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Conformational and functional similarities between glutaredoxin and thioredoxins

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The tertiary structures of thioredoxin from *Escherichia coli* and bacteriophage T4 have been compared and aligned giving a common fold of 68 C_α atoms with a root mean square difference of 2.6 Å. The amino acid sequence of glutaredoxin has been aligned to those of the thioredoxins assuming that glutaredoxin has the same common fold. A model of the glutaredoxin molecule was built on a vector display using this alignment and the T4 thioredoxin tertiary structure. By comparison of the model with those of the thioredoxins, we have identified a molecular surface area on one side of the redox-active S-S bridge which we suggest is the binding area of these molecules for redox interactions with other proteins. This area comprises residues 33 – 34, 75 – 76 and 91 – 93 in *E. coli* thioredoxin; 15 – 16, 65 – 66 and 76 – 78 in T4 thioredoxin and 12 – 13, 59 – 60 and 69 – 71 in glutaredoxin. In all three molecules, this part of the surface is flat and hydrophobic. Charged groups are completely absent. In contrast, there is a cluster of charged groups on the other side of the S-S bridge which we suggest participates in the mechanisms of the redox reactions. In particular, a lysine residue close to an aromatic ring is conserved in all molecules.

Key words: protein structure/folding units/structural homology/small redox proteins.

Introduction

Thioredoxin and glutaredoxin are small ubiquitous proteins containing a redox-active cysteine dithiol/cystine disulfide. They function as protein disulfide reductants in a variety of enzymatic reactions (Thelander and Reichard, 1979; Holmgren, 1976, 1981; Buchanan, 1983). In addition, thioredoxin from *Escherichia coli* also participates in phage T7 infection as an essential subunit of the phage induced DNA polymerase (Mark and Richardson, 1976).

Complete amino acid sequences are presently known for four different thioredoxin or glutaredoxin molecules: thioredoxins from *E. coli* (Holmgren, 1968), phage T4 (Sjöberg and Holmgren, 1972) and *Corynebacterium nephridii* (Meng and Hogenkamp, 1981), and glutaredoxin from *E. coli* (Höög *et al.*, 1983). Three-dimensional structures based on X-ray crystallographic results have been reported for thioredoxin from *E. coli* (Holmgren *et al.*, 1975) and from phage T4 (Söderberg *et al.*, 1978). It was then found that the tertiary structures are highly similar in spite of large differences in amino acid sequence. A related fold has also been observed in glutathione peroxidase (Ladenstein *et al.*, 1979). The thioredoxin sequences from *E. coli* and *C. nephridii* exhibit 49% identity, which in all probability reflects similar tertiary structures, while the amino acid sequences of T4 thioredoxin and *E. coli* glutaredoxin exhibit 32% identity. Consequently, these proteins belong to a common super-family (Höög *et al.*, 1983) of functionally related molecules with different sequences in a common fold.

In the present study, we have compared these molecules in order to deduce structurally invariant features of functional significance. Some of these observations have been previously reported in a preliminary form (Brändén *et al.*, 1983). For the comparison, we have also adapted the primary structure of glutaredoxin into the known conformational model of T4 thioredoxin in order to predict the three-dimensional structure of glutaredoxin.

Results and Discussion

Comparison of the tertiary structures of E. coli and T4 thioredoxin

The structure of T4 thioredoxin can be described in terms of two folding units. An N-terminal $\beta/\alpha/\beta$ structural unit is joined to a C-terminal $\beta/\beta/\alpha$ unit by a loop of residues involving a helix (Söderberg *et al.*, 1978). *E. coli* thioredoxin has an N-terminal β/α unit in addition to the structural units of T4 thioredoxin (Holmgren *et al.*, 1975). In both structures, the strands form a continuous twisted pleated sheet with $\beta 4$ antiparallel to the parallel strands (Figure 1 and Table I). Helices $\alpha 1$ and $\alpha 3$ are on one side of the sheet, $\alpha 2$ and $\alpha 4$ on the opposite side. The redox-active S-S bridge is at the N-terminal end of helix $\alpha 2$ in both structures.

