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Interaction of thioredoxins with target proteins: Role of particular structural elements and electrostatic properties of thioredoxins in their interplay with 2-oxoacid dehydrogenase complexes

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Abstract

The thioredoxin action upon the 2-oxoacid dehydrogenase complexes is investigated by using different thioredoxins, both wild-type and mutated. The attacking cysteine residue of thioredoxin is established to be essential for the thioredoxin-dependent activation of the complexes. Mutation of the buried cysteine residue to serine is not crucial for the activation, but prevents inhibition of the complexes, exhibited by the *Clamydomonas reinhardtii* thioredoxin *m* disulfide. Site-directed mutagenesis of D26, W31, F/W12, and Y/A70 (the *Escherichia coli* thioredoxin numbering is employed for all the thioredoxins studied) indicates that both the active site and remote residues of thioredoxin are involved in its interplay with the 2-oxoacid dehydrogenase complexes. Sequences of 11 thioredoxin species tested biochemically are aligned. The thioredoxin residues at the contact between the $\alpha 3/3_{10}$ and $\alpha 1$ helices, the length of the $\alpha 1$ helix and the charges in the $\alpha 2$-$\beta 3$ and $\beta 4$-$\beta 5$ linkers are found to correlate with the protein influence on the 2-oxoacid dehydrogenase complexes (the secondary structural elements of thioredoxin are defined according to Eklund H et al., 1991, *Proteins 11*:13–28). The distribution of the charges on the surface of the thioredoxin molecules is analyzed. The analysis reveals the species specific polarization of the thioredoxin active site surroundings, which corresponds to the efficiency of the thioredoxin interplay with the 2-oxoacid dehydrogenase systems. The most effective mitochondrial thioredoxin is characterized by the strongest polarization of this area and the highest value of the electrostatic dipole vector of the molecule. Not only the magnitude, but also the orientation of the dipole vector show correlation with the thioredoxin action. The dipole direction is found to be significantly influenced by the charges of the residues 13/14, 51, and 83/85, which distinguish the activating and inhibiting thioredoxin disulfides.

Keywords: electrostatic dipole vector; homology modeling; long-range electrostatic interactions; protein–protein recognition; structural analog; thioredoxin; 2-oxoacid dehydrogenase complex

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Abbreviations: E1, 2-oxoacid dehydrogenase; E1p, pyruvate dehydrogenase; E1o, 2-oxoglutarate dehydrogenase; E2, dihydrolipoate acyltransferase; E3, dihydrolipoate dehydrogenase; ThDP, thiamin diphosphate; lip, lipoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, N-ethylmaleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol; v, reaction velocity in the absence of effectors; v_a, reaction velocity affected by an effector; $S_{0.5}$, thioredoxin concentration required for the half-maximal effect; 3D, three-dimensional.

Thioredoxins comprise a family of small (about 12 kDa) proteins with a conserved redox active disulfide bridge in their active site. A number of closely related yet functionally different thioredoxins have been discovered up to date, with their number and functions continuing to increase (Eklund et al., 1991; Follmann & Häberlein, 1996; Jacquot et al., 1997a). Most of the studied biological effects of thioredoxins are dependent on their general thiol-disulfide oxidoreductase activity. It has been recently established that mitochondrial 2-oxoacid dehydrogenase complexes are activated by thio-

redoxin (Bunik et al., 1997a). The complexes catalyze irreversible oxidation of 2-oxoacids with production of acyl-CoA's and NADH via Reactions 1–5:

$$\mathrm{HOOC-C(R)=O + ThDP\text{-}E1 \rightarrow CO_2 + OH-C^{-}(R)-ThDP\text{-}E1} \quad (1)$$

$$\mathrm{OH-C^{-}(R)-ThDP\text{-}E1 + lip(S\text{-}S)\text{-}E2 \rightarrow ThDP\text{-}E1 + O=C(R)-S\text{-}lip(SH)\text{-}E2} \quad (2)$$

$$\mathrm{O=C(R)-S\text{-}lip(SH)\text{-}E2 + HS\text{-}CoA \leftrightarrow O=C(R)-S-CoA + lip(SH)_2\text{-}E2} \quad (3)$$

$$\mathrm{lip(SH)_2\text{-}E2 + E3(S\text{-}S)\text{-}FAD \leftrightarrow lip(S\text{-}S)\text{-}E2 + E3(SH)_2\text{-}FAD} \quad (4)$$

$$\mathrm{E3(SH)_2\text{-}FAD + NAD^{+} \leftrightarrow E3(S\text{-}S)\text{-}FAD + NADH + H^{+}.} \quad (5)$$

Thioredoxin has been shown to interplay with the complex-bound dihydrolipoate intermediate at very low concentrations of both thioredoxin (10^{-6} M) and the dehydrogenase system (10^{-9} M) (Bunik et al., 1997a). Although the complex formation between proteins is usually rather slow at these concentrations, thioredoxin was able to compete with the rapid electron transfer Reactions 4 and 5 without any preincubation with the target. This work aims to identify the structural features of the thioredoxin molecule, responsible for its high reactivity toward the 2-oxoacid dehydrogenase complexes.

The thioredoxin cross-reactivity is usually lower with a non-physiological target as compared to the physiological one, but the structural requisites for the thioredoxin-target recognition are not well understood. No stable native complexes between thioredoxins and their target enzymes have been available for characterization, whereas some of the cross-linked complexes either are difficult to resolve (Lennon et al., 1997) or include only a small peptide fragment of the target (Qin et al., 1995). An alternative approach to revealing the requisites for recognition may involve the comparative functional study of the enzyme reactivity to a specific ligand and its structural analogs. A similar strategy is applied here. Mitochondrial 2-oxoacid dehydrogenase complexes are affected by mitochondrial thioredoxin and a number of homologous thioredoxin structures, including associated mutants and chemically modified species. Biochemical characterization of the effects is added by multiple alignment of the thioredoxin sequences and comparison of their 3D structures. The data obtained provide new knowledge about the mechanism of the thioredoxin action upon the dehydrogenase complexes, specificity of the mitochondrial protein in affecting the 2-oxoacid oxidation and structural determinants of the thioredoxin-target recognition.

Results

The effects of thioredoxin upon dehydrogenases of 2-oxoacids have been revealed under the two sets of conditions (Bunik et al., 1997a). First, thioredoxins induce an increase in the catalytic rate of the 2-oxoacid oxidation at low NAD^+ concentrations. Second, thioredoxins protect the complexes from the catalysis-induced inactivation upon excessive accumulation of the dihydrolipoate intermediate; the activity loss can be followed at high NAD^+ concentrations after preincubation of the complexes with 2-oxoacid and CoA. Both systems were used in this work to compare the effects of a number of thioredoxin species upon the pig heart 2-oxoacid dehydrogenase complexes.

