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Structural differences between oxidized and reduced thioredoxin monitored by two-dimensional ^1H NMR spectroscopy

H. Jane Dyson, Arne Holmgren* and Peter E. Wright

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037, USA and
**Department of Physiological Chemistry, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden*

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Two-dimensional high resolution NMR techniques have been applied to study the structural differences between the oxidized and reduced forms of *Escherichia coli* thioredoxin in solution. Sequential proton resonance assignments indicate only limited conformational changes; major chemical shift differences are found for a few residues in a β-strand immediately preceding the active site S-S bridge and the active site itself. Additional resonance shifts are observed for several residues distant in the primary sequence. The X-ray structure of oxidized thioredoxin shows that these residues form a flat hydrophobic surface, close to the active site S-S bridge, which is probably involved in interactions with other protein molecules.

Thioredoxin; Redox protein; Thiol-disulfide; 2D NMR; Protein conformation

1. INTRODUCTION

Thioredoxin is a ubiquitous small protein [1] with a redox-active disulfide bridge in its oxidized form (Trx-S_2). The reduced form of thioredoxin (Trx-$(SH)_2$) contains a dithiol and is an effective protein disulfide reductase and also a specific hydrogen donor for enzymes such as ribonucleotide reductase and methionine sulfoxide reductase [1]. In addition, *Escherichia coli* Trx-$(SH)_2$ is an essential subunit of phage T_7 DNA polymerase [2] and is required for assembly of the filamentous phages f1 and M13 [3].

The three-dimensional structure of Trx-$(SH)_2$ is so far unknown, but is of great importance in explaining the functional properties of thioredoxin. The structure of *E. coli* Trx-S_2 has been determined by X-ray crystallography to 2.8 Å resolution [4]. The active center disulfide within the sequence -Trp-Cys-Gly-Pro-Cys-Lys- is located on a protrusion of the molecule at the C-terminal end of a β-strand and is followed by an α-helix [4]. Attempts to reduce Trx-S_2 in crystals or to crystallize Trx-$(SH)_2$ have so far been unsuccessful (Holmgren, A., unpublished). Evidence for a localized conformational change upon reduction of Trx-S_2 has been obtained from tryptophan fluorescence and circular dichroism spectroscopy [5,6] and from a limited NMR study [7]. In the present investigation we have applied high-resolution 2D-NMR techniques to study the structural differences between Trx-S_2 and Trx-$(SH)_2$ in solution. Sequential proton resonance assignments have been completed for both the oxidized and reduced protein, and studies are under way to determine their structures in solution using distance geometry and molecular dynamic calculations. These results will be published in full elsewhere. We report here NMR evidence for a limited and specific conformational difference between the two oxidation states of thioredoxin.

Correspondence address: P.E. Wright, Dept of Molecular Biology, Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

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2. MATERIALS AND METHODS

2.1. *Protein preparation*

The *E. coli* strain SK3981 containing the thioredoxin gene *(trxA)* cloned on a derivative of the plasmid pBR325 [8] was a kind gift from Dr Sidney Kushner, Athens, GA, USA. Cells were grown in a 100 l fermentor [8] and harvested in stationary phase. Thioredoxin was prepared by a modification of the method of Holmgren and Reichard [9], omitting the acid and heat steps. Lyophilization was not used and the protein solution after the DEAE-cellulose chromatography and other steps was concentrated by ultrafiltration using Diaflo UM2 membranes. Following the Sephadex G-50 column, a final isocratic chromatography step on DE32-cellulose at pH 4.79 with 0.07 M acetic acid–0.04 M ammonia was used. This removes traces of more acidic materials to give electrophoretically homogeneous material of full specific activity [9]. Finally the thioredoxin solution was prepared for NMR spectroscopy by solvent exchange on a short Sephadex G-25 column equilibrated with 0.1 M potassium phosphate at the desired pH, in either ^2H_2O or 90% H_2O/10% ^2H_2O. pH values for ^2H_2O solutions are uncorrected meter readings. Protein concentration was determined using the molar extinction coefficient of 13700 $cm^{-1} \cdot M^{-1}$ at 280 nm [9] and was typically about 4 mM. The Trx-S_2 solution was reduced by the addition of 1 M dithiothreitol, freshly prepared in the same buffer used for the protein solution, to a final concentration of 10 mM. The protein and dithiothreitol solutions were equilibrated under argon and the addition was done using sealed tubes under argon. Finally the NMR tube was sealed with a rubber septum under argon.