These structures were initially aligned by superimposing the main chain hydrogen bonds in the pleated sheet area. Twenty alpha carbon atoms from the four common strands were originally equivalenced and aligned by the program HOMO giving a root mean square (rms) difference of 1.0 Å. This alignment was then used to maximize the number of structurally homologous C_α atoms in continuous sequence regions of the two molecules. Finally, 68 C_α atoms could be aligned with an rms difference of 2.6 Å. These regions which are listed within boxes in Table I include all secondary structural elements in T4 thioredoxin and account for all residues except those that correspond to gaps in the other molecules. With this procedure, we found that the S-S bridges were also aligned, providing objective evidence that they are at similar positions in these thioredoxin molecules with respect to the overall structures.

The main differences between the two structures, apart from the amino terminal β/α unit of *E. coli* thioredoxin, are in the loop regions between the elements of secondary structure. These differences are listed in Table I and illustrated in Figure 1. The aligned residues define a structural core of the molecules where C_α atoms can be superimposed with an rms difference of 1.8 Å. This core is listed in Table II.

Proposed three-dimensional structure of E. coli glutaredoxin

The alignments of glutaredoxin and T4 thioredoxin sequences

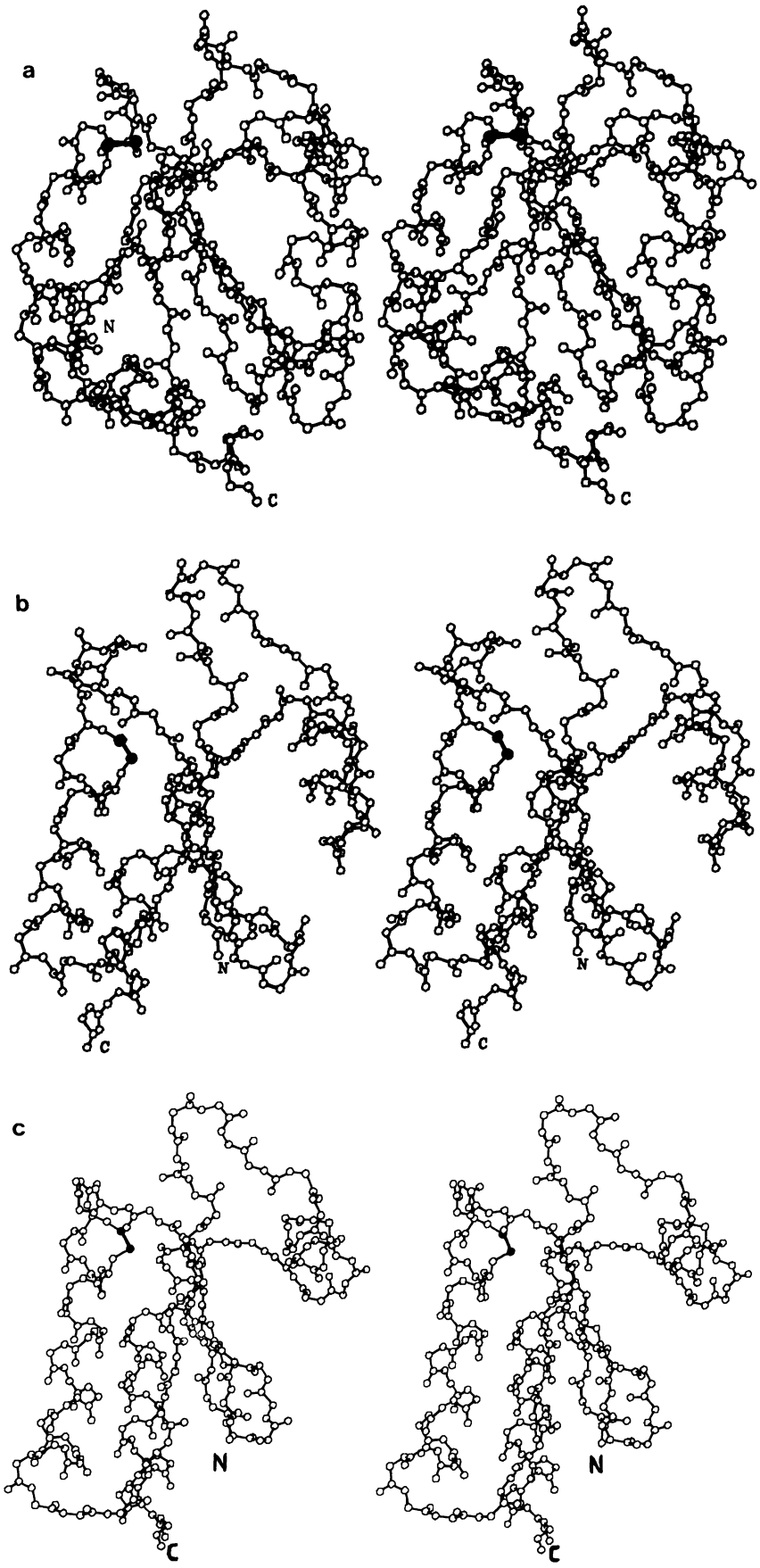
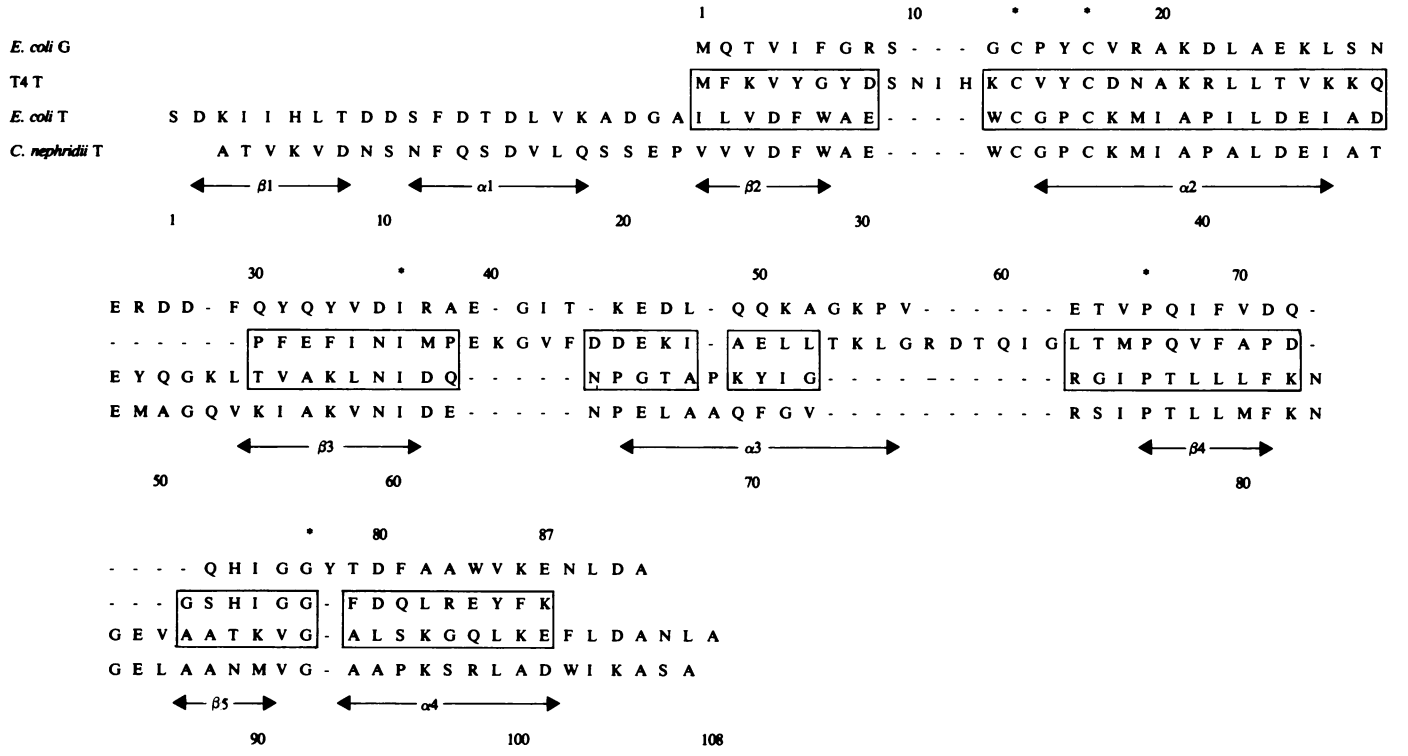


Fig. 1. Stereodiagrams of the main chain conformations of **(A)** thioredoxin from *E. coli*, **(B)** thioredoxin from bacteriophage T4, **(C)** the proposed model of glutaredoxin. The molecules are viewed in similar directions with respect to the common tertiary fold. The sulphur atoms and the S-S bridge are indicated in black. N and C denote the N-terminal and C-terminal residues, respectively.