Study of different thioredoxins showed that they may not only activate (mitochondrial, pea *m*, and *Chlamydomonas reinhardtii h* thioredoxins), but also inhibit (*C. reinhardtii m* thioredoxin) the 2-oxoacid:NAD^+-reductase activity at low NAD^+ concentrations (Fig. 1). In spite of the opposite effect, the $S_{0.5}$ of thioredoxin *m* from *C. reinhardtii* was close to the values inherent in the *Escherichia coli* and pea *m* proteins (Table 1), suggesting similar binding affinity. The mitochondrial thioredoxin was an order of magnitude more effective than other proteins (Table 1). Thioredoxins *h* exhibit specific saturation kinetics: S-shaped curves suggesting inefficiency of the low concentrations of these thioredoxins were obtained with the protein from *C. reinhardtii* (Fig. 1A) and with *Arabidopsis thaliana h5* thioredoxin (not shown). The latter caused no effect below 20 μM; with the concentration increased up to 115 μM, it still showed no more

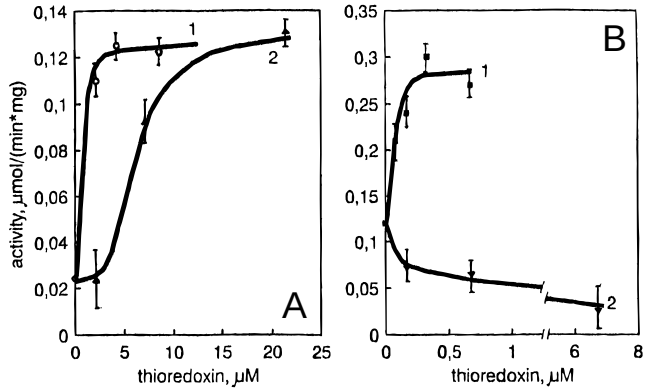

Fig. 1. Influence of different thioredoxins on the activity of 2-oxoglutarate dehydrogenase complex measured at (**A**) 5 and (**B**) 30 μM NAD^+. Thioredoxins employed are pea *m* (1**A**), *C. reinhardtii h* (2**A**), mitochondrial (1**B**), *C. reinhardtii m* (2**B**).

Table 1. *Influence of different thioredoxins on pyruvate and 2-oxoglutarate dehydrogenase complexes*[a]

Thioredoxin	Effect	$S_{0.5}$ (μM) 2-Oxoglutarate dehydrogenase complex	$S_{0.5}$ (μM) Pyruvate dehydrogenase complex
Mitochondrial	Activation	<0.25	<0.1
E. coli	Activation	1.1 ± 0.5	0.8 ± 0.2
m pea	Activation	1.2 ± 0.4	2.2 ± 0.8
h C. reinhardtii	Activation	4.5 ± 1.5	3.8 ± 0.7
f pea	None	—	—
m C. reinhardtii	Inhibition	1.0 ± 0.5	n.d.[b]

[a]The complex activity was measured in the medium with saturating 2-oxoacid and CoA at 5 or 30 μM NAD^+ in case of activation or inhibition, respectively

[b]Not determined.

than 30% of the maximum activation elicited by other species. Tested up to 20 μM, pea *f* thioredoxin had no distinct effect.

Similar differences in the thioredoxin efficiencies were revealed from the thioredoxin protection of the complexes inactivated by 2-oxoacid and CoA (Table 2). The residual activity after preincubation with the substrates was increased already at an extremely low (0.05 μM) concentration of mitochondrial thioredoxin and the protection was complete at 0.5 μM, whereas 3–5 μM concentration was required for the full protection by *E. coli*, pea *m*, and *C. reinhardtii h* thioredoxins. Three of the five known thioredoxins *h* from *A. thaliana* caused only partial effect, with no additional protection achieved at further increase in the thioredoxin concentrations. Other thioredoxins (*A. thaliana h2*, *h3*, pea *f*, *C. reinhardtii m*) were not protective. Thus, from the results of the two independent sets of data thioredoxins could be ordered according to their ability to influence 2-oxoacid dehydrogenase complexes as shown in Tables 1 and 2.

The differential role of the thioredoxin attacking and buried cysteine residues in activation of the 2-oxoacid dehydrogenase complexes was investigated using combination of site-directed mutagenesis and chemical modification. Mutation of the buried cysteine in *C. reinhardtii h* thioredoxin to serine did not result in an inactive specie, though increased the concentration required for the maximum activation (Table 3). In fact, the same activation level was reached with 7 μM of the wild-type and 17 μM of the C35S mutant thioredoxins (Table 3, 16 μM NAD^+). The C35S mutant was also found to be active in the DTT:insulin reduction, catalyzing this reaction at a rate of 0.7 ΔD_{650}/min·mg as compared to 2.7 ΔD_{650}/min·mg of the wild-type protein. Modification of the attacking cysteine of the C35S mutant with NEM or NBD-Cl abolished both the DTT:insulin oxidoreductase activity and activation of the 2-oxoacid dehydrogenase complexes. Even an inhibition of the complexes by the modified thioredoxins was observed instead of activation (Table 3). Thus, the attacking cysteine residue of thioredoxin is required for activation of the 2-oxoacid dehydrogenase complexes. The activation is dependent on the thiol-disulfide oxidoreductase activity of thioredoxin. Mutation of the buried cysteine residue to serine does not cancel this activity and hence the activation, though it decreases the efficiency of both. Although surprising, the catalytic action of the C35S protein is in accord with the data on a partial activity of the analogous thioredoxin reductase mutant (Williams et al., 1989). Thus, substitution of the buried cysteine residue by serine is shown to result in the catalytically competent thiol-disulfide oxidoreductases.

Specific action of the attacking cysteine residue in interplay with the 2-oxoacid dehydrogenase complexes was further supported by the loss of the thioredoxin-induced activation upon the D26A substitution. Participating in the charge stabilization in the thioredoxin active site (Eklund et al., 1991), this aspartic acid residue inputs to the unique reactivity of the attacking cysteine. Noteworthy, its substitution resulted in an inhibitory specie as has been also observed with the C32-modified C35S mutant (Table 3). This indicates that the thioredoxin structures with the specifically deteriorated reactivity of the attacking cysteine residue can be bound, with the binding inhibiting the 2-oxoacid oxidation. The

Table 2. *Thioredoxin protection of the 2-oxoglutarate dehydrogenase complex from the 2-oxoglutarate, CoA-induced inactivation*[a]

Thioredoxin	v_a/v (%) at thioredoxin concentration (μM) 0	0.05	0.5	3	5	10	20	80
None	30 ± 10							
Mitochondrial		59 ± 5	100 ± 8					
E. coli			32 ± 10	100 ± 12				
m pea			22 ± 10		100 ± 4			
h C. reinhardtii			23 ± 10	60 ± 7	100 ± 3			
*h*4 *A. thaliana*		22 ± 10	51 ± 7	68 ± 8		66 ± 8		
*h*5 *A. thaliana*			32 ± 10				60 ± 5	70 ± 5
*h*1 *A. thaliana*		33 ± 10		52 ± 15		51 ± 15		
f pea			33 ± 10		41 ± 12	41 ± 10		
*h*2 *A. thaliana*		22 ± 10		25 ± 14		30 ± 14		
*h*3 *A. thaliana*			43 ± 10				31 ± 15	11 ± 10
m C. reinhardtii		40 ± 10	30 ± 10	17 ± 14	19 ± 10			

[a]Conditions are described in Materials and methods.

