Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide

The recent high-resolution solution structures of human and *Escherichia coli* thioredoxin in their oxidized and reduced states support a catalytic model of protein disulfide reduction involving binding of a target protein and nucleophilic attack by the active-site Cys32 thiolate to form a transition state mixed disulfide.

Structure 15 March 1995, **3**:239–243

Thioredoxin is a truly ubiquitous protein, present in all species from Archebacteria to man. It has a large and growing number of known functions (reviewed in [1]) in addition to its role in DNA synthesis by virtue of its acting as a hydrogen donor for the essential enzyme ribonucleotide reductase. In most of its functions, thioredoxin serves as a general protein disulfide oxido-reductase. It interacts with a broad range of proteins either for electron transport in substrate reduction or regulation of activity via thiol–redox control [1] by a seemingly simple redox mechanism based on reversible oxidation of two cysteine thiol groups to a disulfide, accompanied by the transfer of two electrons and two protons. The net result is the covalent interconversion of a disulfide and a dithiol. Protein disulfide reduction by the thioredoxin system is illustrated in Fig. 1.

The structure of thioredoxin has been the subject of many investigations since elucidation of the amino acid sequence of the *Escherichia coli* protein in 1968 showed it to comprise 108 amino acid residues [2], including the now classical active-site sequence, Trp-Cys32-Gly-Pro-Cys35 [1]. It has been a long-standing goal to observe the three-dimensional structures of both the oxidized and reduced forms of thioredoxin. This has recently been achieved through the publication of high-resolution solution structures of both the reduced [Trx-$(SH)_2$] and oxidized (Trx-S_2) forms of human [3] and *E. coli* [4] thioredoxins. These structures present new opportunities for understanding the catalytic activity of thioredoxin as a protein disulfide reductase. They are also of wider interest in view of the number of proteins with a thioredoxin fold (see the accompanying article in this issue of *Structure* [5] for a more detailed discussion of the thioredoxin fold) and the increasing functional importance of thiol–redox regulation of protein activity in different biological systems.

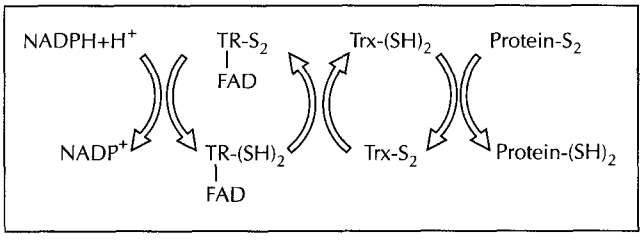

Fig. 1. Mechanism of NADPH-dependent protein disulfide reduction by the thioredoxin system. Thioredoxin reductase (TR) catalyzes reduction of oxidized thioredoxin (Trx-S_2) by NADPH using FAD and its redox-active disulfide. Reduced thioredoxin [Trx-$(SH)_2$] then directly reduces the disulfide in the substrate protein [1]. Using NADPH (100 μM), TR (0.1 μM) and Trx-S_2 (2 μM) plus a substrate protein, disulfide reduction is measured spectrophotometrically at 340 nm.

Measuring thioredoxin activity

Insulin is a favorite disulfide substrate for assays of thioredoxin with an apparent Michaelis–Menten constant (K_M) in the micromolar range [6,7]. The first evidence for a local conformational change in thioredoxin on reduction of the active-site disulfide was a three-fold increase in tryptophan fluorescence emission [8], now known to originate from a decreased quenching of Trp28 [4]. Trp28 fluorescence is thus an exceptionally useful probe for directly measuring rates of thiol-disulfide exchange reactions with thioredoxin. Trx-$(SH)_2$ reduces insulin disulfides at pH 7 with a rate constant of 10^5 M^{-1} s^{-1}, which is about five orders of magnitude faster than insulin reduction by dithiothreitol (DTT), a well-known dithiol reductant [7]. Also, reduction of Trx-S_2 by DTT (10^3 M^{-1} s^{-1}) is about three orders of magnitude faster than reduction of insulin disulfides by DTT. As a result, thioredoxin catalyzes reduction of insulin disulfides by DTT [7]. In general, thioredoxin will catalyze thiol-disulfide exchange between one protein dithiol and another protein disulfide acceptor [1]. Even faster disulfide reduction rates (10^6 M^{-1} s^{-1}) are observed with the disulfide in oxidized ribonucleotide reductase which is involved in electron transfer to the active site [1]. How can these fast rates with thioredoxin be explained and what is the nature of the conformational change?