Table I. Amino acid sequence alignment of *E. coli* glutaredoxin (1) and thioredoxin from bacteriophage T4 (2), *E. coli* (3) and *Corynebacterium nephridii* (4)



The alignment is based on three-dimensional structural equivalence of T4 and *E. coli* thioredoxins. This alignment is different in the middle region from that given by Höog *et al.*, 1983. α_n and β_n refer to secondary structural elements of T4 and *E. coli* thioredoxins (Holmgren *et al.*, 1975; Söderberg *et al.*, 1978). Structurally equivalent residues are within boxes. Invariant residues of all four molecules are indicated by an asterisk. Top numbers refer to the T4 thioredoxin sequence and bottom numbers to *E. coli* thioredoxin.

are obvious at the two ends of the molecules. In the middle, the sequences are so different that more than one possible alignment exists. One such alignment was previously used to establish the evolutionary relationship between the molecules (Höög *et al.*, 1983). Here we have chosen an alignment that preserves the character of the residues in secondary structural elements in T4 thioredoxin as close as possible to the residues in glutaredoxin. Thus, for strand β_3 the glutaredoxin sequence Tyr-Gln-Tyr-Val-Asp-Ile was aligned with the sequence Phe-Glu-Phe-Ile-Asn-Ile in T4 thioredoxin. The sequence alignment in this middle region (residues 30–62 in T4 thioredoxin) shown by Höög *et al.* (1983) gives one identity more than the conformational alignment but gives deletions of all residues corresponding to strand β_3 in T4 thioredoxin.

Our conformational alignment produces deletions and insertions in the loop regions. Thus, the four extra residues following helix $\alpha 2$ in glutaredoxin compared with T4 thioredoxin could form an extra turn of this helix. If this is true these residues can be readily accommodated in our model of glutaredoxin and their charged side chains are external. At the other end of the pleated sheet where the S-S bridge is located, an anti-parallel loop is present. This loop is longer in T4 thioredoxin by one residue in each strand at positions 40 and 44. The long loop after helix $\alpha 3$ in T4 thioredoxin is not present in glutaredoxin and the chain can here be directly connected via a few residues to strand $\beta 4$. The loop between strands $\beta 2$ and $\alpha 2$ which is shorter in *E. coli* thioredoxin than in T4 thioredoxin is also shorter in glutaredoxin. There is a β-bulge after a reverse turn between $\beta 4$ and $\beta 5$ in T4 thioredoxin since there are five residues in the bend. Interestingly, there is a one-residue deletion in this bend in glutaredoxin and a normal reverse turn can thus be formed.

The C-terminal helix is one turn longer in glutaredoxin and is thus more similar to $\alpha 4$ in *E. coli* thioredoxin to which the sequence is also clearly homologous. In T4 and *E. coli* thioredoxins, helix $\alpha 4$ has different directions relative to the pleated sheet. To make proper interactions with the rest of the molecule, the direction of this helix was changed slightly from that in the T4 molecule towards that in the *E. coli* molecule. These changes and some others not described in detail here have the consequence that the shape of the glutaredoxin molecule is quite different from both thioredoxin molecules.

Homologous residues between different thioredoxins and glutaredoxin

There are 51 conserved residues between *E. coli* and *C. nephridii* thioredoxin. Only ~20 of these are buried in the interior of the molecule, providing hydrophobic packing interactions between helices and sheets. Most of the remaining 30 invariant side chains are clustered on one part of the surface, as illustrated in Figure 2. This cluster comprises residues 26–46, 57–64, 73–77 and 91–93. These facts indicate that this surface region is of special functional importance. All residues with C_α atoms within 10 Å from the S-S bridge are in this cluster.

There are 22 conserved residues between glutaredoxin and T4 thioredoxin (Table I). Ten of the conserved residues exposed at the surface are clustered in one region comprising

residues 14–17 (which include the S-S bridge), 64–67 and 74–77 (all in the numbering system of T4 thioredoxin). These residues are aligned to 32–35, 74–77 and 90–93 in *E. coli* thioredoxin, and are thus part of the same cluster as described in the previous paragraph.