2.2. *NMR measurements*

All NMR measurements were performed using a Bruker AM500 spectrometer equipped with digital phase-shifting hardware. Chemical shifts are referred to an internal standard of dioxan. Proton resonance assignments were made using phase-sensitive double-quantum-filtered COSY [10], double-quantum [11,12] and NOESY [13] spectra. Spin-system assignments were considerably aided by the use of RELAY [14] and TOCSY [15,16] spectra. Spectra were routinely acquired with 4096 complex datapoints, with 500–700 t_1 points. Spectral widths were typically 7042 Hz in ω_2, for a digital resolution of 3.4 Hz/pt in ω_2 and 6.9 Hz/pt in ω_1. Spectra were Fourier transformed using a SUN workstation fitted with a Sky Warrior array processor, with software provided by Dr D. Hare and modified by Dr J. Sayre. Phase-shifted sine bell window functions were used in both dimensions. For NOESY, TOCSY and double-quantum spectra in 90% H_2O/10% ^2H_2O, considerable improvement was obtained by linear baseline-correction and by suppression of t_1 ridges [17].

3. RESULTS

Complete sequence-specific resonance assignments for both oxidized and reduced thioredoxin were obtained by the sequential assignment procedure [18] using a strategy described elsewhere [19]. (We are aware that Le Master and Richards have recently completed backbone sequential assignments for oxidized *E. coli* thioredoxin using isotopic substitution methods (Le Master, D. and Richards, F., submitted.) Assignments of backbone proton resonances of residues in the active site region of oxidized and reduced thioredoxin are summarized in table 1, together with those for short sections of the polypeptide chain preceding and following this region. Details of the sequence-specific assignments will be reported elsewhere. Assignment of the resonances of the amino acid residues in the active site itself was difficult, for several reasons. Our assignment strategy [19] focuses on relayed connectivities involving the amide protons since their resonances are well-dispersed. The proline residue of the active site has, of course, no amide proton, so the connectivities are interrupted at this point. The amide protons of the other residues in the active site exchange rapidly with solvent protons and were extremely difficult to observe. Most could only be seen in spectra recorded at low temperature and pH (298 K and pH 5.7). Even under these condi-

Table 1

Thioredoxin chemical shifts

Residue	Oxidized chemical shift (ppm)		Reduced chemical shift (ppm)	
	NH	CαH	NH	CαH
Gly 21	8.08	3.99,3.99	8.09	3.99,3.99
Ala 22	8.38	5.19	8.40	5.19
Ile 23	8.96	5.13	8.97	5.13
Leu 24	9.20	5.21	9.21	5.21
Val 25	9.74	4.51	9.73	4.54
Asp 26	8.86	5.21	8.84	5.24
Phe 27	8.93	5.31	9.17	5.31
Trp 28	8.62	5.14	8.61	5.30
Ala 29	6.54	3.29	7.14	3.47
Glu 30	9.28	4.16	9.04	4.11
Trp 31	6.65	4.49	6.71	4.51
Cys 32	6.82	4.75	6.51	4.64
Gly 33	9.58	4.05,4.31	9.54[a]	3.98,4.31
Pro 34		(4.04?)		
Cys 35	8.21	4.41	7.81	4.09
Lys 36	7.92	4.07	7.82	4.09
Met 37	7.61	4.37	7.62	4.25
Ile 38	7.19	4.38	7.18	4.38
Ala 39	7.18	3.83	7.17	3.82

[a] At 298 K, pH 5.72

All figures are quoted at pH 5.72 and 308 K, referenced to the dioxan signal at 3.75 ppm, except where indicated

tions, the amide proton resonance of Gly-33 is saturated when the H_2O resonance is irradiated and is observed only using composite pulse sequences for H_2O suppression. For the reduced proton weak nuclear Overhauser effect (NOE) cross peaks were observed between the Gly NH and the Gly $C^{\alpha}H$ resonances. No other NOEs have been observed for this glycine amide resonance. Amide proton exchange, although slower than for Gly-33, is also a problem for Cys-32, Cys-35 and Trp-31.

