering a pH range from 5 to 8 were run at 10°C on a LKB Multiphor apparatus.

2.9. Enzyme assays

Target enzymes were purified as described [8,26] and the thioredoxin activities measured by the two stage assay [26].

3. Results and discussion

3.1. Expression and solubility of mutant thioredoxins

In recombinant thioredoxin *f* [13] Cys73 was replaced by alanine, serine or glycine and the constructs expressed in *E. coli*. The proteins were produced in large amounts and deposited in inclu-

sion bodies like the original recombinant thioredoxin *f*. The C73A and C73S mutants could be easily solubilized and purified. Up to 50 mg pure protein/l of bacterial culture was obtained. Their correct folding was verified by size exclusion chromatography on a FPLC-Superdex-75 column. Although the C73G mutant was synthesized in large amounts as evidenced by the amount of inclusion bodies that could be isolated and by SDS-PAGE followed by immunoblotting, it was difficult to renature and purify and only very small quantities of pure protein were obtained. The C73G mutation might be in a very critical region for protein folding and due to the increased flexibility of Gly prevent proper folding.

Interestingly, the deletion mutant, Tfdel, was produced as soluble protein in the cytoplasm, however, with smaller yield than the other mutants. In order to boost the production yield of Tfdel, we have tested various culture media and conditions. A temperature shift from 37 to 22°C increased the relative amount of Tfdel about 4-fold. These conditions were finally adopted for all further productions of Tfdel which yielded 1–2 mg Tfdel/l of bacterial culture. The truncated thioredoxin *f* is much more soluble than the recombinant or the native chloroplast protein. Therefore,

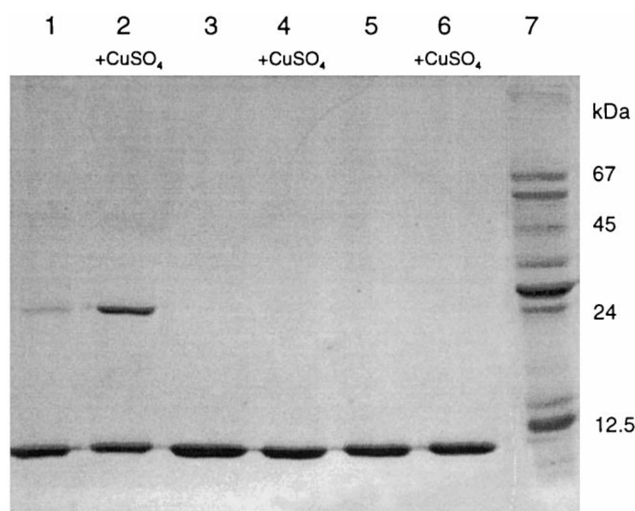

Fig. 2. SDS-PAGE separation of recombinant and mutant thioredoxin *f* in the absence of reducing agent. Lanes 1 and 2: recombinant thioredoxin *f*; lanes 3 and 4: C73A mutant; Lanes 5 and 6: C73S mutant. Samples in lanes 2, 4 and 6 had been preincubated for 2 h with 2 mM $CuSO_4$. Lane 7: molecular mass marker proteins: bovine serum albumin 67 kDa, catalase 60 kDa, ovalbumin 45 kDa, DNase 31 kDa, carboanhydrase 29 kDa, PMSF-trypsinogen 24 kDa, RNase 13 kDa, horse heart cyt.c 12.5 kDa.

it can be highly concentrated by ultrafiltration, rendering it suitable for crystallographic analyses [14].

The difference in solubility amongst the various analyzed *f*-type thioredoxins could primarily be due to structural differences in the N-terminal part (see Fig. 1). The following observations suggest that a shortened N-terminal part favors the solubility: Both, Tfdel (spinach) and recombinant pea thioredoxin *f* [28] with shorter N-terminus as well as another shorter recombinant construct [29] are very soluble contrary to the recombinant spinach thioredoxin *f* having an extended N-terminus. Similarly, thioredoxin *m* (spinach) which lacks the extended N-terminal part is also very soluble.