The thioredoxin mechanism based on the structure of Trx-S_2

The first three-dimensional structure of any thioredoxin was obtained by X-ray crystallography in 1975 [9]. *E. coli* Trx-S_2 was shown to have its active site disulfide located on a unique protrusion at the C-terminal end of a

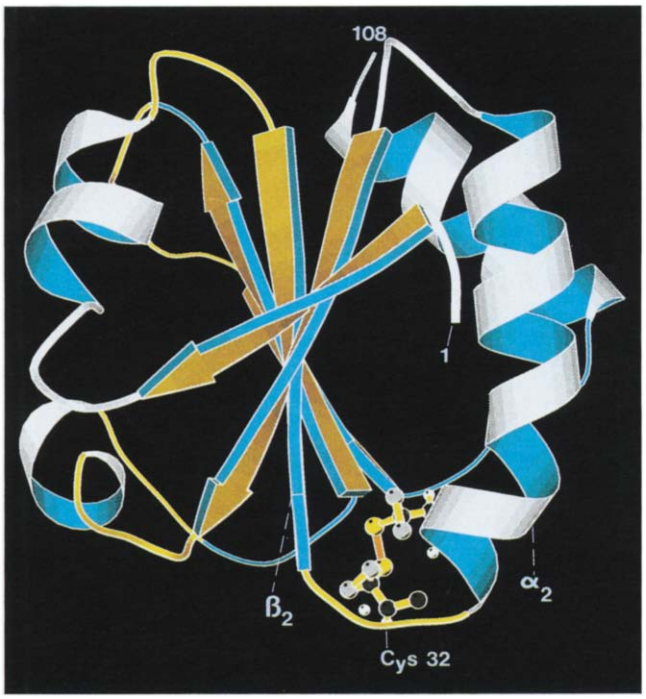

Fig. 2. Folding of thioredoxin. The secondary structure of thioredoxin from coordinates for *E. coli* Trx-S_2 obtained by NMR in solution [4]. Note that the redox-active disulfide in the active site (Cys32–Cys35) is located on a protrusion between the strand β_2 and the helix α_2. Only the sulfur of Cys32 is exposed to solvent.

β-strand (β_2) followed by a long α-helix (α_2). This structure (see Fig. 2) has since been refined to 1.7 Å resolution [10] revealing details of the know well-known thioredoxin fold [11] also present in other proteins like protein disulfide-isomerase which has two thioredoxin domains and makes, rather than breaks, disulfides [11].

Despite the successful X-ray work with oxidized thioredoxin the structure of the reduced form remained elusive. This was because crystals of *E. coli* thioredoxin were only obtained in the presence of Cu^{2+} ions [12] which act to cross-link two molecules by coordination at Ser1, Asp2 and Asp10 [9,10]. This requirement for Cu^{2+} precludes crystallization of reduced thioredoxin because Cu^{2+} is an oxidizing substrate [6]. However, chemical modification showed that only Cys32, the N-terminal active-site cysteine residue, was alkylated by iodoacetate in the native protein at pH 7, and that the apparent pK_a value was 6.7 as determined from the pH dependence, whereas the other cysteine residue (Cys35) showed a higher pK_a value. This led to a postulated mechanism of action of thioredoxin [13] as shown in Fig. 3. The basic concepts of this mechanism are: firstly, reduced thioredoxin has a hydrophobic surface area, conserved in the thioredoxin-glutaredoxin family [14], which binds to a substrate protein (X in Fig. 3) making a complex; secondly, in the hydrophobic environment of the complex, the thiolate of Cys32, acting as a nucleophile, attacks the target protein to form a covalently linked mixed disulfide transition state; finally, attack of the now deprotonated thiolate of Cys35 on the disulfide generates a dithiol in the target protein and a disulfide in thioredoxin. Conformational changes in thioredoxin and the target protein occur during binding and the subsequent electron-transfer steps.