4. DISCUSSION

The 'fingerprint' regions (NH-$C^{\alpha}H$ cross peaks) of the COSY spectra of Trx-S_2 and Trx-$(SH)_2$ are shown superimposed in fig.1. The majority of the cross peaks are coincident or shift at most a few hundredths of a ppm between the oxidized and reduced states. However substantial chemical shift changes do occur for some resonances and suggest that significant conformational changes occur in the active site and in some other regions of the protein. Some changes in side chain chemical shifts are also observed (not shown). The location of the backbone chemical shift changes which accompany oxidation or reduction is most easily visualized from fig.2, which plots the differences in NH and $C^{\alpha}H$ chemical shifts between Trx-S_2 and Trx-$(SH)_2$ versus residue number. It is evident from fig.2 that the largest backbone proton chemical shift changes, and hence probably the largest conformational changes, occur in the active site itself, residues 32–35, and in residues 27–31 immediately

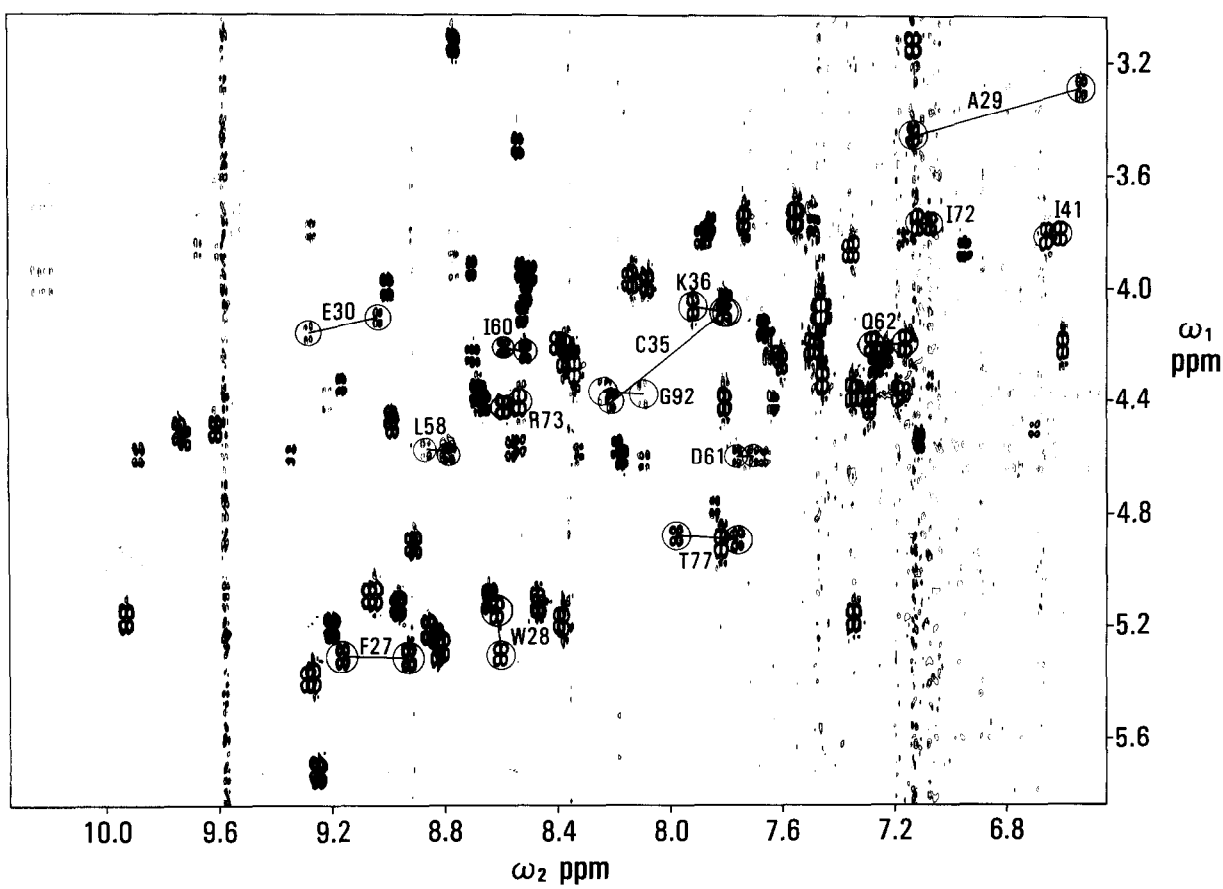

Fig.1. Contour plot of a region of the double-quantum-filtered COSY spectrum of reduced thioredoxin, superimposed on the same region of the spectrum of oxidized thioredoxin. The region contains the NH-$C^{\alpha}H$ cross peaks. Both spectra were acquired in 90% ^1H_2O/10% ^2H_2O at 308 K and pH 5.7.

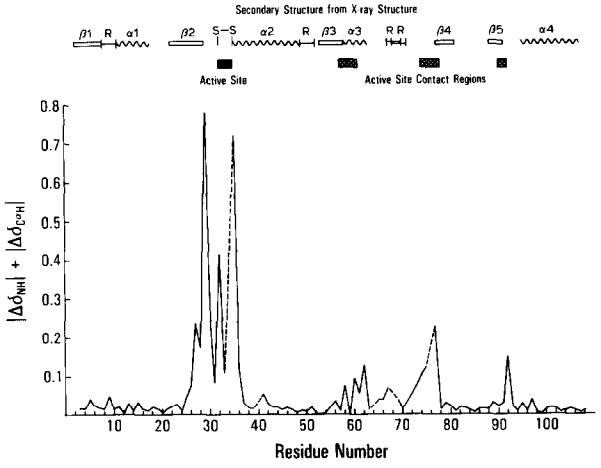