Table II. Distances between aligned C_α atoms in the common structural core of T4 and *E. coli* thioredoxins

T4 T	*E. coli* T	Distance (Å)	T4 T	*E. coli* T	Distance (Å)
Met-1	Ile-23	1.9	Ile-34	Leu-58	0.4
Phe-2	Leu-24	0.9	Asn-35	Asn-59	1.2
Lys-3	Val-25	0.5	Ile-36	Ile-60	2.1
Val-4	Asp-26	0.3	Met-37	Asp-61	2.0
Tyr-5	Phe-27	0.8	Pro-38	Gln-62	1.7
Gly-6	Trp-28	1.9	Glu-46	Gly-65	2.4
Tyr-7	Ala-29	0.8	Lys-47	Thr-66	0.8
Asp-8	Glu-30	2.5	Ile-48	Ala-67	2.5
Lys-13	Trp-31	1.2	Ala-49	Lys-69	2.6
Cys-14	Cys-32	1.5	Leu-63	Arg-73	2.7
Val-15	Gly-33	1.6	Thr-64	Gly-74	2.7
Tyr-16	Pro-34	1.8	Met-65	Ile-75	1.2
Cys-17	Cys-35	1.9	Pro-66	Pro-76	1.0
Asp-18	Lys-36	2.0	Gln-67	Thr-77	0.6
Asn-19	Met-37	1.9	Val-68	Leu-78	0.4
Ala-20	Ile-38	2.2	Phe-69	Leu-79	1.2
Lys-21	Ala-39	2.1	Ala-70	Leu-80	2.2
Arg-22	Pro-40	1.2	Gly-73	Ala-87	2.7
Leu-23	Ile-41	1.1	Ser-74	Ala-88	0.9
Leu-24	Leu-42	1.7	His-75	Thr-89	0.4
Thr-25	Asp-43	0.7	Ile-76	Lys-90	2.2
Val-26	Glu-44	1.4	Gly-77	Val-91	2.2
Lys-27	Ile-45	2.1	Gly-78	Gly-92	1.8
Lys-28	Ala-46	2.7	Phe-79	Ala-93	1.2
Pro-30	Thr-54	2.9	Asp-80	Leu-94	2.7
Phe-31	Val-55	1.6	Glu-84	Gln-98	1.8
Glu-32	Ala-56	1.2	Tyr-85	Leu-99	3.2
Phe-33	Lys-57	0.8	Phe-86	Lys-100	2.0

It is apparent from this comparison that similarly positioned regions of the three-dimensional structures have been conserved in these two pairs of homologous molecules. When the sequences of all four molecules are compared using the alignment according to structural equivalence in Table I, we find that there are five invariant residues. These are Ile-60, Pro-76 and Gly-92 (numbers refer to *E. coli*) in addition to the two cysteine residues. Pro-76 and Gly-92 are both within the conserved cluster of surface residues. Pro-76 is situated between Gly-92 and the S-S bridge along the surface of the molecule. This proline is the closest side chain to the S-S bridge in T4 and *E. coli* thioredoxins. In both these structures, we find that this proline has the less usual *cis*-configuration which exposes the carbonyl oxygen at the surface. Ile-60 has a partly buried side chain, 6–7 Å from the S-S bridge. Since the similarities among *E. coli* thioredoxin, T4 thioredoxin and *E. coli* glutaredoxin constitute overall conformational properties rather than isolated residue identities around the redox-active dithiols, the structures are unlikely to represent convergent relationships. Instead the similarities suggest divergence from ancestral connections. However, differences are extensive and relationships distant.

Functional implications of the environment of the redox-active S-S bridge

The common functional denominator for the thioredoxin and glutaredoxin molecules is their redox-reactivity with ribonucleotide reductase (Thelander and Reichard, 1979). In several other interactions all these molecules differ considerably. Such functional differences, which may be important for the following discussion, are listed in Table III. The redox-active sulphur atoms in all three structures are accessible from one side of the molecule but are more shielded and not directly accessible from the opposite side. On the shielded side, the sulphur atoms are screened by the β-carbon atoms which face a number of charged groups (Figure 3). On the accessible side they instead face an essentially hydrophobic surface region (Figure 4).

Shielded side. There are several charged side chains between the sulphur atoms of the S-S bridge and the solution on the shielded side. They comprise Glu-30, Lys-36 and Lys-57 in