Table 3. *Involvement of the particular amino acid residues of the C. reinhardtii thioredoxin h in its activation of the 2-oxoglutarate dehydrogenase complex*

Thioredoxin *h*	Concentration (μM)	v_a/v (%) at NAD^+ (μM)		
		5	16	30
Wild-type oxidized	17	240 ± 40	—	300 ± 40
Wild-type reduced	17	260 ± 10	—	—
	7	—	180 ± 20	—
C35S	17	180 ± 20	180 ± 15	190 ± 20
C35S, NEM-modified	9	60 ± 7	—	—
	17	45 ± 10	—	—
C35S, NBD-Cl-modified	17	50 ± 10	80 ± 10	—
	34	—	10 ± 6	—
D26A	17	—	—	80 ± 8
W31A	17	—	—	110 ± 10
A70Y	17	—	—	132 ± 11
W12F	17	—	—	98 ± 9

latter suggests thioredoxin to occupy the catalytically important area within the 2-oxoacid dehydrogenase systems. In contrast, mutation of another invariant group of the thioredoxin active site, W31A, decreases the thioredoxin-dependent activation of the complexes, but causes no inhibition even at high concentration of the mutant (17 μM, Table 3). This agrees with the assumption that W31 participates in fixing a target for the catalysis (Eklund et al., 1991). Its substitution to alanine deteriorates the formation of the thioredoxin-target complex. However, the reactivity of C32 in the formed complex is not changed and therefore no inhibition is observed.

The C35S mutant of *C. reinhardtii h* thioredoxin also protects from the substrate-induced inactivation (Table 4). Compared to the C35S protein, a greater loss of the protective effect is observed with the D26A and W31A mutants. Thus, the protection does not disappear upon substitution of the buried cysteine to serine, but requires the high reactivity of the attacking cysteine, dependent on D26, and the proper thioredoxin-target interaction, mediated by W31.

In contrast to its little significance for the activation (Table 3) and protection (Table 4), mutation of the buried cysteine (C35S) prevented the inhibition by *C. reinhardtii* thioredoxin *m* (Table 5). Because the inhibition was observed with the disulfide form of the wild-type protein, stabilization of the reduced form of the attacking cysteine (C32) in the C35S mutant could be responsible for the disappearance of inhibition. Indeed, the activity of 2-oxoglutarate dehydrogenase complex at 6 μM NAD^+ was decreased by 30% with 1 μM disulfide, but increased by 50% with 1 μM dithiol forms of the thioredoxin. Also, the full protection (96 ± 4% of the residual activity) was achieved in the presence of the reduced *C. reinhardtii m* thioredoxin, in contrast to no protection observed with the disulfide form (Table 2). Therefore, the reduced state of the attacking cysteine in the C35S mutant explains the loss of inhibition. Taking into account that the effect of the dithiol forms of the *C. reinhardtii m* thioredoxin and other species does not differ, while the effects of their disulfides do, a greater selectivity of the dehydrogenase complexes to the thioredoxin disulfides may be suggested. Probably, the highly reactive dithiol group in the reduced thioredoxins enables accomplishing their effects less specifically.

Substitutions of the conserved residues of the thioredoxin active site (C35, D26, W31) and chemical modification of C32 substan-

Table 4. *Effect of the C.reinhardtii thioredoxin h mutations on its protection of the 2-oxoglutarate dehydrogenase complex from the 2-oxoglutarate, CoA-induced inactivation*[a]

Thioredoxin *h* of *C. reinhardtii*	v_a/v (%) at thioredoxin concentration (μM)		
	0	3	20
None	30 ± 10		
Wild type		60 ± 7	—
C35S		55 ± 12	93 ± 4
D26A		41 ± 15	—
W31A		32 ± 14	—
A70Y		28 ± 14	—
W12F		14 ± 10	—

[a]Conditions are described in Materials and methods.

Table 5. *Involvement of the particular amino acid residues of the C. reinhardtii thioredoxin m in its inhibition of 2-oxoglutarate dehydrogenase complex*[a]

Thioredoxin *m* of *C. reinhardtii* (7 μM)	v_a/v (%)
Wild-type	29 ± 5
C35S	100 ± 5
F12W	64 ± 5
Y70A	55 ± 5

[a]Reaction rates were measured at 30 μM NAD^+.

tiated the role of the catalytically essential amino acids of thioredoxin in its reactivity to the 2-oxoacid dehydrogenase complexes. Nevertheless, even with the different active site motif CPPC (Fig. 2), the *A. thaliana h4* and *h5* thioredoxins displayed a stronger effect (Table 2) than *h1* and *h2* thioredoxins having the regular CGPC. This indicates that structural elements beyond those at the active site influence the thioredoxin reactivity, too. To reveal such elements, the sequences of the employed thioredoxins (Holmgren, 1968; Jacquot et al., 1992; Lepiniec et al., 1992; Lopez-Jaramillo et al., 1994; Rivera-Madrid et al., 1995; Stein et al., 1995; Spyrou et al., 1997) were aligned. The four sequences of mammalian mitochondrial thioredoxins available from the SWISSPROT database, i.e., rat, mouse, ox, and human, possess 96–100% identity, with no more than four substitutions (V6I, M47V, I68L, I81M). The highly conservative structure of the mitochondrial protein suggested no strong deviations in the sequence of this thioredoxin in other mammals as well, enabling us to use one of the known sequences for the alignment. Figure 2 shows that all of the more efficient thioredoxins have Y70, while thioredoxins of low or no efficiency (*h* and pea *f*) do not. Indeed, Y70A substitution in *C. reinhardtii* thioredoxin *m* brought about a decrease in its inhibitory action (Table 5), suggesting deteriorated interaction with the target. However, the reverse substitution in *C. reinhardtii* thioredoxin *h* (A70Y) did not improve the efficacy of the latter. The A70Y mutant of *C. reinhardtii* thioredoxin *h* elicited an even lower ability to activate the complexes (130%) when compared to the wild-type protein (300%) (Table 3). Although no dependence of the thioredoxin efficacy on the tyrosine residue at this position was thus found, the A70Y and Y70A mutants showed that the substituted residues influence the thioredoxin function. According to the resolved structures of the *E. coli, C. reinhardtii m* and *h* thioredoxins (Katti et al., 1990; Saarinen et al., 1995; Jacquot et al., 1997a), Y70 interacts through van der Waals contacts with the phenylalanine residue in the α1 helix of the thioredoxin molecule (F12 in *E. coli* thioredoxin, Fig. 3). Mutations at this position (W12F in *C. reinhardtii h* and F12W in *C. reinhardtii m* thioredoxins) also deteriorated the thioredoxin effects (Tables 3–5), pointing to the significance of the structural interaction between F12 and Y70 for the thioredoxin interplay with the dehydrogenase complexes. This is further supported by the fact that *A. thaliana* thioredoxins, which cause a low or no effect, form this couple by more bulky and hydrophobic residues (W12W70 or W12F70, Fig. 2) than those (F12Y70 or W12A70) present in effective thioredoxins.