Fig.2. Sum of the absolute value of the differences between the chemical shifts of $C^\alpha H$ and NH resonances in the spectra of oxidized and reduced thioredoxin. Dotted lines indicate the presence of proline residues, for which comparable data are not available. The secondary structure elements from a 2.8 Å resolution X-ray structure [4] are shown as well as active site contact regions.

preceding the active site and shown by the X-ray structure [4] to be at the end of a β-strand. We note that NMR spectra provide no indications of any conformational changes involving residues in the α-helix following the active site loop.

In addition to the chemical shift differences observed for residues at and immediately preceding the active site, fig.2 reveals several other short regions of the polypeptide chain for which significant NH and $C^\alpha H$ chemical shift differences occur between Trx-S_2 and Trx-$(SH)_2$. These involve residues 58, 60–62, 73–77 and 92. Interestingly the X-ray structure [4] shows that all of these residues are close to the active site loop in Trx-S_2. Thus NMR spectroscopy identifies several residues in the active site of Trx and in its immediate vicinity whose backbone NH and $C^\alpha H$ resonances are sensitive to oxidation state changes. The observed chemical shift changes could arise from one or a combination of backbone conformational changes, changes in hydrogen bonding to backbone atoms, or side chain conformational changes. The number and magnitude of the chemical shift changes, including several side chain proton resonances, suggest that at least some degree of conformational rearrangement is involved. A quantitative description of these structural changes must await completion of distance geometry and molecular dynamics calculations