This protein–protein binding mechanism may explain how thioredoxin can reduce a disulfide in a protein which appears sterically inaccessible to a small molecule like DTT. Activation of malate dehydrogenase from spinach chloroplasts with thioredoxin m (one of several chloroplast thioredoxins) is a good example [15]. It is also an example of a specific thioredoxin-dependent reaction that mediates light-driven regulation of photosynthetic enzymes by ferredoxin and a specific reductase.

The high-resolution NMR work on thioredoxin in solution, initially focused on the reduced form of the human [16] and *E. coli* [17] proteins. Cloning of human thioredoxin resulted from a novel functional role of this protein as a secreted cytokine [18], isolated from either conditioned medium of human T-lymphotropic virus type I transformed T cells or from Epstein–Barr virus transformed B cells [19,20]. Like other mammalian thioredoxins [1], the human protein contains additional structural cysteine residues with sulfhydryl groups [11]; one of these, Cys73, is located on the surface close to the active site and in an equivalent position to Gly74 in *E. coli* thioredoxin [3,4]. Oxidation of these structural sulfhydryl groups inactivates the mammalian thioredoxins causing them to aggregate and form multimers, a process that has been suggested to autoregulate activity [1,21]. To analyze the human oxidized thioredoxin [3], the triple Cys→Ala mutant protein C62A, C69A, C73A was constructed [22]. This had equivalent activity to the wild-type thioredoxin in the DTT-coupled insulin disulfide

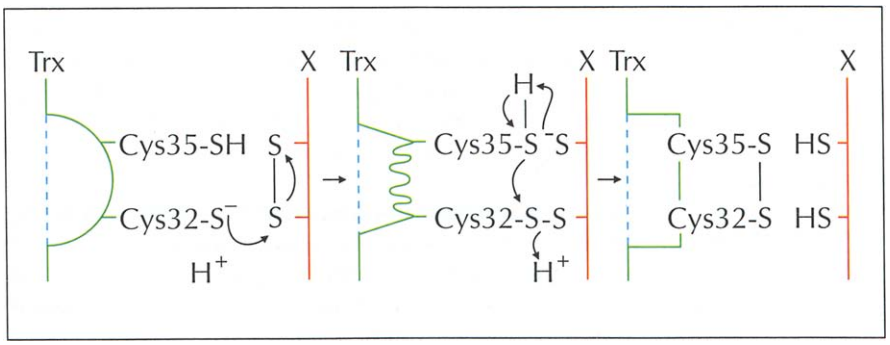

Fig. 3. Proposed mechanism of thioredoxin-catalyzed protein disulfide reduction [1,13]. Reduced thioredoxin [Trx-$(SH)_2$] binds to a target protein via its hydrophobic surface area. Nucleophilic attack by the thiolate of Cys32 results in formation of a transient mixed disulfide, which is followed by a nucleophilic attack of the deprotonated Cys35 generating Trx-S_2 and the reduced protein. Note that conformational changes in thioredoxin (and the target protein) occur during the reaction.

reduction assays [21]. It should be noted that the human thioredoxin analyzed also contains the substitution Met74→Thr [18,19,22]. Analysis of oxidized *E. coli* thioredoxin [3] was straightforward.

Mechanistic implications of conformational changes between reduced and oxidized thioredoxin

The papers on human and *E. coli* thioredoxins [3,4] contain a good deal of detailed information about the structures that falls outside the scope of this minireview. Here, I will focus on the essence of the conformational change and, in particular, its mechanistic implications. Despite only 25% sequence identity between *E. coli* and human thioredoxins, the active site Trp-Cys-Gly-Pro-Cys motif and other critical residues such as Pro76 and Gly92 of the *E. coli* protein are conserved in the human structure [3,4,11]. The major conclusions are discussed below.