A specific feature of all thioredoxins *h* is an elongation of the α1 helix owing to the three residue insertion between the 19th and 20th amino acids of the *E. coli* thioredoxin (Fig. 2). This may contribute to a decreased efficiency of these thioredoxins (Table 2). The elongation of the α1 helix is usually accompa-

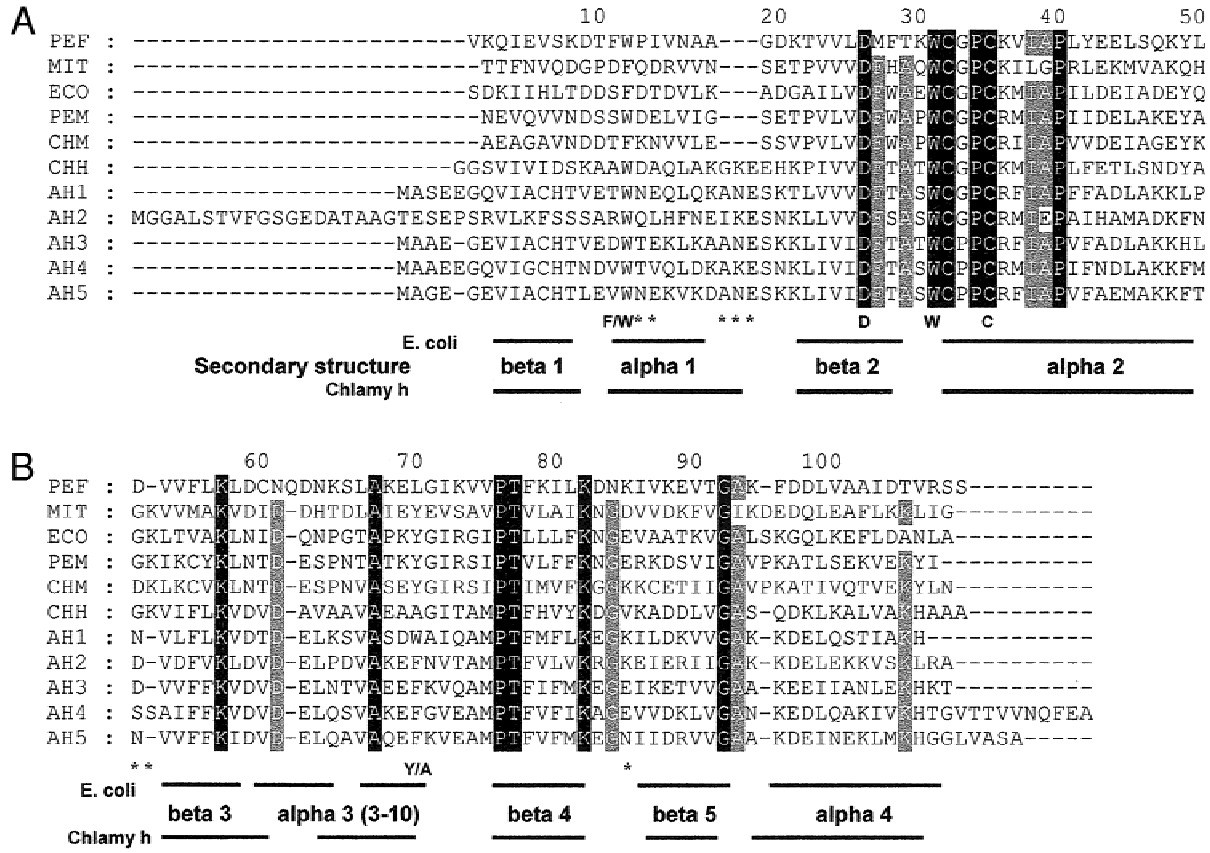

Fig. 2. Multiple sequence alignment of the thioredoxins studied in this work. **A:** *E. coli* thioredoxin numbering is given. The degree of the residue conservation corresponds to the shading intensity. **B:** The employed mutants and other residues considered are marked by the amino acid code and asterisks, respectively. The secondary structure elements of thioredoxins from *E. coli* and *C. reinhardtii h* are presented below the alignment. The three letter abbreviations at the left refer to the sequences of pea *f*, mitochondrial, *E. coli*, pea *m, C. reinhardtii m, C. reinhardtii h, A. thaliana h1, h2, h3, h4, h5* thioredoxins (from up to down).

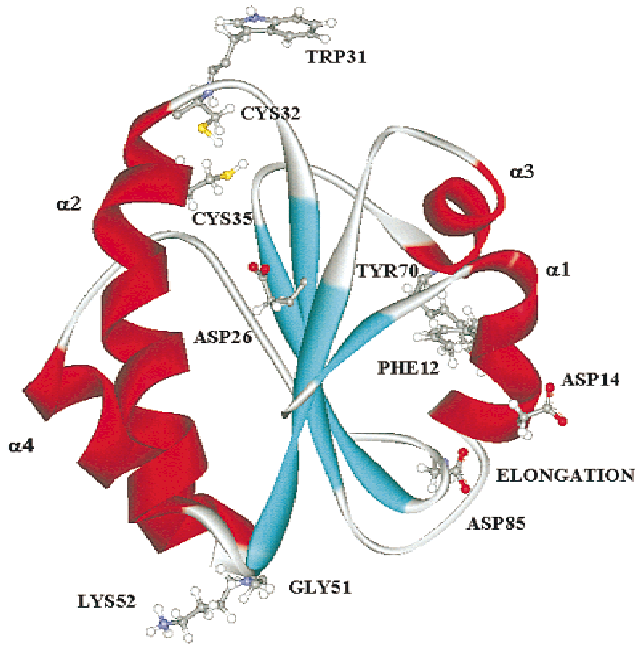

Fig. 3. The thioredoxin essential residues and structural elements, which correlate with the reactivity toward the 2-oxoacid dehydrogenase complexes. Numbered of the residues correspond to the mitochondrial thioredoxin. Helices are red and strands green-blue. The elongation of the α1 helix is indicated.

nied by shortening the turn between the α2 helix and β3 strand due to the absence of K52 (Fig. 2). All thioredoxins with K52, including the only one of *h* type (from *C. reinhardtii)*, elicit 100% amplitude of activation. Absence of K52 correlates with a low (*A. thaliana h1*, *h4*, *h5)* or no (*A. thaliana h3*, *h2*, pea *f*) effect. Thus, the positive charge of K52 in the α2-β3 linker may input to the amplitude of thioredoxin action on the 2-oxoacid dehydrogenase complexes.