3.2. Electrophoretic analysis of thioredoxin *f*

It has been found that Cys73 does form covalent homodimers which can be demonstrated by SDS-PAGE. If thioredoxin samples are prepared and separated in the absence of a reducing compound a protein band at about 24 kDa is observed (Fig. 2; lane 1). Immunoblotting reveals that this band is thioredoxin *f* (data not shown). The 24 kDa band is more prominent in samples that have been repeatedly frozen and thawed and can be increased to about 30% of the total protein by preincubation with 2 mM $CuSO_4$ as oxidizing agent (Fig. 2; lane 2). It has not been possible to increase the amount of dimer any further even after overnight incubations with $CuSO_4$. In contrast, there is no dimer observed with the C73X mutants of thioredoxin *f* (Fig. 2; lanes 3–6) which clearly demonstrates that the dimer is formed through a disulfide linkage involving Cys73. The dimer formation can be reversed by incubation with reduced glutathione at concentrations reported for the chloroplast stroma [30]. In vivo, reduced glutathione in the chloroplast might prevent the formation of dimers. A thioredoxin *f* dimer has been reported earlier, however, this dimer could be separated by high ionic strength which excludes the presence of a disulfide bond [9].

As expected, isoelectric focusing analysis revealed that the replacement of Cys73 does not modify the isoelectric point. It was found to be 6.1, identical to the recombinant as well as the native thioredoxin *f*. However, the removal of the N-terminal extension containing two glutamic acid residues increases the p*I* to 7.1. This result is of

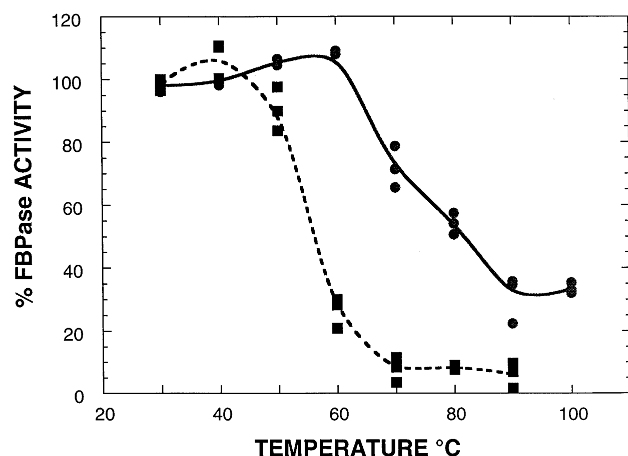

Fig. 3. Thermal stability of recombinant and truncated thioredoxin *f*. The thioredoxin samples were heated for 3 min at the indicated temperatures and then aliquots tested for activation of FBPase. ■, recombinant thioredoxin *f*; ●, truncated thioredoxin *f*.

significance with respect to the N-terminus of native spinach thioredoxin *f*. Its N-terminal residue was found to be a blocked methionine [11]. The N-terminal methionine could be Met1 or Met10 in Fig. 1, resulting in two polypeptides differing by two negative charges. Electrofocusing analysis of native and recombinant thioredoxin *f* yielded an identical p*I* for both proteins. It suggests that the native protein contains the additional charged residues and therefore starts at the first methionine [13]. The present observation, that the removal of the N-terminal extension containing two negative charges shifts the p*I* up by one pH unit, is again a strong argument in favor of the earlier conclusion that the mature native protein starts with Met1 (Fig. 1).

3.3. Thermal stability

The thermal stability is one of the typical properties of thioredoxins. However, whereas the bacterial type thioredoxins are generally very heat stable, some plant thioredoxins, including thioredoxin *f* [7], are less heat stable [18,19]. To find out whether this instability is influenced by the N-terminal extension, the heat denaturation of the recombinant and deleted thioredoxin *f* was compared. The truncated thioredoxin *f* turned out to be significantly more heat stable. From the curves in Fig. 3, one can deduce a half-denaturation temperature of 55°C for the recombinant thioredoxin *f* with the long N-terminus, and of 75°C for Tfdel. This let us conclude that the extended N-terminus must be, at least in part, responsible for the reduced thermal stability of spinach thioredoxin *f*.

3.4. Reactivity of mutant thioredoxin f

Thioredoxin *f* is known to be the activator protein for FBPase, but it is also capable to reduce and thereby activate NADP-dependent malate dehydrogenase quite well [24,31]. To assess whether the replacement of Cys73 by another amino acid, or the removal of the N-terminal tail, have an influence on the biological activity the reactivity of the modified proteins with FBPase and malate dehydrogenase as target enzymes was tested. The replacement of Cys73, which is close to the active site, significantly reduces the reactivity with both target enzymes, however, both reach full activity (Table 2). This result is in line with what we had observed following chemical modification of Cys73 with *N*-ethylmaleimide [15]. The drop in activity with both enzymes implies that the same surface area of thioredoxin *f* is involved in the activation. The C73S mutation resulted in a larger loss of activity than the C73A mutation. This is somewhat surprising since the C73S mutation is rather conservative and should yield a protein

Table 2
Activation parameters of recombinant and mutant thioredoxins

	FBPase activation		MDH activation	
	$S_{0.5}$ (μM)	$S_{0.5}$ ratio (mutant/control)	$S_{0.5}$ (μM)	$S_{0.5}$ ratio (mutant/control)
Thioredoxin *f*	0.2	1	4.9	1.5
Thioredoxin *m*	n.d.	n.d.	3.3	1
C73A	0.9	3	25.4	8
C73S	3	12	64.5	19
C73G	9.6	38	n.d.	n.d.
Tfdel	0.5	2	n.d	n.d.

Table 3
Elution parameters of recombinant and mutant thioredoxins on the HiTrap fructose 1,6-bisphosphatase (FBPase)-Sepharose column

Thioredoxin	Retention volume (ml)	Retention volume difference to control (ml)	Elution molarity (mM NaCl)
Recombinant thioredoxin *f*	8.47	0	54
Thioredoxin *m*	1	Not retained	0
Mutant C73A	8.23	−0.24	49
Mutant C73S	7.62	−0.85	32
Truncated thioredoxin *f*	9.4	+0.93	80

structurally and functionally very similar to the recombinant, but maybe slightly more polar. The C73A mutation favors a hydrophobic environment close to the active site. It had been demonstrated that hydrophobic interactions in addition to some charged residues are involved in the activation of FBPase by thioredoxin *f* [32].

The truncated thioredoxin *f* had only a slightly reduced reactivity indicating that the N-terminal extension is not involved in the activation, but could be significant in positioning the FBPase (see below).

3.5. Affinity chromatography

The activation results suggest that the interaction between the mutant thioredoxins and FBPase is somehow impaired. To probe the affinity between the thioredoxins and the target enzymes, a FBPase-affinity column has been used and the elution parameters of the different thioredoxins have been determined under strictly controlled conditions. Table 3 summarizes the results obtained with the affinity column. Both Cys73 mutants display lower affinities than the recombinant thioredoxin *f*. The C73A mutant is slightly more retained by the FBPase than the C73S mutant. This behavior is in line with the $S_{0.5}$ values reported in Table 2 which indicate that the C73A mutant has a higher affinity to the FBPase. Surprisingly, the truncated thioredoxin *f* interacted significantly stronger with the column bound enzyme than the recombinant thioredoxin *f*, whereas its $S_{0.5}$ was 2 times higher. The stronger binding to the FBPase could be due to the removal of the two negative charges in the N-terminal extension.

In conclusion, the replacement of the exposed Cys73 has no influence on the expression of this protein in *E. coli*, but prevents formation of covalent homodimers. Mutation of Cys73 also reduces the reactivity with target enzymes suggesting that this residue is important for the protein–protein interaction. The removal of the N-terminal extension renders the protein soluble and heat stable without markedly influencing its catalytic activity, but enhances its affinity for the FBPase. The modified physical properties of the truncated thioredoxin *f* made this form a very useful material for crystallographic analyses [14].

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