For *E. coli* thioredoxin, the structures of the oxidized and reduced forms are extremely similar. Their backbones are essentially identical except for slight differences in the active site including Cys32 and Cys35. The side chain of Cys32 is tilted towards the solution in the reduced form to accommodate the increase in sulfur–sulfur distance upon formation of two sulfhydryl groups. Changes are seen in other side chains and in the hydrogen-bonding pattern [4]. Fig. 4 illustrates the surfaces of oxidized and reduced thioredoxin as space-filling models. Clearly, only the sulfur of Cys32 is exposed to solvent. The low pK_a value of Cys32 [13,23,24] is suggested to arise from the partial positive charge from the α-helix dipole moment of helix α_2 [25].

What then, is the difference between the oxidized and reduced states illustrated in Fig. 4? A target protein with a disulfide bond should bind well to the surface of Trx-$(SH)_2$, but not to Trx-S_2. Such complexes have not yet been studied. However, a striking example of a true difference in recognition is observed with phage T7 DNA polymerase. This enzyme, widely used for DNA sequencing, consists of a 1:1 complex of Trx-$(SH)_2$ and the 80 kDa phage T7 gene 5 protein [26]. The complex has a dissociation constant of ~5 nM and thioredoxin confers high processivity on the enzyme. No redox function is required for processivity and active site Cys32/35→Ser double mutants of thioredoxin are also functional [26]. In contrast, no activity or binding of Trx-S_2 to the gene 5 protein is observed (the binding affinity is reduced by at least four orders of magnitude; [26] and A Holmgren, unpublished data). The hydrophobic surface area shown in Fig. 4 is clearly involved in determining target specificity because a mutant thioredoxin in which Gly92 (colored magenta in Fig. 4) is substituted by an aspartate residue, thereby introducing a negative charge, lacks all gene 5 binding activity [26]. Interestingly, the Gly92→Asp variant obtained from an *E. coli* mutant with a T7 negative phenotype [27] was the first natural mutant of thioredoxin to be isolated. However, the Gly92→Asp thioredoxin