The highly homologous sequences of the pea and *C. reinhardtii m* thioredoxins were compared to find determinants of the inhibitory action of the latter. With the most of amino acid residues conserved and many substitutions being functionally equivalent (like S to T, V to I, etc.), only a limited number of potentially significant groups were found to differ. Checking these groups in other thioredoxins pointed to the systematic features that distinguish the inhibitory thioredoxin *m* of *C. reinhardtii* from the well-activating (mitochondrial, *E. coli*, pea *m*, and *C. reinhardtii h*) species (Fig. 2). First, the latter possess a negative charge (D13 or D14) next to the functionally important F/W12 residue, while the positive charge at this place (K13) is specific for the inhibitory thioredoxin. Second, an uncharged glycine residue precedes K52 in the α2-β3 linker of the activating species, but it is replaced by the negatively charged aspartate residue in the inhibiting thioredoxin. Third, the β4-β5 linker has the negatively charged D/E85 or D83 in activating thioredoxins and positively charged K85 in that inhibiting. Because all these residues are located on the surface opposite to the catalytic site (Fig. 3), our findings testify to the influence of the remote electrostatic charges on the thioredoxin reactivity to the 2-oxoacid dehydrogenase complexes.

The electrostatic features of thioredoxins were further analyzed using their 3D structures. Those for the *E. coli* and *C. reinhardtii h* proteins were available from the X-ray and NMR data (Katti et al., 1990; Mittard et al., 1997). The 3D models of other species were constructed by homology modeling using the known sequences (Holmgren, 1968; Jacquot et al., 1992; Lepiniec et al., 1992; Lopez-Jaramillo et al., 1994; Rivera-Madrid et al., 1995; Stein et al., 1995; Spyrou et al., 1997), high degree of the sequence homology, similar spatial organization and conserved active sites and surroundings of thioredoxins. Figure 4 shows the part of the thioredoxin surface around the active site, which interacts with the target protein in the cocrystallized binary complex of the target peptide with human thioredoxin (Qin et al., 1995). Diversity in the electrostatics of this area in different thioredoxins is obvious. There are mostly negative (Fig. 4A), positive (F), and intermediary (B–E) surfaces. Comparison of the thioredoxin action (Table 1) with the data of Figure 4 indicates that the unspecific cross-reactivity, i.e., that between the mammalian enzymes and bacterial or plant thioredoxins, is provided only by the intermediary surfaces (Fig. 4B–E). Those highly polarized (Fig. 4A,F) seem to respond much more specific, providing either the highest efficiency in a biologically relevant couple (both thioredoxin and the complexes from mitochondria) or no interaction at all between the unrelated species (plant thioredoxin *f* and mammalian complexes). The highest reactivity of mitochondrial thioredoxin (Table 1) corresponds

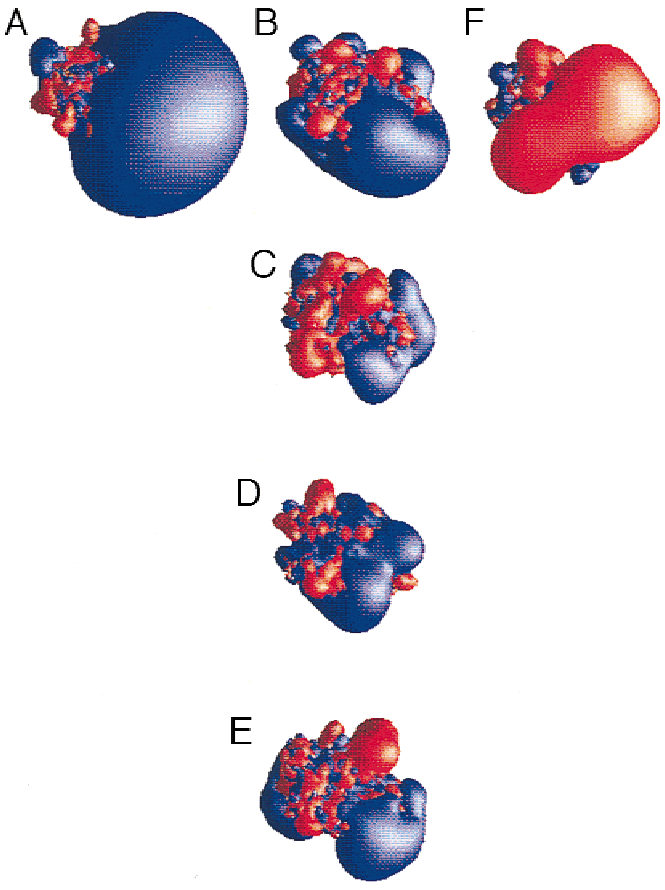

Fig. 4. Electrostatics potential plot produced with the program GRASP for the thioredoxin proteins from (**A**) rat mitochondria, (**B**) *E. coli*, (**C**) pea *m*, (**D**) *C. reinhardtii h*, (**E**) *C. reinhardtii m*, and (**F**) pea *f*. Isopotential lobes are shown for −2 kt (blue) and +1 kt (red). The view-axis points directly onto the substrate binding site.

to the area containing more negative charges, which are distinctly separated from those positive (Fig. 4A). A less polarized charge distribution in the *E. coli, m* and *h* thioredoxins (Fig. 4B–E) coincides with the decreased affinities (Table 1). The correlation between the affinity and specific electrostatic pattern of the thioredoxin active site surroundings is better than that with the net molecule charge or *pI* value. In fact, the net charge of *E. coli* thioredoxin is closer to that of mitochondrial protein and much lower than those of *m* and *h* thioredoxins (Table 6). In spite of this, the efficiency of mitochondrial protein is much higher than that of *E. coli, m* and *h* species (Fig. 1; Tables 2, 3). Hence, neither the similar *pI* of mitochondrial and *E. coli* thioredoxins, nor the different *pI*'s of the *E. coli* and *m* thioredoxins (Table 6) could explain the difference in $S_{0.5}$ values in the former case and the similarity in the latter one (Table 1).