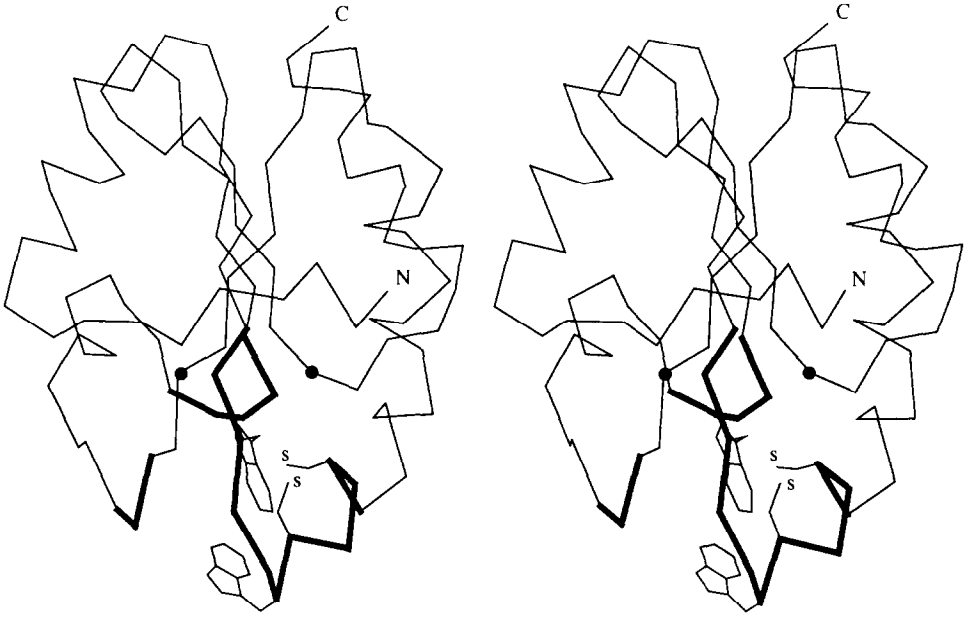

Fig.3. Stereoscopic view of the α-carbon chain of *E. coli* oxidized thioredoxin, derived from X-ray coordinates [4] kindly supplied by Dr Hans Eklund. The side chains of Trp-28 and Trp-31 are shown. Thick lines and circles indicate residues for which substantial amide and $C^\alpha H$ resonance shifts are observed (fig.2).

which are being undertaken in this laboratory. We note however that the sequential NOE connectivities observed between backbone protons are qualitatively similar for Trx-S_2 and Trx-$(SH)_2$, ruling out any major rearrangement of secondary structure.

The regions identified by NMR which show significant chemical shift differences between Trx-S_2 and Trx-$(SH)_2$ (see fig.2) correlate strikingly with independent results based on alignment of X-ray structures [20] and mutational analysis of *E. coli* thioredoxin [1,21,22]. Previously a flat and hydrophobic surface in *E. coli* Trx-S_2, comprised of residues 33–34, 75–76 and 91–93, was implicated as important for redox interactions with thioredoxin reductase and ribonucleotide reductase [20]. This result was based on functional properties and conformational similarities between thioredoxin from *E. coli* and bacteriophage T_4 and also extended to a proposed model for *E. coli* glutaredoxin [20]. Analysis of a number of mutant forms of thioredoxin obtained both in vivo and in vitro [21,22] further emphasized the importance of the residues comprising the hydrophobic surface for the binding of *E. coli* Trx-$(SH)_2$ to the gene 5 protein of phage T_7 DNA polymerase and for its role in assembly of filamentous phages. Only the reduced form of thioredoxin is active as a subunit of phage T_7 DNA polymerase and the oxidized form shows no activity or competition. Finally, we note that residues corresponding to the peaks in fig.2 are conserved exactly in thioredoxins from different species. Even for calf thymus thioredoxin, which with 104 residues is only 28% homologous to *E. coli* thioredoxin, residues Val-25, Asp-26, Phe-27, Ala-29, Trp-31 to Met-37, Asp-60, Pro-76, Gly-92 and Ala-93 of *E. coli* thioredoxin are all conserved [23].

In summary, the present NMR experiments show that a number of residues, forming a surface at one end of thioredoxin near the active site, are sensitive to reduction of the disulfide bridge. In combination with the available biochemical evidence, these results suggest that conformational changes in this region of the molecule play a major functional role.

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