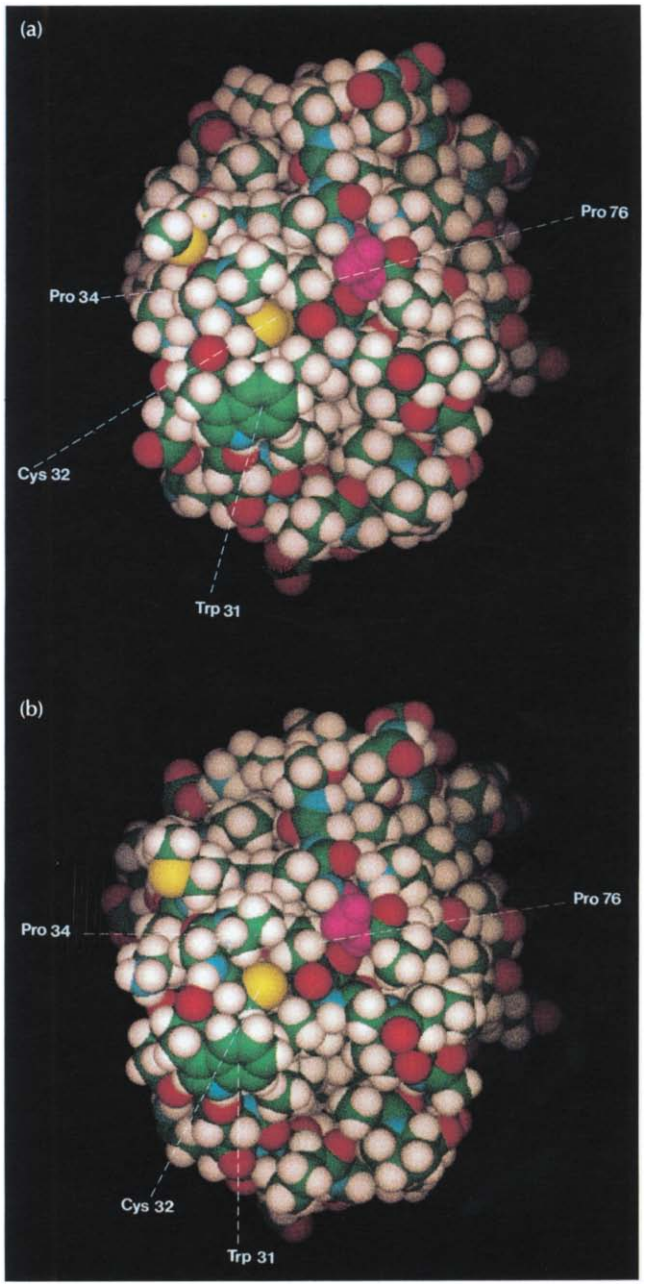

Fig. 4. Molecular models of the active-site surface of *E. coli* thioredoxin in **(a)** the oxidized state and **(b)** the reduced state. The view in this figure is from the back (180° rotation) of the structure presented in Fig. 2. The sulfur of Cys32 (yellow) and the conserved residues Trp31, Pro34 and Pro76 are indicated. All atoms in Gly92 are shown colored magenta.

mutant retains ~10% of wild-type activity in assays with thioredoxin reductase and insulin (displaying both a lower V_{max} and a higher K_M if assayed by the method shown in Fig. 1 [27]).

The topic of substrate recognition by thioredoxin is addressed in a paper in this issue of *Structure* [28] which presents the structure of a mixed disulfide transition state intermediate complex between human thioredoxin and a peptide fragment from one of its target proteins, the transcription factor NFκB.

It remains to be determined whether the functional differences between the two oxidation states, which have such similar active-site surfaces (see Fig. 4), arise from local conformational flexibility in and around the disulfide loop [29]. Higher mobility is observed in the reduced form, in the microsecond to millisecond timescale, consistent with conformational subsets of structures in the reduced form that may be important for binding target proteins. The exact nature of the structural flexibility remains to be determined.

The structures of the oxidized and reduced C62A, C69A, C73A, M74T human thioredoxin are also very similar (with a backbone atomic root mean square difference of ~0.9 Å) and the packing of the side chains within the protein core is almost identical [3]. However, subtle differences are observed in the local conformation of the active site and surrounding regions. These can be attributed to a slight shortening of the C^{α}–C^{α} distance by ~0.1 Å between Cys32 and Cys35 upon formation of the disulfide bond and a change in the χ_1 angle of Cys35 from −50.9° in the reduced state to −39.1° in the oxidized state [3]. A small change in the position of the Trp31 side chain is also observed. The changes in the active site are propagated to the neighboring helices α_2 and α_4, whose orientations with respect to the underlying β-sheet are slightly different in the oxidized and reduced forms [3]. This includes the loops (74–76 and 90–92) that make up the active-site surface (see Figs 2 and 4 for orientation). The cleft between the active site and the two opposing loops is increased by ~10% in the oxidized state relative to the reduced state. Apart from movement of the helices, another clear difference between the results obtained with *E. coli* thioredoxin and human thioredoxin concerns the sulfur–sulfur distance in the reduced state, which is 3.1 Å in human thioredoxin [3] but 3.8 Å in the *E. coli* protein [4]. A hydrogen bond between the Cys35 amide proton and the Cys32 S^{α}, which is present in oxidized human thioredoxin, is retained in the reduced state and may explain the low pK_a value (6.3) measured for Cys32 in human thioredoxin [30].