A high degree of charge separation causing the molecule polarization is observed not only in the active site surroundings of mitochondrial thioredoxin (Fig. 4A), but also in other projections of this molecule. This results in the highest magnitude of the protein electrostatic dipole vector (Fig. 5; Table 6). It is remarkable that the dipole vectors inherent in thioredoxins which activate/protect have a similar direction (angle difference less than 90°). Moreover, in this case the thioredoxin apparent affinities ($S_{0.5}$, Table 1) are proportional to the total dipole values (Table 6), with both parameters forming the same row (first four lines in Tables 1, 6). The dipole vectors of the *A. thaliana h* thioredoxins are similar to those of activatory species both in orientation and magnitude (not shown). This is in accord with the affinities of the active *A. thaliana h* proteins to be in the micromolar concentration range, as can be estimated from the maximum effect reached already at 3 μM (*h1, h4* proteins, Table 2). The pea *f* thioredoxin has no influence and does not seem to bind to the 2-oxoacid dehydrogenase complexes so far. This corresponds well to the reversed polarization of its active site surroundings (Fig. 4F) and an opposite direction of its dipole vector compared to that of mitochondrial thioredoxin (Fig. 5). The dipole vector inherent in inhibitory *C. reinhardtii m* thioredoxin is almost orthogonal to the mitochondrial thioredoxin dipole (Fig. 5). Noteworthy that after substitution of the residues distinguishing the inhibitory thioredoxin *m* (K13, D51, K85) by those present in activatory thioredoxins (D13, G51, E85), the dipole of *C. reinhardtii m* thioredoxin significantly changes its direction, approximating that in activatory species (Fig. 5, vector with asterisk). This suggests that the "inhibitory" residues may affect the thioredoxin function by influencing the dipole orientation.

Table 6. *Electrostatic parameters of different thioredoxins*[a]

Thioredoxin	pI	Molecule net charge	Total dipole value (Debye)
Mitochondrial	4.9	−6	561
E. coli	4.7	−5	287
m pea	5.4	−2	285
h C. reinhardtii	5.9	−2	205
f pea	8.2	+1	326
m C. reinhardtii	5.1	−2	241

[a]pI's and the net charge of the molecules were obtained from http://expasy.hcuge.ch/sprot/protparam. Dipole values were calculated as described in Materials and methods.

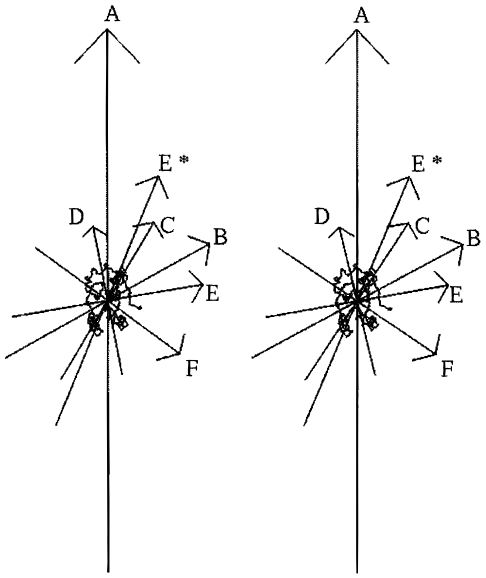

Fig. 5. Stereo view of the dipole vectors of the superpositioned structures of (A) rat mitochondrial, (B) *E. coli*, (C) pea *m*, (D) *C. reinhardtii h*, (E) *C. reinhardtii m*, and (F) pea *f* thioredoxins. The dipole vector of *C. reinhardtii m* thioredoxin with the substituted residues (see text) is marked by asterisk (E*).

The correlation between the thioredoxin affinity (Table 1) and its integral electrostatic properties (Figs. 4, 5; Table 6) indicates that both the electrostatics of the active site surroundings and orientation and magnitude of the electrostatic dipole of the whole molecule may contribute to the thioredoxin-target interplay.

Discussion

Comparison of a number of thioredoxin species has revealed the highest potency of the mitochondrial thioredoxin to affect the mitochondrial process of the 2-oxoacid oxidation. This observation points to the specific protein–protein interactions involved, providing enzymological evidence for physiological significance of the thioredoxin-dependent regulation studied. The regulation is basically dependent on the thioredoxin invariant residues, which input directly to the protein thiol-disulfide oxidoreductase activity (Eklund et al., 1991): C32, C35, D26, W31. However, remote residues (Figs. 2, 3) have been found to affect the thioredoxin interplay with the complexes as well. Although the redox properties of the catalytic disulfide in thioredoxins may be influenced by the residues distant from the active site (Gane et al., 1995), the functional groups considered here are not likely to result in significant changes of the thioredoxin standard redox potentials. The values determined for the *C. reinhardtii h* and pea *f* thioredoxins (−290 mV, Hirasawa et al., unpubl. results) do not differ very much from that known for the *E. coli* protein (−280 mV, Schirmer & Schulz, 1987). Thus, the remote groups discussed (Figs. 2, 3) contribute to general structural features rather than to the thioredoxin redox properties. Taking into account that the thioredoxin interplay with the complexes correlates with the elements on the opposite to the catalytic side of the thioredoxin molecule, i.e., the α1 helix, charges in the α2-β3 and β4-β5 linkers (Fig. 3), this area may be suggested to "anchor" thioredoxin on the surface of the 2-oxoacid dehydrogenase complex, while the thioredoxin active

site faces the complex-bound lipoate (Fig. 6). The known homology between dihydrolipoamide dehydrogenase (E3) and thioredoxin reductase (Gasdaska et al., 1995; Arscott et al., 1997), the thioredoxin action competitive to the E3-catalyzed oxidation of dihydrolipoate intermediate (Bunik et al., 1997a) and the thioredoxin binding involving catalytically important areas of the 2-oxoacid dehydrogenase complexes (Bunik et al., 1997a; this work) may point to the "anchoring" near the lipoyl channel of the E3 component. The same row of preference to different thioredoxins, exhibited by both pyruvate and 2-oxoglutarate dehydrogenase complexes (Table 1), also indicates that their common component, E3, is involved. The required juxtaposition of the catalytic dithiol/disulfide couples may be not possible and/or the active site of E3 may be sterically blocked, when the charges in the "anchoring" area of the thioredoxin molecule differ, as in *C. reinhardtii m* thioredoxin (Fig. 6, dotted line).

While the interface electrostatics is important for the short-range interactions stabilizing the protein–protein complexes, polarization of the whole protein molecules is known to help their docking into binding sites (DePaskalis et al., 1993; Demchuk et al., 1994) through the correct pre-orientation already at the long-range distances (Janin, 1997). Proper alignment of dipoles increases the probability of successful collisions, i.e., the collisions that favor stabilization of the complex by the corresponding short-range interactions. The increased number of such collisions is equivalent to a decrease in effective concentrations of interacting proteins. This is in accord with the ability of the thioredoxin with the highest dipole magnitude (the mitochondrial protein) to work at the lowest concentrations (down to 10^{-7} M, Table 1). Thus, the proper direction and magnitude of dipoles increase the thioredoxin affinity to the complexes. However, they do not ensure the complex formation unless the corresponding short-range interactions are realized. The latter depend on the specific electrostatic pattern of the thioredoxin active site surroundings (Fig. 4) and may involve other regions of the thioredoxin molecule as well (Figs. 3, 5). On the other hand, the thioredoxin-target interplay is determined not only by binding, but also by the following catalysis. Not an affinity, but catalytic incompetence seems to be responsible for the low effects elicited by *A. thaliana* thioredoxins (Table 2). Their inefficient catalysis correlates with the absence of K52, the increased length of the α1 helix and the different contact of the latter with the $\alpha 3/3_{10}$ helix through the residues 12 and 70 (Fig. 3). Since the $\alpha 3/3_{10}$ helix belongs to the catalytically important interface presented in Figure 4, changes in the α1 helix may influence the geometry of this area through the interaction between the helices. Role of this interaction in the thioredoxin function is supported by the fact that in human thioredoxin, Y70 is substituted by one of the additional cysteine residues (see multiple alignment in Jacquot et al., 1997a) involved in the redox regulation of this protein (Gasdaska et al., 1997).

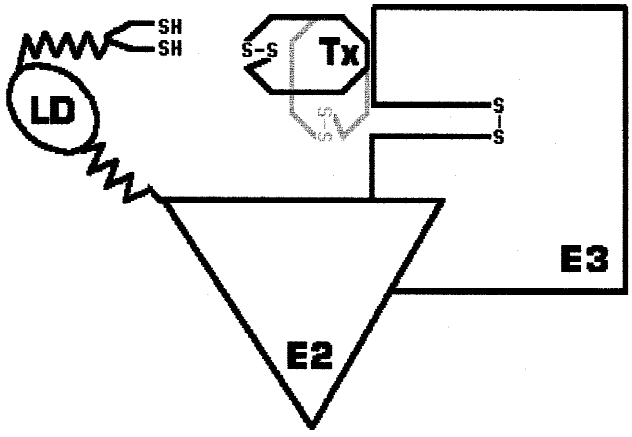

Fig. 6. Hypothetical scheme of the thioredoxin interaction with 2-oxoacid dehydrogenase complexes. LD, lipoyl domain; Tx, thioredoxin. Inhibitory mode of thioredoxin interaction is shown by the dotted line.

Given the identical mechanism of the thioredoxin-catalyzed thiol-disulfide oxidoreduction, provided by the conserved structure of the thioredoxin active site and surroundings, selective action of a thioredoxin should stem from specific recognition upon formation of the thioredoxin-target complex. If the recognizing and catalytic groups comprise the same area, the high reactivity of the essential cysteine residues may interfere with the cognitive process, and therefore with the specificity of oxidoreduction. In this case the importance of long-range interactions is increased, since they allow the recognition to start before the highly reactive catalytic groups are brought together. The spatial separation of the recognizing and catalytic groups as shown in Figure 6 represents an additional way to regulate the thioredoxin selectivity, which may be realized upon the thioredoxin interaction with biological structures of extended dimensions. Thus, not only the active site area, but also the integral structure participates in the thioredoxin-target recognition. Proteins with improper molecular electrostatics do not efficiently interact. Those with the proper electrostatics do, but their complex may be catalytically inactive owing to other factors, influencing juxtaposition and reactivity of the catalytic groups. Only when both integral and specific criteria are satisfied, the catalytically competent thioredoxin-target complex is efficiently formed. Such multilevel control is especially important when several thioredoxins are supposed to specifically influence the corresponding disulfide/dithiol-containing proteins within the same cellular compartment. It is obvious that the effective interaction in this case is defined by competition. Structural adaptations in the physiologically relevant couple, in particular, the specific pattern of the binding surface(s) and proper general electrostatics and geometry, provide the kinetically and thermodynamically competent interplay between the specially designed molecules. In its turn, the high efficiency of the biologically important interaction competitively inhibits any unspecific cross-reactivity.

Materials and methods

2-Oxoglutarate and pyruvate dehydrogenase complexes were isolated from pig heart according to (Stanley & Perham, 1980) with modifications given in (Bunik & Follmann, 1993). Thioredoxin from pig heart mitochondria was isolated by a modification of the method described earlier (Bodenstein-Lang et al., 1989; Bunik et al., 1997b). The preparation of mitochondrial thioredoxin used in this work showed a single band of 11.8 kDa in the SDS slab gel electrophoresis under nonreducing conditions and high thioredoxin activity (4–7 ΔD_{650}/min·mg protein, measured at 0.01–0.03 mg/mL of thioredoxin) in the insulin reduction test according to (Holmgren, 1979).

Expression and purification of *C. reinhardtii* thioredoxins *m* and *h* and of *A. thaliana* thioredoxins *h* has already been described (Rivera-Madrid et al., 1995; Stein et al., 1995). Briefly, *E. coli* BL21(DE3) cells were transformed by a recombinant mutagenic

plasmid. A single colony was then used to inoculate 3 mL of Luria Browth medium supplemented with 50 μg/mL ampicillin. The culture was in turn amplified at 37 °C to 400 mL (for ca. 9 h) in the presence of ampicillin, transferred into 5 L of Luria Browth with no ampicillin and left shaking for 24 h at 30 °C. Cells were then harvested by centrifugation at 5,000 × *g* for 5 min, resuspended in a minimal volume of 0.03 M Tris-HCl, pH 7.9, and stored frozen. This protocol alleviates the need for induction by isopropyl thiogalactoside and protein yields are often enhanced in these conditions (Jacquot et al., 1997b, 1997c). It is believed that in a complex medium such as Luria Browth, lactose can be an alternate inducer to isopropyl thiogalactoside (Hofman et al., 1995). After thawing, the cells were broken by four passages through a French press cell at a pressure of 18,000 psi and the resulting suspension centrifuged at 50,000 × *g* for 1 h. The supernatant was then heated for 5 min at 70 °C and solid ammonium sulfate was added to bring it to 40% saturation. After centrifugation, the thioredoxin fraction was brought to 80% ammonium sulfate and the precipitate collected by centrifugation (30 min, 300,000 × *g*). The pellet was dissolved with 0.03 M Tris-HCl, pH 7.9, and the sample applied on the top of a Sephadex G50 column (5 × 80 cm) equilibrated in the same buffer. The column was eluted overnight by gravity flow at 4 °C. The thioredoxin fractions were then applied on a DEAE Sephacel column (2.5 × 25 cm), which was washed with excess buffer and eluted with a linear gradient 250–250 mL, 0–400 mM NaCl. The thioredoxin peak was collected and concentrated/dialyzed by ultrafiltration on an Amicon cell fitted with a YM 10 membrane. Thioredoxin samples were kept at −20 °C at a concentration of 10–20 mg/mL.

Mutagenesis and cloning of the cDNA sequences of *C. reinhardtii* thioredoxins *h* and *m* were done as described below. Plasmid pET-Ch1 (Stein et al., 1995) was used as a template with the following oligonucleotides for mutating *C. reinhardtii* thioredoxin *h* WT into D26A

		Triplet change	
D26A up	5′ AAGCCGATTGTTGTCGCCTTCACTGCTACGT 3′	GAC	GCC
D26A do	5′ ACGTAGCAGTGAAGGCGACAACAATCGGCTT 3′	CTC	CGC
pETup	5′ AGTAGGTTGAGGCCGTTGAGCACCGCCGCCGC 3′		
pETdo	5′ GCCGCCGCCCAGTCCTGCTCGCTTCGCTAC 3′		

To facilitate the subsequent cloning of the fragments, two "cloning primers" (pETup and pETdo) homologous to plasmid sequences 334 bp upstream of the *Nco*I site and 217 bp downstream of the *BamH*I site were used. The D26A mutant was generated in a two-step method. First, two overlapping mutated fragments of ca 450 bp were produced (primers pETup and oligo D26A do and primers pETdo and oligo D26A up). The two mutated fragments were in turn used as templates together with oligos pETup and pETdo to generate a 880 bp cDNA fragment. The mutated fragment was purified by agarose gel electrophoresis and digested with *BamH*I and *Nco*I. The resulting 340 bp fragment which contained the mutated thioredoxin *h* sequence was then ligated into pET-3d. *E. coli* XL 1 cells were then transformed and analysed for the recombinant plasmid. Midipreps of plasmids of the positive colonies were then sequenced and shown to contain the appropriate mutation. All the work involving plasmid preparation, cloning and sequencing was carried out as in (Sambrook et al., 1989) or as described by the suppliers. Similar mutagenesis and cloning strategy was followed to produce the W12F, W31A, C35S, and A70Y of *C. reinhardtii* thioredoxin *h*, using appropriate mutagenic oligonucleotides. The F12W, C35S, and Y70A mutants of *C. reinhardtii* thioredoxin *m* were generated in a similar way, using the previously described plasmid pET-Ch2 as a template (Stein et al., 1995).

Thioredoxins were reduced and chemically modified as follows. *C. reinhardtii* thioredoxins *m* (1.6 mg/mL) and *h* (4 mg/mL) were incubated with DTT (10 mM) for 40 min at 4 °C in 0.03 M Tris-HCl, pH 7.9, and 0.1 M potassium phosphate, pH 7.0, respectively. The C35S mutant of *C. reinhardtii* thioredoxin *h* (2.2 mg/mL) was modified with NBD-Cl (2.4 mM) in the presence of 1 mM DTT in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C for 40 min. The addition of DTT prevented the formation of a nonfluorescent adduct (presumably O-NBD-derivative) with λ_{max} = 406 nm. Modified in the presence of DTT, the mutant showed the characteristic spectrum of the S-NBD-derivative (λ_{max} = 425 nm), fluorescent at 520 nm. Modification of its single SH group was supported by quantitative determination of 1–1.2 NBD-adduct per molecule. The molar extinction coefficient 13,000 M^{-1} cm^{-1} for the NBD- derivative of free cysteine was used for the calculation (Birkett et al., 1970). The C35S mutant (0.8 mg/mL) was treated with NEM (5 mM) for 35 min in 0.1 M potassium phosphate, pH 7.0, at 25 °C. Modification was followed by the disappearance of the DTNB-titratable thiols from 1 to 0 per molecule. This was accompanied by the decrease in the DTT-insulin reductase activity of the C35S mutant (0.07 mg/mL) from 0.7 to 0 ΔD_{650}/min·mg protein. Reduced or modified thioredoxins were separated from the reagent excess using a HiTrap™ 5 mL desalting column (Pharmacia, Uppsala, Sweden), eluted with 0.1 M potassium phosphate, pH 7.0, at 4 mL/min at room temperature. Thioredoxin concentrations were determined from their absorption at 280 nM applying molar absorption coefficients from http://expasy.hcuge.ch/sprot/protparam.

2-Oxoglutarate and pyruvate dehydrogenase complexes were assayed in standard reaction mixtures as described in (Bunik et al., 1997a). To study the thioredoxin effects, DTT was omitted from the assay. The level of CoA reduction was therefore controlled in separate titration experiments with DTNB or enzymatically, with the limiting CoA concentrations and an excess of 2-oxoglutarate dehydrogenase complex and other substrates. CoA preparations used were reduced to 95% and more. In the thioredoxin activation experiments, an assay was started with the dehydrogenase complex. Thioredoxin-dependent protection was measured after 4 min preincubation of the enzyme complex in an assay mixture containing 2-oxoacid (2 mM) and CoA (0.05 mM); the initial rate of reaction was then determined after addition of NAD^+ (2.5 mM). Unless otherwise indicated, thioredoxins were used in their disulfide forms. Kinetic measurements were carried out at 25 °C on an *Uvicon* spectrophotometer (Kontron Instruments, Neufahrm, Germany) with a 5 s delay after mixing. Dispersion of the v_a/v values given includes experimental errors in determination of both v_a and v according to:

$$\frac{v_a \pm \Delta v_a}{v \pm \Delta v} = \frac{v_a}{v} \pm \sqrt{[(\Delta v_a/v)^2 + (v_a \cdot \Delta v/v^2)^2]}.$$

All modeling procedures were performed using the program SYBYL 6.3 (Tripos Ass., 1994) running on an IRIS Indy workstation. Homology modeling of rat mitochondrial thioredoxin was

described in detail (Raddatz et al., 1997). The 3D structures of thioredoxin from *E. coli* and thioredoxin *h* from *C. reinhardtii* were obtained from the Protein Data Bank Brookhaven (Bernstein et al., 1977), http://www.pdb.bnl.gov/ under the entries 2TRX and 1TOF, respectively. Initial construction of the model structures of thioredoxin from other sources, addition of loop regions and missing side chains and preliminary refinement were carried out using the Swiss-Model Automated Protein Modeling service (http://expasy.hcuge.ch). Full geometry optimization was performed with the program AMBER 4.0 (Kollman & Weiner, 1991) running on a CONVEX-220 using the Kollman all atom force field (Weiner et al., 1984). The structures were solvated in a shell of TIP3P-water (Jorgensen et al., 1983) and geometry optimized by 5,000 steps conjugate gradient energy minimization. Electrostatics potential surfaces and dipole vectors were computed with the program GRASP (Honig & Nicholls, 1995). The calculated dipole vectors where visualized with the SYBYL program package. All amino acids were considered to have standard protonation states at pH 7.0. Sequence alignment was computed using the program CLUSTALW, available at the Internet Server of the Baylor College of Medicine (BCM) (http://dot.imgen.bcm.tmc.edu:9331) and visualized with the program GeneDoc (Nicholas & Nicholas, 1997).

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