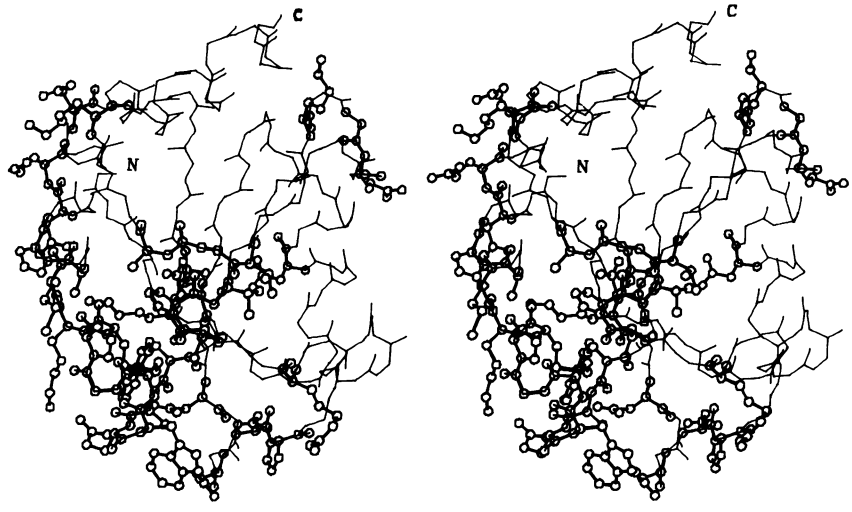


Fig. 2. Stereodiagram of the main chain of *E. coli* thioredoxin and those surface side chains that are identical in *C. nephridii* thioredoxin.

E. coli and *C. nephridii* thioredoxins, while they comprise Asp-8, Asp-18, Lys-21 and, further away, Lys-13 and Arg-22 (Figure 3) in T4 thioredoxin. Glu-30 and Asp-8 are structurally homologous residues as well as Lys-36 and Asp-18. In glutaredoxin, we find from our proposed model of the tertiary structure that Arg-8, Lys-18 and Asp-19 are in this region. These are homologous to Asp-8, Lys-21 and Arg-22 in T4 thioredoxin.

Lys-57 in *E. coli* and *C. nephridii* are far apart in the sequence alignment from Lys-21 in T4 thioredoxin and Lys-18 in glutaredoxin but the ϵ-amino groups actually occupy similar positions in space with respect to the S-S bridge. These

Table III. Functional differences and similarities between thioredoxins and glutaredoxin[a]

Protein	Oxidized by			Reduced by			Enzymatic activity	
	E. coli RDR	T4 RDR	Calf thymus RDR	*E. coli* TR	Rat liver TR	GSH plus *E. coli* GR	GSH-disulfide transhydrogenase	protein dithiol-disulfide reductase[f]
E. coli T	++	−	++	+++	+	−	−	+++
E. coli G	+++[b]	+++	(+)	−	−	+++	+++	−
T4 T	(+)[c]	+++	−	++[d]	−	+++	+[e]	−

[a]Abbreviations: RDR, ribonucleotide reductase; TR, thioredoxin reductase; GR, glutathione reductase; T, thioredoxin; G, glutaredoxin. +++, ++, +, activity in decreasing order; −, no activity.
[b]Apparent K_m value 0.13 μM *versus* 1.3 μM for *E. coli* T (Holmgren, 1979a).
[c]Some activity at >10 μM (Berglund, 1969).
[d]Apparent K_m value 12 μM *versus* 4.5 μM for *E. coli* T (Berglund, 1969).
[e]Apparent specific activity 5% of *E. coli* G with 2-hydroxyethyl disulfide (Holmgren, 1978).
[f]With insulin as substrate (Holmgren, 1978; Holmgren, 1979b).

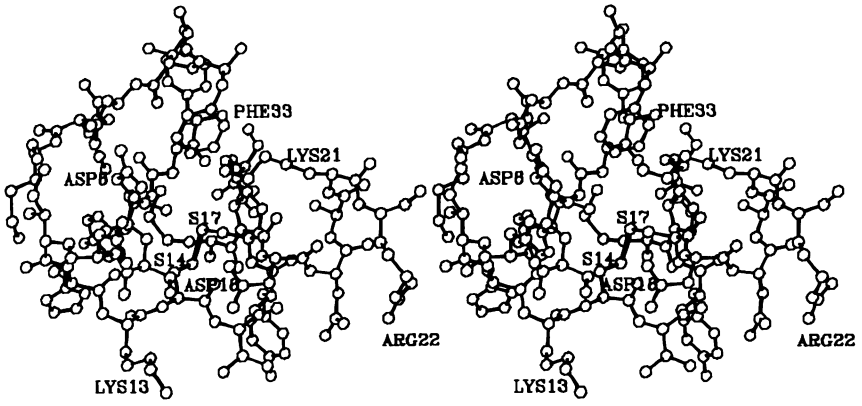


Fig. 3. Stereodiagram showing the distribution of charged groups in the vicinity of the redox-active S-S bridge in T4 thioredoxin.

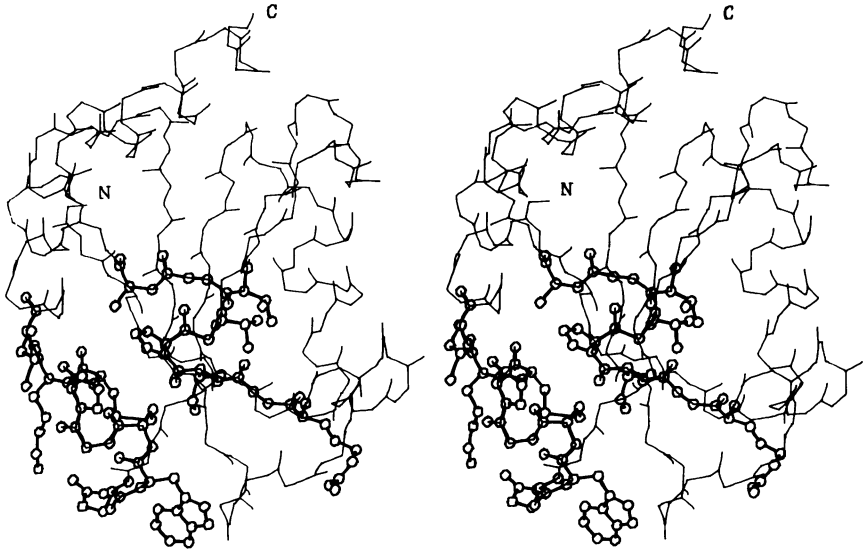


Fig. 4. Stereodiagram illustrating a hydrophobic surface area in *E. coli* thioredoxin proposed to interact with other proteins in redox reactions. The main chains are shown in thin lines. Side chain atoms of residues 32–35, 75–76 and 91–93 are drawn as circles.

are the only residues with completely conserved net charge in this polar surface of the molecules. In all four structures, there is a structurally invariant aromatic side-chain close to the ϵ-amino acid groups of these lysine residues. In *E. coli* and *C. nephridii* thioredoxin, the aromatic residue is Trp-28, in T4 thioredoxin Phe-33 and in glutaredoxin Tyr-33. The importance of this arrangement is further strengthened by the known amino acid sequence parts of yeast and spinach thioredoxins. In yeast thioredoxins I and II, Trp-28 is substituted by Phe and Tyr, respectively (Hall *et al.*, 1971), and in spinach thioredoxin f by a Phe (Tsugita *et al.*, 1983).

The charged area and the aromatic ring are probably of mechanistic importance. The nature of the charges as well as their spatial distribution may well be related to differences in the redox reactions of thioredoxins and glutaredoxins (Table III). However, structural details of these mechanisms cannot be deduced yet since both the X-ray structures for thioredoxins and the predicted model for glutaredoxin represent the oxidized or disulphide form of these molecules. It is known from spectroscopic measurements (Stryer *et al.*. 1967; Holmgren, 1972; Berglund and Holmgren, 1972; Holmgren and Roberts, 1976; Höög *et al.*, 1983) that there are minor structural differences between the oxidized and reduced forms of thioredoxins and glutaredoxins.

Accessible side. The invariant residues Pro-76 and Gly-92 are part of the hydrophobic surface area on the accessible side of the S-S bridge. In *E. coli* thioredoxin, the hydrophobic region comprises residues Gly-33, Pro-34, Ile-75, Pro-76, Val-91, Gly-92 and Ala-93 (Figure 4). In the other molecules, corresponding residues are either identical or conservatively substituted (Table I), with the exception of Pro-34 which is substituted by a tyrosine in T4 thioredoxin and glutaredoxin. Charged groups are completely absent in this region in all molecules and essentially no polar side chains occur. Some main chain carbonyl oxygen atoms in this region are exposed.

We suggest that the hydrophobic area is responsible for redox interactions of thioredoxins and glutaredoxin with other proteins. In particular, the absence of charged groups seems to be important and at least two biochemical supports for this suggestion are known. (i) Thioredoxin from the *E. coli* mutant *tsnC* 7007 was isolated as a defective subunit of T4 DNA polymerase (Holmgren *et al.*, 1978). The mutation in thioredoxin 7007 results in a substitution of Asp for Gly at position 92 (Holmgren *et al.*, 1981). This single site substitution has drastic consequences on the interaction of thioredoxin with thioredoxin reductases as illustrated by a 7-fold higher K_m and a 3-fold lower V_{max}. (ii) *E. coli* thioredoxin-T′ is generated by restricted tryptic cleavage at Arg-73 close to Pro-76 and is a non-covalent reconstituted complex of the resulting two polypeptide fragments. The proteolysis introduces two new charges in the main chain at positions 73 and 74 and thioredoxin-T′ has a very low activity with thioredoxin reductase (Slaby and Holmgren, 1979).

The existence of one major protein interaction area is in agreement with the known reaction mechanisms of thioredoxins, which have been shown to occur via ping-pong mechanisms in several cases (Thelander, 1974; Holmgren, 1979c). An interaction area based on a flat non-polar surface where charged groups are absent is also in agreement with the broad specificity of these molecules. In this respect, *E. coli* thioredoxin is the least specific redox protein and interacts readily with ribonucleotide reductases from a large variety of species (Thelander and Reichard, 1979). Several other proteins are also known to be reduced by *E. coli* thioredoxin (Holmgren, 1981, and Table III). In all these systems, the mechanism of disulphide reduction probably occurs via the formation of a transient mixed disulphide, suggesting that thioredoxin and its substrates form a protein complex. The non-polar area of thioredoxin may well participate in transient binding to other proteins during catalysis.

The interaction between *E. coli* thioredoxin and the gene 5 subunit of phage T7 DNA polymerase, on the other hand, is different. T7 DNA polymerase consists of a stoichiometric association of these two polypeptide chains, which can be isolated as a complex by chromatographic procedures (Mark and Richardson, 1976). Furthermore, the gene 5 subunit interaction may not involve the above-mentioned surface area of *E. coli* thioredoxin, since the T7 DNA polymerase complex shows thioredoxin activity in assays with ribonucleotide reductase or insulin (Randahl, Holmgren and Slaby, unpublished).

Conclusion

All known thioredoxin and glutaredoxin sequences can be analyzed according to a similar tertiary fold. By using primary and tertiary structure alignments, we have been able to identify regions which appear important for redox interactions. The following features characterize these regions. There is a surface area on one side of the S-S bridge defined mainly by hydrophobic residues. We propose that this area forms the intermolecular contact area between thioredoxins/glutaredoxin and other protein redox partners. Charged groups are completely absent from this hydrophobic area. On the other side of the S-S bridge, there is in all molecules a cluster of charged residues which are probably involved in the redox mechanisms.

Materials and methods

Models for *E. coli* and T4 thioredoxins were built from electron density maps on a Vector General 3404 interactive display connected to a Vax 11/750 computer using the FRODO system of programs (Jones, 1978, 1982). The crystal forms of both these proteins contain two independent molecules in the asymmetric unit. Models for the two molecules were built for T4 thioredoxin from an isomorphous electron density map and have been refined to a crystallographic R-value of 0.29 at 2.0 Å resolution. One of these molecules was used for the subsequent work. For *E. coli* thioredoxin, we calculated an average isomorphous map of the two molecules in the asymmetric unit by modifying the Fourier coefficients according to procedures outlined by Agard and Stroud (1982) for a pure translational relation. This map has been interpreted in terms of the known sequence. The model obtained has been used with no further refinement.

Models for *C. nephridii* thioredoxin and glutaredoxin were built using FRODO on the vector display, utilizing the sequence homology with *E. coli* and T4 thioredoxin, respectively. With this method, residues can be renamed according to the amino acid sequence and atoms introduced with proper stereochemistry from a dictionary. Main chain atoms are thereby kept and side chain atoms are positioned as close to the original side chain as possible. Close contacts are avoided preferably by rotating the side chains. This was easily done in *C. nephridii* thioredoxin. In a few cases it was necessary to remodel the main chain slightly for glutaredoxin. Residues can be deleted and new ones can be introduced from the dictionary. Inserted residues were modelled manually to the most plausible conformation. At the end, the models were energy minimized using EREF to give a chemically relevant structure (Jack and Levitt, 1978).

Fourier maps were calculated with the PROTEIN programs of W. Steigemann. Three-dimensional alignments (Ohlsson *et al.*, 1974) of structurally homologous atoms were made by a least-squares minimization of distances between equivalent atoms, using the program HOMO by M.G. Rossmann. Stereodiagrams were generated by the program PLUTO written by S. Motherwell, modified for proteins by E. Dodson and plotted on a Hewlett-Packard plotter.

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