In summary, the active-site surfaces of human and *E. coli* thioredoxin are highly conserved. The changes observed upon oxido-reduction are subtle and local and require the full might of present-day multidimensional NMR techniques to be detected. However, there do appear to be genuine, albeit small, differences between the human and *E. coli* proteins. The kinetics of the redox activity with insulin are very similar for both thioredoxins suggesting that nature has solved the mechanism of thioredoxin in slightly different ways. An alternative explanation is that the mutations C62A, C69A, C73A and M73T cause changes in the native structure. Clearly, further studies detailing the binding interactions of proteins with thioredoxin will be of great interest. It should also be noted that glutaredoxin has a binding site for glutathione [31] and uses this molecule either for reduction of the active-site disulfide or in substrate recognition. Apparently, the active-site surface in thioredoxin is designed to fit many proteins. Thioredoxin thus uses a chaperone-like mechanism of conformational changes to bind a diverse group of proteins and fast thiol-disulfide exchange chemistry in a hydrophobic environment [32] to promote high rates of disulfide reduction.

Acknowledgements: Thanks to Drs Jane Dyson and Peter Wright for many helpful discussions and also to Garry Gippert and Mei-Fen Jeng at the Scripps Research Institute, La Jolla, for preparation of structure models. This work was supported by grants from the Swedish Medical Research Council (13X-3529) and the Swedish Cancer Society (961).

References

1. Holmgren, A. (1985). Thioredoxin. *Annu. Rev. Biochem.* **54**, 237–271.
2. Holmgren, A. (1968). Thioredoxin: 6. The amino acid sequence of the protein from *Escherichia coli* B. *Eur. J. Biochem.* **6**, 475–484.
3. Qin, J., Clore, G.M. & Gronenborn, A.M. (1994). The high-resolution three-dimensional solution structures of the oxidized and reduced states of human thioredoxin. *Structure* **2**, 503–522.
4. Jeng, M.-F., *et al.*, & Dyson, H J. (1994). High-resolution solution structures of oxidized and reduced *Escherichia coli* thioredoxin. *Structure* **2**, 853–868.
5. Martin, J.L. (1995). Thioredoxin — a fold for all reasons. *Structure* **3**, 245–250.
6. Holmgren, A. (1979). Reduction of disulfides by thioredoxin. Exceptional reactivity of insulin and suggested functions of thioredoxin in the mechanism of hormone action. *J. Biol. Chem.* **254**, 9113–9119.
7. Holmgren, A. (1979). Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J. Biol. Chem.* **254**, 9627–9632.
8. Stryer, L., Holmgren, A. & Reichard, P. (1967). Thioredoxin. A localized conformational change accompanying reduction of the protein to the sulfhydryl form. *Biochemistry* **6**, 1016–1020.
9. Holmgren, A., Söderberg, B.-O., Eklund, H. & Brändén, C.-I. (1975). Three-dimensional structure of *Escherichia coli* thioredoxin-S_2 to 2.8 Å resolution. *Proc. Natl. Acad. Sci. USA* **72**, 2305–2309.
10. Katti, S.K., LeMaster, D.M. & Eklund, H. (1990). Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution. *J. Mol. Biol.* **212**, 167–184.
11. Eklund, H., Gleason, F.K. & Holmgren, A. (1991). Structural and functional relations among thioredoxins of different species. *Proteins* **11**, 13–28.
12. Holmgren, A. & Söderberg, B.-O. (1970). Crystallization and preliminary crystallographic data for thioredoxin from *Escherichia coli* B. *J. Mol. Biol.* **54**, 387–390.
13. Kallis, G.B. & Holmgren, A. (1980). Differential reactivity of the functional sulfhydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. *J. Biol. Chem.* **255**, 10261–10265.
14. Eklund, H., *et al.*, & Brändén, C.-I. (1984). Conformational and functional similarities between glutaredoxin and thioredoxins. *EMBO J.* **3**, 1443–1449.
15. Buchanan, B.B. (1991). Regulation of CO_2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Perspective on its discovery, present, and future development. *Arch. Biochem. Biophys.* **288**, 1–9.
16. Forman-Kay, J.D., Clore, G.M., Wingfield, P.T. & Gronenborn, A.M. (1991). High-resolution three-dimensional structure of reduced recombinant human thioredoxin in solution. *Biochemistry* **30**, 2685–2698.
17. Dyson, H.J., Holmgren, A. & Wright, P.E. (1989). Assignment of the proton NMR spectrum of reduced and oxidized thioredoxin: sequence-specific assignments, secondary structure and global fold. *Biochemistry* **28**, 7074–7087.
18. Tagaya, Y., *et al.*, & Yodoi, J. (1989). ATL-derived factor (ADF), and IL-2R/Tac inducer homologous to thioredoxin: possible involvement of dithiol-reduction in the IL-2 receptor induction. *EMBO J.* **8**, 757–764.
19. Wollman, E.E., *et al.*, & Fradelizi, D. (1988). Cloning and expression of a cDNA for human thioredoxin. *J. Biol. Chem.* **263**, 15506–15512.
20. Wakasugi, N., *et al.*, & Tursz, T. (1990). Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I and Epstein–Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc. Natl. Acad. Sci. USA* **87**, 8282–8286.

21. Ren, X., Björnstedt, M., Shen, B., Ericson, M. & Holmgren, A. (1993). Mutagenesis of structural half-cystine residues in human thioredoxin and effects on regulation of activity by selenodiglutathione. *Biochemistry* **32**, 9701–9708.
22. Forman-Kay, J.D., Clore, G.M., Stahl, S.J. & Gronenborn, A.M. (1992). ^{1}H and ^{15}N resonance assignments and secondary structure of the human thioredoxin C62A, C69A, C73A mutant. *J. Biomol. NMR* **2**, 431–445.
23. Dyson, H.J., Tennant, L.L. & Holmgren, A. (1991). Proton-transfer effects in the active site region of *Escherichia coli* thioredoxin using two-dimensional ^{1}H NMR. *Biochemistry* **30**, 4262–4268.
24. Li, H., Hanson, C., Fuchs, J.A., Woodward, C. & Thomas, G.J.,Jr (1993). Determination of the pKa values of active-center cysteines, cysteines-32 and -35, in *Escherichia coli* thioredoxin by Raman spectroscopy. *Biochemistry* **32**, 5800–5808.
25. Hol, W.G.J. (1985). The role of the alpha-helix dipole in protein function and structure. *Prog. Biophys. Mol. Biol.* **45**, 149–195.
26. Huber, H.E., Russel, M., Model, P. & Richardson, C.C. (1986). Interaction of mutant thioredoxins of *Escherichia coli* with the gene 5 protein of phage T7. The redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity. *J. Biol. Chem.* **261**, 15006–15012.
27. Holmgren, A., Kallis, G.-B. & Nordström, B. (1981). A mutant thioredoxin from *Escherichia coli* tsnC 7007 that is non-functional as a subunit of phage T7 DNA polymerase. *J. Biol. Chem.* **256**, 3118–3124.
28. Qin, J., Clore, G.M., Kennedy, W.M.P., Huth, J.R. & Gronenborn, A.M. (1995). Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NFκB. *Structure* **3**, 289–297.
29. Stone, M.J., Chandrasekhar, K., Holmgren, A., Wright, P.E. & Dyson, H.J. (1993). Comparison of backbone and tryptophan side-chain dynamics of reduced and oxidized *Escherichia coli* thioredoxin using ^{15}N NMR relaxation measurements. *Biochemistry* **32**, 426–435.
30. Forman-Kay, J.D., Clore, G.M. & Gronenborn, A.M. (1992). Relationship between electrostatics and redox function in human thioredoxin: characterization of pH titration shifts using two-dimensional homo- and heteronuclear NMR. *Biochemistry* **31**, 3442–3452.
31. Bushweller, J.H., Billeter, M., Holmgren, A. & Wüthrich, K. (1994). The NMR solution structure of the mixed disulfide between *E. coli* glutaredoxin (C14S) and glutathione. *J. Mol. Biol.* **235**, 1585–1597.
32. Singh, R. & Whitesides, G.M. (1990). Degenerate intermolecular thiolate–disulfide interchange involving cyclic five-membered disulfides is faster by 10^3 than that involving six- or seven-membered disulfides. *J. Am. Chem. Soc.* **112**, 6304–6309.

Arne Holmgren, Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden.