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Effect of disulfide bridge formation on the NMR spectrum of a protein: Studies on oxidized and reduced *Escherichia coli* thioredoxin

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SUMMARY

As a prelude to complete structure calculations of both the oxidized and reduced forms of *Escherichia coli* thioredoxin (M_r 11 700), we have analyzed the NMR data obtained for the two proteins under identical conditions. The complete aliphatic ^{13}C assignments for both oxidized and reduced thioredoxin are reported. Correlations previously noted between ^{13}C chemical shifts and secondary structure are confirmed in this work, and significant differences are observed in the C^{β} and C^{γ} shifts between *cis*- and *trans*-proline, consistent with previous work that identifies this as a simple and unambiguous method of identifying *cis*-proline residues in proteins. Reduction of the disulfide bond in the active-site Cys^{32}-Gly-Pro-Cys^{35} sequence causes changes in the ^1H, ^{15}N and ^{13}C chemical shifts of residues close to the active site, some of them quite far distant in the amino acid sequence. Coupling constants, both backbone and side chain, show some differences between the two proteins, and the NOE connectivities and chemical shifts are consistent with small changes in the positions of several side chains, including the two tryptophan rings (Trp^{28} and Trp^{31}). These results show that, consistent with the biochemical behavior of thioredoxin, there are minimal differences in backbone configuration between the oxidized and reduced forms of the protein.

INTRODUCTION

Thioredoxin is a ubiquitous protein with a large number of known functions, almost all related to the thiol-disulfide redox chemistry of an active-site pair of cysteine residues (Holmgren, 1985). Thioredoxin acts as a general protein disulfide-oxidoreductase (Moore et al., 1964; Blombäck et

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al., 1974; Holmgren, 1984), and its active-site sequence is closely related to that of protein disulfide-isomerase (Edman et al., 1985; Krause et al., 1991; Lundström et al., 1992). Moreover, it is active as a dithiol hydrogen donor in bacterial and yeast sulfate reduction (Black et al., 1960; Wilson et al., 1961) and in ribonucleotide reduction (Laurent et al., 1964; Thelander and Reichard, 1979). Viral systems also utilize thioredoxin or the functionally and structurally related glutaredoxin (Holmgren, 1989). Thioredoxin functions have been discovered in a number of bacteriophage systems including T7 and M13 or f1 (Modrich and Richardson, 1976; Russel and Model, 1986). Phage T4 induces a glutaredoxin (thioredoxin) of its own (Berglund, 1969), the structure of which has been determined (Söderberg et al., 1978).

The oxidized and reduced forms of the thioredoxin from *E. coli* are very similar in structure, as revealed by the similarity of their NMR spectra (Holmgren and Roberts, 1976; Dyson et al., 1988,1989). However, thioredoxin also has several functions that are structural rather than catalytic and apparently depend not simply on the presence or absence of free thiol groups at the active site, but also on other factors related to the integrity of the disulfide bond. For example, in the phage T7 system, only reduced thioredoxin functions as an essential subunit of the T7-induced DNA polymerase; oxidized thioredoxin is totally inactive in this system (Holmgren et al., 1981; Nordström et al., 1981; Adler and Modrich, 1983; Reutimann et al., 1985; Huber et al., 1986; Slaby and Holmgren, 1989). Similarly, in the assembly of filamentous phage only reduced thioredoxin is active (Russel and Model, 1986). While the presence of free thiol groups is an important factor in this difference in behavior, mutants of thioredoxin where one or both cysteines were replaced with serine or alanine are also functional, although with large changes in K_D (Huber et al., 1986). Therefore, subtle structural and dynamic differences probably exist between the two oxidation states of thioredoxin, causing the observed differences in function.

A crystal structure of oxidized *E. coli* thioredoxin was obtained for crystals containing Cu(II) (Holmgren and Söderberg, 1970; Holmgren et al., 1975). These conditions were unsuitable for the formation of crystals of the reduced form (Holmgren and Söderberg, 1970), and no other conditions have been found under which crystallization of either form of thioredoxin occurred. A solution structure of the reduced form of *E. coli* thioredoxin was therefore obtained from ^1H NMR data (Dyson et al., 1990). A high-resolution NMR structure of human thioredoxin has also been published (Forman-Kay et al., 1991). Very little difference was observed between the refined 2.0 Å X-ray crystal structure of oxidized thioredoxin (Katti et al., 1990) and the NMR solution structure of reduced thioredoxin (Dyson et al., 1990). Significant differences in chemical shifts were observed between the two forms (Dyson et al., 1988,1989; Chandrasekhar et al., 1991), but the backbone fold obtained for reduced thioredoxin was virtually identical with that of the X-ray structure, and very little difference in well-ordered side chains was seen. Since the chemical difference between the two forms lies only in the integrity of a disulfide bond between residues separated by two amino acids in the polypeptide chain, it is not very surprising that great structural differences are not observed. Minimal changes in conformation between the two states of the protein would be conducive to a rapid interconversion rate in thiol-disulfide exchange reactions, as observed for thioredoxin (Holmgren, 1979a,b). A significantly greater heat stability is, however, observed for oxidized thioredoxin (Hiraoki et al., 1988).

The functional differences between the two forms of thioredoxin could also arise because of a difference in the flexibility of the active-site region. The absence of the disulfide bond in reduced thioredoxin could result in the accessibility of certain conformational states, not necessarily those

of lowest energy, that are necessary for binding interactions with the gene 5 protein of T7 DNA polymerase or a protein disulfide acting as a substrate. As a measure of the chain flexibility of the two forms of thioredoxin, we have examined their ^{15}N relaxation behavior (Stone et al., 1993). The two forms are virtually identical in their dynamics in the picosecond range; a small but significant difference was seen in the microsecond time-scale dynamics of the polypeptide backbone in the vicinity of the active site (Stone et al., 1993).

The availability of thioredoxin samples, uniformly labeled with ^{15}N and ^{13}C, has allowed us to reconsider the question of subtle conformational differences between the oxidized and reduced forms of the protein. So far, structural comparisons have been made between an X-ray structure of the oxidized form, crystallized in the presence of Cu(II) and ethanol or 2,4-pentanediol at pH close to the isoelectric point (~4.5) (Holmgren and Söderberg, 1970; Holmgren et al., 1975; Katti et al., 1990) and an NMR structure of the reduced form in the absence of Cu(II) at pH 5.7, calculated from 2D ^{1}H data alone (Dyson et al., 1990). Thioredoxin from *E. coli* (M_r 11 700) is one of the largest proteins so far examined in this way; even for smaller systems, three- and higher-dimensional spectroscopy is now routinely used. Proton-detected heteronuclear spectroscopy of uniformly labeled samples has proved to be particularly useful (Clore and Gronenborn, 1991); the additional resolution provided by the heteronucleus allows the inclusion of greater numbers of NOE and other constraints in the structure calculations, giving better resolution. In the case of thioredoxin, the differences between the oxidized and reduced forms are sufficiently subtle that the highest resolution structures are required to probe them. To this end, we are at present engaged in the calculation of structures for both forms of the protein under identical conditions, using information from 3D heteronuclear spectra.

The structural definition of the active site of thioredoxin is a particularly difficult task because of the generally solvent-exposed backbone of the active-site region itself. The sequence contains a proline residue (Pro^{34}) and the amide proton of the glycine at position 33 apparently exchanges rapidly with solvent, so that it is difficult to observe. Thus, although a large number of unambiguous NOE and dihedral angle constraints are now available for both forms of *E. coli* thioredoxin, they refer mainly to the remainder of the molecule, other than the active site. We therefore present in this paper an evaluation of the NMR data, including the previously unpublished ^{13}C assignments, for the two forms of the protein, aimed at pinpointing observable differences between them that can subsequently be used to compare the two high-resolution solution structures.

MATERIALS AND METHODS

Uniformly labeled thioredoxin was obtained from an overproducing strain of *E. coli* as described previously (Chandrasekhar et al., 1991). For the production of thioredoxin, ~90–95% uniformly labeled with both ^{13}C and ^{15}N, an algal homogenate (Martek Co.) was used as a basis for an enriched medium, since the yield of cells and of thioredoxin from a minimal medium containing labeled glucose did not give sufficient amounts of material for NMR experiments. Purification of thioredoxin from cell extracts and preparation of samples of the reduced protein using dithiothreitol were performed as previously described (Dyson et al., 1989).

NMR experiments were carried out at 308 K on Bruker spectrometers operating at 600 or 500 MHz for protons. A ^{15}N 3D NOESY-HMQC experiment (Clore et al., 1989) was performed for

each form of the protein to obtain the NOE information discussed in this paper. Thioredoxin samples (usually ~4 mM) were dissolved in 90% ^1H_2O/10% ^2H_2O, pH 5.70 ± 0.02, and the probe temperature was 308 K except where noted. The mixing time was 150 ms. A total of 64 (ω_1–ω_3) planes were acquired, over a ^{15}N spectral width of 2041 Hz. Spectral widths were 4032 Hz (data size 2K points) and 8064 Hz (data size 256 real points) in ω_3 and ω_1, respectively. The data were acquired with a combined States-TPPI phase incrementation protocol (Marion et al., 1989) and processed using the program FTNMR (Hare Research, Inc.).

Three-dimensional heteronuclear correlated spectra of samples dissolved in ^2H_2O (~4 mM, pH (uncorrected) 5.70) were used to assign the ^{13}C resonances of both oxidized and reduced thioredoxin, and included constant-time versions of 3D HCCH-COSY (Ikura et al., 1991), 3D HCCH-TOCSY (Bax et al., 1990), and 3D NOESY-HSQC (Clore et al., 1989) spectroscopy. Standard pulse sequences were used throughout. The spectra generally consisted of 64 (ω_1–ω_3) planes, acquired with 128 × 2 free induction decays and 1024 points in the acquisition dimension, using a combined States-TPPI method of phase incrementation. Spectral widths were 7500 Hz in ω_3 and 3600 Hz in ω_1. A spectral width of 5810 Hz was chosen in ω_2 to give maximum digital resolution of the folded data. Data were Fourier transformed in ω_3 and ω_1, using a 90° sine-bell window function, with the program FTNMR; a low-pass filter was used to suppress the water resonance in some cases. The ω_2 transform was performed on an SGI Personal Iris 4D25 computer, using a program written in-house. ^{13}C and ^{15}N chemical shifts were indirectly referenced to TMS and NH_3, respectively, as described previously (Live et al., 1984; Bax and Subramanian, 1986; Fairbrother et al., 1992).

Dihedral angle information and stereospecific assignments were obtained using several spectroscopic methods to obtain coupling constants, with confirmatory evidence from intraresidue NOEs. The method of Neri et al. (1990) was used to obtain the $^3J_{HN\alpha}$ coupling constant for all non-proline and non-glycine residues in the two forms of the protein, giving information on the ϕ dihedral angle. A few residues were severely overlapped in the ^{15}N HSQC spectrum, so that $^3J_{HN\alpha}$ coupling constants could not be obtained in this way; these were estimated from the 2QF-COSY spectrum in 90% ^1H_2O/10% ^2H_2O and are shown in Table 2 preceded by the symbol '~'. For residues with very small $^3J_{HN\alpha}$ coupling constants, the data were insufficient to give an accurate estimate of the actual value, but an upper limit could reliably be determined in these cases and is indicated as such in Table 2. To obtain $^3J_{\alpha\beta}$ coupling constant information, we took several approaches. The ^1H E.COSY spectrum (Griesinger et al., 1985) was significantly overlapped in the α-β region of the spectrum, but a number of cross peaks were sufficiently well resolved to be used to assign $^3J_{\alpha\beta}$ values. Where information could not be obtained from the E.COSY spectrum, the ^1H 2QF-COSY spectrum was used to evaluate large values of the $^3J_{\alpha\beta}$ coupling constant; small coupling constants usually cannot be obtained in this way. These values were corroborated using the 2D ^{15}N HSQC-TOCSY spectrum (Clore et al., 1991) and qualitative assignments of large and small coupling constants were made in several cases where both E.COSY and 2QF-COSY were uninformative. Complementary coupling constant information was obtained from the $^3J_{N\beta}$ coupling constant. Several methods have been suggested for measuring this quantity, a number based on the principle of E.COSY (Montelione et al., 1989; Wagner, 1990). We have not found these experiments to be very practical for a protein of the size of thioredoxin, due to overlap and linewidth problems. Instead, we have successfully employed a direct measurement of $^3J_{N\beta}$ (Chary et al., 1991). Based on the values of $^3J_{\alpha\beta}$ and $^3J_{N\beta}$ obtained for

each residue, stereospecific assignments of β-methylene protons were made; a number of these were averaged. Stereospecific assignments were confirmed with reference to intraresidue NOEs, obtained from the 3D ^{15}N and ^{13}C NOESY spectra. Stereospecific assignment of valine methyl groups was also possible in a number of cases where intraresidue NOEs could be employed.

Proton–proton NOE differences between the spectra of reduced and oxidized thioredoxin were assessed by comparison of normalized cross-peak volumes from four different spectra of each protein, a phase-sensitive 2D NOESY in 90% ^1H_2O/10% ^2H_2O (τ_m = 150 ms), a phase-sensitive 2D NOESY in 90% ^1H_2O/10% ^2H_2O (τ_m = 150 ms) obtained by using a composite pulse $45°_{(x)}$-τ-$45°_{(-x)}$ for the observe pulse (Clore and Gronenborn, 1983), a 2D NOESY in 99% ^2H_2O (τ_m = 150 ms) and a 3D ^{15}N HSQC spectrum (Clore et al., 1991) in 90% ^1H_2O/10% ^2H_2O (τ_m = 150 ms). All of these experiments were performed under the same conditions of temperature and pH (308 K and pH 5.7), except for the 2D NOESY (composite pulse) experiments, which were performed at 298 K. The cross-peak volumes were normalized by multiplication by a factor, derived from a comparison of the values obtained for a number of standard cross peaks, involving residues that are not affected by the formation of the disulfide bond between residues 32 and 35. The cross peaks used were generally either between protons in the β-sheet, distant from the active site, or between side-chain protons; intraresidue cross peaks were avoided in order to minimize systematic errors due to different levels of spin diffusion and zero-quantum effects in the various spectra.

RESULTS

Resonance assignments

As for the ^{15}N spectrum (Chandrasekhar et al., 1991), the assignment of the ^{13}C spectrum was greatly assisted by the availability of complete ^1H resonance assignments (Dyson et al., 1989). In most cases, the ^{13}C assignments could be obtained directly from the HCCH-TOCSY spectrum by correlating the proton spin systems. One correction should be made to the original proton resonance assignments (Dyson et al., 1989): the connectivities between the four methylene groups of Lys^{57} were scrambled and the $C^\varepsilon H$ was wrongly assigned. The assignment of the lysine side-chain carbon and proton resonances was made from the HCCH-TOCSY spectrum, as shown in Fig. 1. This was the only error detected in the original assignments. One of the ^{15}N assignments (Chandrasekhar et al., 1991) was also corrected on the basis of 3D experiments: the assignment for Ser^{95} is 114.7 ppm for both forms of the protein, not 120 ppm as reported previously. The complete assignments of the ^{13}C spectrum for oxidized and reduced thioredoxin are presented in Table 1.

Coupling constants and stereospecific assignments

Coupling constants and stereospecific assignments of the resonances of prochiral $C^\beta H$ protons were obtained using several different 2D spectra. A ^{15}N-edited TOCSY experiment (Clore et al., 1991) was used together with information from E.COSY and 2QF-COSY spectra to obtain values for the $^3J_{\alpha\beta}$ coupling constants. An example is shown in Fig. 2. Both the g_2t_3 and t_2g_3 rotamers give rise to the same pattern of $^3J_{\alpha\beta}$ coupling constants, one > 10 and one < 5 Hz. There are several measurements that can be used to distinguish between these two rotamers, including the heteronuclear E.COSY-type experiment (Montelione et al., 1989) and the ^{15}N-edited experiment selective for $^3J_{N\beta}$ couplings (Chary et al., 1991). The actual value of the $^3J_{N\beta}$ coupling constant is

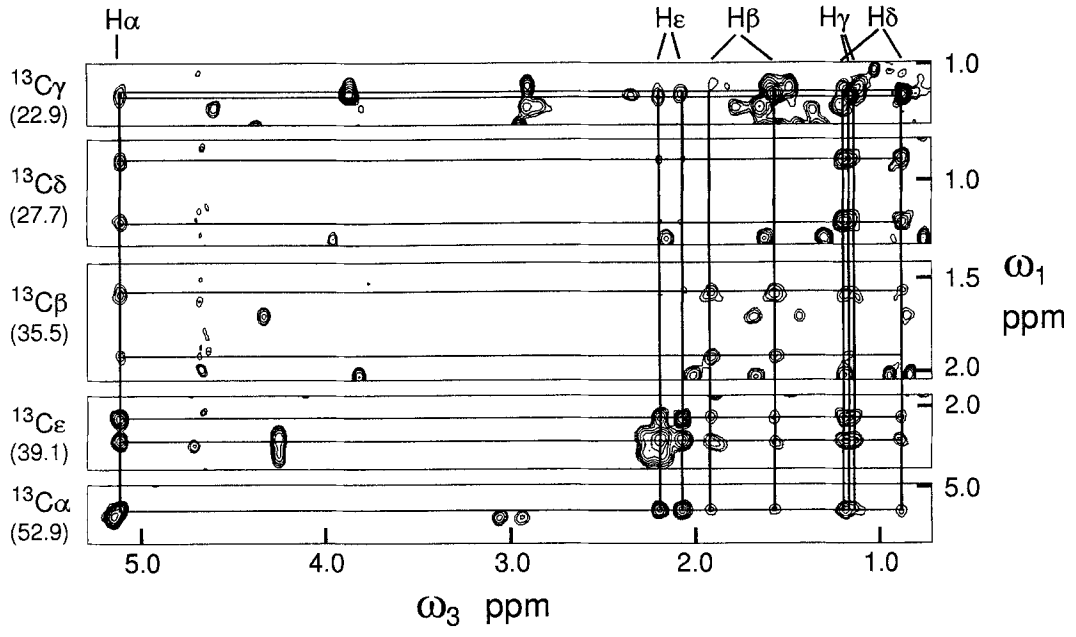

Fig. 1. Some slices from a 500 MHz 3D HCCH-TOCSY spectrum (τ_m = 21 ms) of a uniformly $^{13}C/^{15}N$ double-labeled sample of oxidized thioredoxin in 99% ^2H_2O at pH 5.7 and 308 K, showing the connectivities of the Lys[57] side chain.

difficult to evaluate from the ^{15}N-edited spectrum, as shown in Fig. 3, but the presence or absence of cross peaks was an invaluable aid in the stereospecific assignment process, unambiguously distinguishing g_2t_3 and t_2g_3 rotamers. The $^3J_{HN\alpha}$, $^3J_{\alpha\beta}$ and $^3J_{N\beta}$ coupling constants obtained for both forms of the protein are given in Table 2, together with preferred χ^1 rotamers and stereospecific assignments. Corroborative evidence for the stereospecific assignments was in most cases obtained from intraresidue NOE connectivities between NH, $C^\alpha H$ and $C^\beta H$. The stereospecific assignment of prochiral $C^\beta H$ protons could generally be made unambiguously from the coupling constants alone for the t_2g_3 rotamer ($\chi^1 = -60°$). When the preferred rotamer is g_2g_3 ($\chi^1 = +60°$) or g_2t_3 ($\chi^1 = 180°$), the coupling constant information has a greater potential for ambiguity, since it relies on the observation of the *absence* of specific cross peaks. Greater use was made in these cases of corroborative evidence from NOE connectivities, but in several cases the information remained ambiguous to such an extent that a stereospecific assignment could not be made with confidence. Such cases have been noted in Table 2.

The χ^1 value of the preferred rotamer and stereospecific assignment of the active-site Cys[32] and Cys[35] residues were previously reported for reduced thioredoxin on the basis of $^3J_{\alpha\beta}$ coupling constants and NOE information (Dyson et al., 1990). The value of 180° (g_2t_3) obtained for Cys[32] is confirmed in the present work, but the value for Cys[35] is −60° (t_2g_3), not 180° as previously reported (Dyson et al., 1990). A comment to this effect has appeared previously (Stone et al., 1993). This change will make no difference to the backbone conformation in the active site, but will change the orientation of the Cys[35] side chain from that reported for the solution structure of reduced thioredoxin (Dyson et al., 1990). This illustrates the uncertainty inherent in stereospecific assignments that rely too heavily on NOE information: in the case of Cys[35], the NH proton of

TABLE 1
^{13}C RESONANCE ASSIGNMENTS (ppm) FOR REDUCED AND OXIDIZED THIOREDOXIN

Residue	Reduced thioredoxin					Oxidized thioredoxin				
	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}
Ser[1]	54.9	61.5				54.7	61.3			
Asp[2]	52.5	38.7				52.5	38.7			
Lys[3]	54.7	31.3	22.9	26.6	39.9	54.7	31.2	22.9	26.6	39.7
Ile[4]	58.2	36.3	26.3	10.8		58.1	36.2	26.2	10.8	
			14.7[a]					14.7[a]		
Ile[5]	58.3	37.2	25.0	10.7		58.5	37.0	25.0	10.7	
			15.3[a]					15.3[a]		
His[6]	53.7	28.4				53.3	27.6			
Leu[7]	51.7	43.9	24.2	26.5 (0.54)[b]		51.8	43.8	24.4	26.4 (0.53)	
				22.6 (0.67)					22.7 (0.66)	
Thr[8]	56.8	70.9	19.8			56.7	70.8	19.8		
Asp[9]	55.9	38.3				55.8	38.3			
Asp[10]	54.2	38.9				54.3	39.0			
Ser[11]	57.1	62.3				56.9	62.2			
Phe[12]	61.3	38.7				61.3	38.6			
Asp[13]	55.8	38.4				55.8	38.3			
Thr[14]	63.5	67.1	19.8			63.6	67.1	19.9		
Asp[15]	54.4	39.2				54.3	39.2			
Val[16]	61.3	30.4	17.9			61.3	30.1	17.9		
			(−0.62)					(−0.62)		
			20.2					20.2		
			(−0.13)					(−0.14)		
Leu[17]	55.8	37.7	25.1	23.1 (1.18)		55.8	37.5	25.1	23.1 (1.17)	
				20.6 (0.89)					20.6 (0.89)	
Lys[18]	52.9	29.6	23.0	26.6	40.1	52.9	29.9	22.9	26.6	40.0
Ala[19]	50.1	18.1				50.0	18.1			
Asp[20]	52.0	39.6				52.1	39.3			
Gly[21]	42.4					42.4				
Ala[22]	49.6	18.2				49.5	18.2			
Ile[23]	57.0	39.2	25.4	11.7		56.9	38.9	25.4	11.7	
			15.7[a]					15.7[a]		
Leu[24]	51.0	43.4	24.9	24.5 (0.88)		50.9	43.4	24.7	24.5 (0.89)	
				22.3 (0.96)					22.4 (0.96)	
Val[25]	59.6	31.9	20.6 (0.26)			59.6	31.7	20.6 (0.26)		
			20.9 (0.82)					21.0 (0.83)		
Asp[26]	49.6	37.4				49.9	37.3			
Phe[27]	56.4	37.4				56.3	37.5			
Trp[28]	53.0	31.5				53.1	31.0			
Ala[29]	49.7	20.1				49.5	19.8			
Glu[30]	56.8	28.3	33.9			57.5	27.7	33.9		
Trp[31]	52.3	26.9				51.8	26.6			
Cys[32]	55.8	24.3				51.4	41.9			
Gly[33]	47.1					46.7				
Pro[34]	63.8	30.4	25.9	49.5		63.0	29.7	26.2	49.7	
Cys[35]	61.9	25.9				61.5	32.5			
Lys[36]	56.7	29.5	23.1	26.8	39.9	57.2	29.3	23.0	26.6	39.9

TABLE 1 (continued)

Residue	Reduced thioredoxin					Oxidized thioredoxin				
	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ϵ}	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ϵ}
Met^{37}	56.1	30.4	30.0		14.8	56.1	30.2	29.8		14.8
Ile^{38}	59.7	37.4	25.1	12.6		59.7	37.2	25.1	12.6	
			15.1[a]					15.1[a]		
Ala^{39}	55.4	13.1				55.0	13.0			
Pro^{40}	63.6	28.9	26.2	48.3		63.6	28.8	25.8	48.2	
Ile^{41}	62.3	35.9	26.5	11.9		62.2	35.9	26.5	11.9	
			16.1[a]					16.2[a]		
Leu^{42}	55.6	38.8	24.5	24.3 (0.63)		55.8	38.7	24.4	24.1 (0.62)	
				20.6 (0.63)					20.7 (0.62)	
Asp^{43}	56.0	38.4				56.0	38.3			
Glu^{44}	57.3	27.5	34.5			57.3	27.3	34.3		
Ile^{45}	60.2	34.2	27.2	8.9		60.2	34.1	27.2	9.0	
			16.1[a]					16.2[a]		
Ala^{46}	53.0	15.9				53.0	15.9			
Asp^{47}	54.8	39.3				54.8	39.0			
Glu^{48}	57.2	28.1	33.8			57.2	28.0	34.2		
Tyr^{49}	56.4	35.3				56.5	35.2			
Gln^{50}	56.5	26.7	31.6			56.5	26.6	31.4		
Gly^{51}	43.5					43.5				
Lys^{52}	54.2	33.0	22.7	27.8	39.8	54.3	32.8	22.5	27.6	39.7
Leu^{53}	52.2	45.5	24.2	25.9 (0.50)		52.1	45.4	24.4	25.9 (0.49)	
				22.1 (0.73)					22.1 (0.73)	
Thr^{54}	59.9	68.4	19.3			60.2	68.2	19.3		
Val^{55}	59.9	30.4	20.2 (0.85)			60.0	30.2	20.2 (0.86)		
			19.8 (0.81)					19.8 (0.80)		
Ala^{56}	47.3	22.1				47.4	22.1			
Lys^{57}	52.9	35.5	22.9	27.8	39.1	52.9	35.5	22.9	27.7	39.1
Leu^{58}	52.2	42.6	26.4	23.7 (0.77)		52.1	42.7	26.3	23.8 (0.77)	
				23.8 (0.93)					23.8 (0.93)	
Asn^{59}	50.0	36.3				49.9	36.0			
Ile^{60}	61.5	36.1	24.2	13.1		61.5	36.0	24.4	13.1	
			17.6[a]					17.6[a]		
Asp^{61}	54.2	38.7				54.3	38.7			
Gln^{62}	54.2	28.3	31.7			54.3	28.2	31.8		
Asn^{63}	49.6	38.7				49.7	38.6			
Pro^{64}	61.8	30.9	24.4	48.1		61.8	30.9	24.3	48.0	
Gly^{65}	44.2					44.2				
Thr^{66}	66.5	66.5	18.4			66.6	66.6	18.4		
Ala^{67}	55.0	13.1				55.0	13.0			
Pro^{68}	63.7	28.9	26.1	48.6		63.8	28.9	26.2	48.5	
Lys^{69}	56.7	30.0	22.9	27.8	39.8	56.7	29.9	23.0	27.7	39.7
Tyr^{70}	56.3	37.5				56.2	37.5			
Gly^{71}	44.6					44.5				
Ile^{72}	59.1	34.5	25.7	9.0		59.0	34.3	25.5	9.2	
			14.5[a]					14.4[a]		
Arg^{73}	53.6	29.6	24.7	41.0		53.6	29.2	24.7	40.8	
Gly^{74}	42.5					42.5				

TABLE 1 (continued)

Residue	Reduced thioredoxin					Oxidized thioredoxin				
	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}
Ile[75]	55.8	39.3	23.1	13.5		56.1	38.8	22.3	14.2	
			17.3[a]					17.8[a]		
Pro[76]	60.9	32.7	22.4	48.9		61.0	32.5	22.2	49.2	
Thr[77]	62.1	70.9	20.0			62.1	71.0	20.0		
Leu[78]	50.9	41.5	24.6	24.4 (0.73)		51.0	41.6	24.4	24.4 (0.73)	
				22.1 (0.81)					22.2 (0.81)	
Leu[79]	51.6	44.1	25.5	24.2 (0.80)		51.5	44.1	25.1	24.2 (0.83)	
				23.2 (0.86)					22.9 (0.87)	
Leu[80]	51.3	42.6	25.5	24.7 (0.53)		51.1	42.6	25.5	24.5 (0.53)	
				22.1 (0.72)					22.1 (0.73)	
Phe[81]	55.3	40.0				55.2	39.7			
Lys[82]	54.2	34.1	23.4	27.8	39.9	54.3	34.0	23.4	27.5	39.7
Asn[83]	52.9	35.5				52.9	35.4			
Gly[84]	44.3					44.3				
Glu[85]	51.9	31.2	33.5			51.8	31.1	33.5		
Val[86]	62.3	29.1	21.1 (0.57)			62.3	29.1	21.2 (0.56)		
			19.9 (0.60)					19.9 (0.60)		
Ala[87]	50.5	18.4				50.4	18.3			
Ala[88]	50.3	20.3				50.3	20.3			
Thr[89]	59.6	70.1	18.6			59.6	70.1	18.8		
Lys[90]	52.2	32.8	21.9	25.7	40.1	52.2	32.7	21.9	25.3	39.7
Val[91]	59.6	32.0	18.7 (0.82)			59.6	31.7	18.8 (0.86)		
			18.7 (0.90)					18.1 (0.91)		
Gly[92]	41.6					41.6				
Ala[93]	50.6	17.0				50.5	17.2			
Leu[94]	52.2	42.2	24.6	24.2 (0.52)		52.1	42.0	24.4	24.2 (0.53)	
				22.1 (0.67)					22.2 (0.68)	
Ser[95]	54.2	63.8				53.9	63.8			
Lys[96]	58.8	29.5	23.7	27.4	39.8	58.8	29.5	24.0	27.3	39.5
Gly[97]	45.1					45.0				
Gln[98]	56.4	27.5	32.7			56.5	27.5	32.7		
Leu[99]	56.0	39.0	25.4	24.6 (0.74)		56.0	39.0	25.3	24.6 (0.74)	
				21.7 (0.87)					21.6 (0.87)	
Lys[100]	58.6	29.2	23.5	26.8	39.2	58.6	29.2	23.3	26.6	39.2
Glu[101]	57.7	27.8	34.5			57.8	27.5	34.1		
Phe[102]	57.9	37.0				57.9	36.9			
Leu[103]	55.8	38.9	23.6	23.8 (0.12)		55.8	38.8	24.0	23.8 (0.13)	
				20.6 (0.60)					20.7 (0.60)	
Asp[104]	55.4	37.3				55.4	37.2			
Ala[105]	51.8	16.8				51.8	16.8			
Asn[106]	52.5	40.1				52.5	39.7			
Leu[107]	52.9	40.5	24.5	21.4 (0.84)		52.9	40.5	24.4	21.3 (0.84)	
				24.6 (0.87)					24.5 (0.87)	
Ala[108]	52.0	17.7				51.9	17.7			

[a] $C^{\gamma}H_3$ chemical shift.

[b] Numbers in parentheses are the proton chemical shifts for the methyl groups of leucine and valine (Dyson et al., 1989), which are included to identify the respective methyl carbon chemical shifts.

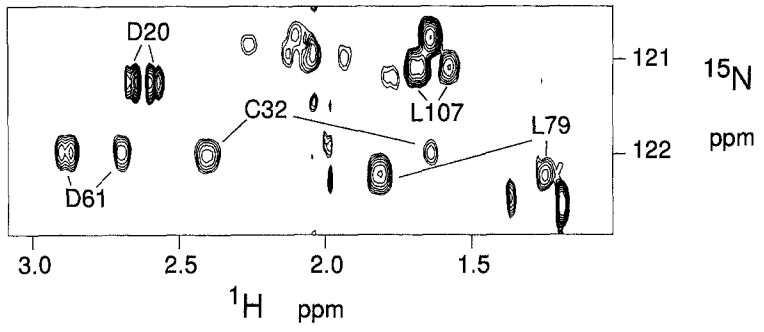

Fig. 2. Portion of a 500 MHz 2D ^{15}N-edited TOCSY spectrum of a uniformly ^{15}N-labeled sample of reduced thioredoxin in 90% $^{1}H_2O$/10% $^{2}H_2O$ at pH 5.7 and 308 K, showing the differences in cross-peak widths for the $C^{\beta}H$, according to the $^{3}J_{\alpha\beta}$ coupling constants. Note the evidence for averaging for Asp^{20} (large coupling constant for both $C^{\beta}H$ cross peaks).

Lys^{36} is overlapped with that of Cys^{35}, which introduces ambiguity into the evaluation of NOE intensities between the two $C^{\beta}H$ protons and the NH in a 2D ^{1}H NOESY spectrum.

NOE differences between oxidized and reduced forms

Differences in structure between oxidized and reduced thioredoxin are inherently difficult to observe on the basis of NOE connectivities. The active-site region is a mobile exterior loop. If a number of conformations were populated, NOEs from each conformation would be observed, with intensities proportional to the population of the particular conformation as well as to the internuclear distance. We therefore looked for significant differences in the intensities of NOEs between protons in the active-site area, as well as for NOEs that might be present in one form of the protein and absent in the other. In order to evaluate intensity differences, the cross-peak volumes were carefully normalized for the four spectra obtained for each form of the protein. The majority of the NOEs showed no significant difference between oxidized and reduced thioredoxin. As has been noted previously (Dyson et al., 1989), a small number of NOE differences occur for residues close to the active site, and in particular those involving the aromatic rings of the two tryptophan residues. While a sizeable number of NOEs showed some difference between the spectra of the two proteins, we looked in particular for unambiguous NOEs that are sufficiently large, so that spin diffusion can be discounted, and that are present in the spectra of either oxidized or reduced thioredoxin, but not in the other. These are shown in Table 3.

DISCUSSION

Carbon chemical shifts and structure of thioredoxin

More than the ^{1}H chemical shift, the ^{13}C chemical shift, particularly of C^{α}, reflects the secondary structure of a polypeptide chain (Wishart et al., 1991). Thioredoxin shows typical behavior, as illustrated in Fig. 4: the chemical shift of the C^{α} for the residues in β-structure lies upfield of the 'random' shift (Howarth and Lilley, 1978; Richarz and Wüthrich, 1978), while for α-helices the values lie downfield. This behavior has previously been qualitatively described for an unpublished set of C^{α} chemical shifts of oxidized thioredoxin (Wishart et al., 1991).

The ^{13}C chemical shifts for oxidized and reduced thioredoxin are very similar; the only signifi-

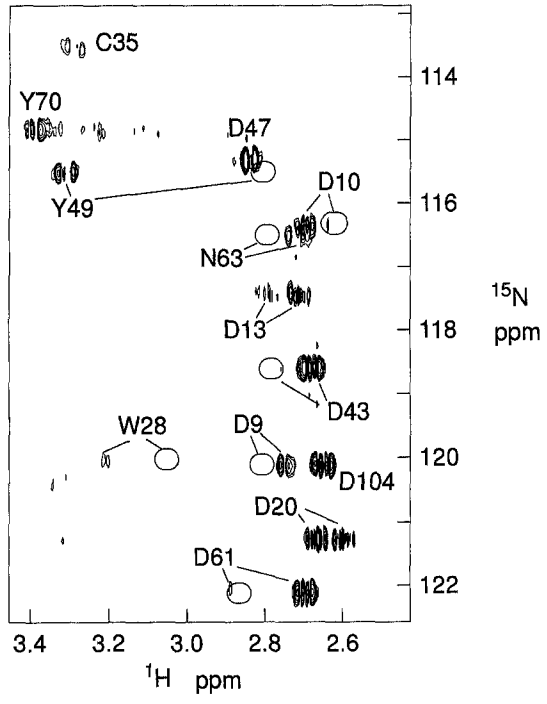

Fig. 3. Portion of a ^{15}N-edited spectrum (Chary et al., 1991) of a uniformly ^{15}N-labeled sample of reduced thioredoxin, pH 5.7, 308 K, showing cross peaks between ^{15}N and $C^{\beta}H$ where the $^{3}J_{N\beta}$ coupling constant is ~ 5 Hz. Cross peaks do not appear when the $^{3}J_{N\beta}$ coupling constant is less than this value. Note the evidence for averaging for Asp^{13} and Asp^{20} (cross peaks present for both $C^{\beta}H$).

cant differences are observed in the active-site loop itself and for Ile^{75} (Table 1). The largest differences occur for the resonances of the two cysteine residues, which is not unexpected since this is the site of the chemical difference between the two forms of the protein. The larger change appears to occur on Cys^{32}, absolute differences of 4.4 ppm for C^{α} and 17.6 ppm for C^{β}, compared to 0.4 ppm for C^{α} and 6.6 ppm for C^{β} of Cys^{35}. These chemical shifts are, however, not strictly comparable. The random-coil shifts for cysteine depend on the chemical state of the sulfur and differ markedly between the disulfide, thiol and thiolate states (Howarth and Lilley, 1978). The differences between the observed chemical shifts and the random-coil values were calculated for the C^{α} and C^{β} carbons of residues in the active-site region of reduced and oxidized thioredoxin, taking into account these differences in random-coil shift, and are shown in Fig. 5. Both of the cysteines in reduced thioredoxin are assumed to be in the thiol form at the experimental pH (5.7), since the lowest pK_a for the cysteine thiols is about 7.0 (Kallis and Holmgren, 1980; Dyson et al., 1991; Li et al., 1993), and the carboxyl group of Asp^{26} is also assumed to be protonated in both oxidized and reduced thioredoxin, since its pK_a is also about 7–7.5 (Dyson et al., 1991; Langsetmo et al., 1991).

The most noticeable general feature of Fig. 5 is the close correspondence, in a reciprocal sense, between the chemical-shift differences for the C^{α} and C^{β} carbons of any given residue. The deviations of the C^{α} carbons are exactly reproduced, with the exception of residues 30–33, by

TABLE 2
THIOREDOXIN COUPLING CONSTANTS AND STEREOSPECIFIC ASSIGNMENTS

Residue	Reduced						Oxidized					
	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo
Lys[3]	7.9	1.69	10		−60	$H^{\beta2}$	7.0	1.66	10		−60	$H^{\beta2}$
		1.91	4	xpk		$H^{\beta3}$		1.92	5	xpk		$H^{\beta3}$
Ile[4]	8.5	1.70	10		−60		8.0	1.68	10		−60	
Ile[5]	9.4	1.72	9		amb[b]		8.0	1.73	8	xpk	amb[b]	
His[6]	9.6	3.16[c]					8.5	3.13	4	xpk	av[d]	
								3.19	8			
Leu[7]	7.6	1.33	abs[e]	xpk	−60	$H^{\beta3}$	7.0	1.33	abs[e]	xpk	−60	$H^{\beta3}$
		1.80	12(Q)[e]			$H^{\beta2}$		1.80	12(Q)[e]			$H^{\beta2}$
Thr[8]	8.0	O/L[f]					7.4	O/L[f]				
Asp[9]	<3.5	2.75	O/L	xpk	amb		<3.5	2.74	O/L	xpk	amb	
		2.80	O/L					2.78	O/L			
Asp[10]	5.6	2.61	8		−60	$H^{\beta2}$	5.8	2.62	9		−60	$H^{\beta2}$
		2.69	5	xpk		$H^{\beta3}$		2.68	5	xpk		$H^{\beta3}$
Ser[11]	9.2	3.69	small(N)[g]		amb		8.0	3.69	abs		+60	$H^{\beta3}$
		4.28	small(N)	O/L				4.28	abs	xpk		$H^{\beta2}$
Phe[12]	1.8	3.19	12		180	$H^{\beta3}$	2.2	3.18	12		180	$H^{\beta3}$
		3.30	4			$H^{\beta2}$		3.30	4			$H^{\beta2}$
Asp[13]	2.4	2.72	smaller	xpk	av		2.2	2.71	smaller	xpk	av	
		2.79	larger	xpk				2.78	larger	xpk		
Thr[14]	5.4	4.05	7(Q)	xpk	+60		5.2	4.06	7(Q)	xpk	+60	
Asp[15]	5.7	2.18	small(N)	xpk	−60	$H^{\beta3}$	5.1	2.17	small(N)	xpk	−60	$H^{\beta3}$
		2.27	large(N)			$H^{\beta2}$		2.25	large(N)			$H^{\beta2}$
Val[16*h]	9.9	0.72	12		180		9.4	0.72	11		180	
		−0.62				Me1[i]		−0.62				Me1[i]
		−0.13				Me2[i]		−0.13				Me2[i]
Leu[17*]	3.7	1.54	3	xpk	−60	$H^{\beta3}$	2.2	1.58	4	xpk	−60	$H^{\beta3}$
		2.38	14			$H^{\beta2}$		2.36	12			$H^{\beta2}$
Lys[18]	9.1	1.82	10		−60	$H^{\beta2}$	8.6	1.82	12		−60	$H^{\beta2}$
		2.10	3	xpk		$H^{\beta3}$		2.09	4	O/L		$H^{\beta3}$
Ala[19]	6.3						6.0					
Asp[20]	7.7	2.59	larger	xpk	av		7.4	2.58	larger	xpk	av	
		2.68	smaller	xpk				2.66	smaller	xpk		
Ala[22]	7.7						6.6					
Ile[23*]	10.4	1.71	10		−60		9.3	1.73	11		−60	
Leu[24*]	9.0	1.27	abs		180	$H^{\beta2}$	8.4	1.26	4		180	$H^{\beta2}$
		2.03	15(Q)			$H^{\beta3}$		2.03	13	O/L		$H^{\beta3}$
Val[25*]	9.8	2.25	12(Q)		180		8.8	2.24	11		180	
		0.26				Me1[i]		0.25				Me1[i]
		0.82				Me2[i]		0.83				Me2[i]
Asp[26*]	8.8	2.23	abs		180	$H^{\beta2}$	8.9	2.20	abs		180	$H^{\beta2}$
		2.89	14(Q)			$H^{\beta3}$		2.89	15(Q)			$H^{\beta3}$
Phe[27*]	9.8	2.75	abs	xpk	−60	$H^{\beta3}$	8.7	2.75	4	xpk	−60	$H^{\beta3}$
		3.35	12(Q)			$H^{\beta2}$		3.30	12			$H^{\beta2}$

TABLE 2
(continued)

Residue	Reduced						Oxidized					
	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo
Trp[28]*	10.2	3.08	abs		+60	$H^{\beta3}$	8.6	2.93	abs	xpk	+60	$H^{\beta2}$
		3.22	abs	xpk		$H^{\beta2}$		3.06	abs			$H^{\beta3}$
Ala[29]*	6.0						5.4					
Glu[30]	2.5	2.07[c]					~3 (O/L)	2.07[c]				
								2.10[c]				
Trp[31]	5.9	3.21	abs		+60	$H^{\beta3}$	5.9	3.19	abs		+60	$H^{\beta3}$
		3.68	abs	xpk		$H^{\beta2}$		3.68	abs	xpk		$H^{\beta2}$
Cys[32]	8.9	1.67	3		180	$H^{\beta2}$	8.4	2.88	abs		180	$H^{\beta2}$
		2.37	11			$H^{\beta3}$		3.18	12(Q)			$H^{\beta3}$
Cys[35]	5.7	3.29	abs	xpk	−60	$H^{\beta3}$	4.6	3.39	abs	xpk	−60	$H^{\beta3}$
		3.79	12(Q)			$H^{\beta2}$		4.34	large(N)			$H^{\beta2}$
Lys[36]	7.7	1.98	O/L	xpk	−60	$H^{\beta3}$	~7	2.00	O/L	O/L	amb	
		2.08	large			$H^{\beta2}$		2.12	O/L	O/L		
Met[37]	5.7	2.20[c]					4.5	2.24[c]				
Ile[38]	7.9	1.90	6	xpk	180		7.5	1.95	7	xpk	180	
Ala[39]	2.6						<3.5					
Ile[41]	7.9	2.03	10	?	−60		6.8	2.02	10	?	−60	
Leu[42]	4.6	1.26	abs	xpk	−60	$H^{\beta3}$	4.9	1.22	abs	xpk	−60	$H^{\beta3}$
		1.87	12(Q)			$H^{\beta2}$		1.90	11(Q)			$H^{\beta2}$
Asp[43]	4.0	2.68	5	xpk	−60	$H^{\beta3}$	3.7	2.68	4	xpk	−60	$H^{\beta3}$
		2.80	11			$H^{\beta2}$		2.78	9			$H^{\beta2}$
Glu[44]	5.0	2.07	abs	xpk	−60	$H^{\beta3}$	4.9	2.07	O/L	xpk	amb	
		2.24	10(Q)			$H^{\beta2}$		2.24	O/L			
Ile[45]*	4.5	2.15	11	xpk	av		4.6	2.15	10	xpk	av	
Ala[46]*	2.2						<2					
Asp[47]	6.6	2.84[i]					6.3	2.83[i]				
Glu[48]	4.3	2.00	smaller	xpk?	amb/av		3.7	2.00	4	?	amb/av	
		2.13	larger					2.13	7			
Tyr[49]	9.2	2.80	12		−60	$H^{\beta2}$	9.1	2.80	10		−60	$H^{\beta2}$
		3.31	3	xpk		$H^{\beta3}$		3.30	4	xpk		$H^{\beta3}$
Gln[50]	<1	2.27	8		−60	$H^{\beta2}$	1.5	2.26	8		−60	$H^{\beta2}$
		2.36	5	xpk		$H^{\beta3}$		2.36	5	xpk		$H^{\beta3}$
Lys[52]	10.9	1.89	11(Q)		amb[j]		8.6	1.90	9		−60	$H^{\beta2}$
		1.95	O/L	xpk?				1.98	5	xpk		$H^{\beta3}$
Leu[53]	7.6	1.30	3		180	$H^{\beta2}$	6.8	1.29	5		180	$H^{\beta2}$
		1.79	10			$H^{\beta3}$		1.79	10			$H^{\beta3}$
Thr[54]*	10.7	3.95	9(Q)		−60		9.9	3.94	9		−60	
Val[55]*	10.1	2.00	11(Q)		180		9.0	2.00	12(Q)		180	
		0.81				Me2[i]		0.80				Me2[i]
		0.85				Me1[i]		0.86				Me1[i]
Ala[56]*	10.4						9.2					
Lys[57]*	10.9	1.60	11(Q)		−60[j]	$H^{\beta2}$	9.4	1.59	10		−60	$H^{\beta2}$
		1.97	abs	xpk?		$H^{\beta3}$		1.93	4	xpk		$H^{\beta3}$

TABLE 2
(continued)

Residue	Reduced						Oxidized					
	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo
Leu[58]*	10.4	0.93	small(N)		180	$H^{\beta2}$	7.7	0.90	small(N)		180	$H^{\beta2}$
		1.33	large(N)			$H^{\beta3}$		1.33	large(N)			$H^{\beta3}$
Asn[59]	7.5	2.21	5		180	$H^{\beta2}$	6.6	2.20	3		180	$H^{\beta2}$
		2.65	12			$H^{\beta3}$		2.68	10			$H^{\beta3}$
Ile[60]*	5.1	2.19	O/L	xpk	+60		2.3	2.22	O/L	xpk	+60	
Asp[61]	8.0	2.70	O/L	xpk	−60	$H^{\beta3}$	8.1	2.69	4	xpk	−60	$H^{\beta3}$
		2.87	11(Q)			$H^{\beta2}$		2.89	9			$H^{\beta2}$
Gln[62]	7.8	1.77	11		−60	$H^{\beta2}$	7.4	1.75	11(Q)		−60	$H^{\beta2}$
		2.20	4	xpk		$H^{\beta3}$		2.24	abs	xpk		$H^{\beta3}$
Asn[63]	10.8	2.72	abs	xpk	−60	$H^{\beta3}$	9.7	2.72	abs	xpk	−60	$H^{\beta3}$
		2.78	12(Q)			$H^{\beta2}$		2.80	12(Q)			$H^{\beta2}$
Thr[66]	3.9	3.75	O/L				6.5	3.75	O/L			
Ala[67]	3.3						<3.5					
Lys[69]	6.5	1.50	3	xpk	−60	$H^{\beta3}$	5.7	1.50	3	xpk	−60	$H^{\beta3}$
		1.82	11			$H^{\beta2}$		1.83	10			$H^{\beta2}$
Tyr[70]	10.5	2.15	12(Q)		−60	$H^{\beta2}$	9.8	2.17	11		−60	$H^{\beta2}$
		3.39	O/L	xpk		$H^{\beta3}$		3.41	4	xpk		$H^{\beta3}$
Ile[72]	5.8	1.55	11		−60		5.6	1.53	10		−60	
Arg[73]	9.8	1.65	9		−60	$H^{\beta2}$	10.2	1.64	10		−60	$H^{\beta2}$
		1.90	5	xpk		$H^{\beta3}$		1.90	3	xpk		$H^{\beta3}$
Ile[75]	9.3	1.77	abs	?	amb		9.1	1.92	abs	xpk	+60	
Thr[77]	11.6	3.90	9(Q)		−60		8.2	3.86	9		−60	
Leu[78]*	10.4	1.21	5	xpk	−60	$H^{\beta3}$	9.6	1.21	5	xpk	−60	$H^{\beta3}$
		1.73	14			$H^{\beta2}$		1.70	11			$H^{\beta2}$
Leu[79]*	10.3	1.30	abs	xpk	−60	$H^{\beta3}$	9.6	1.37	4	xpk	−60	$H^{\beta3}$
		1.87	12(Q)			$H^{\beta2}$		1.86	12			$H^{\beta2}$
Leu[80]*	9.9	1.03	abs		180	$H^{\beta2}$	8.7	1.04	5		180	$H^{\beta2}$
		1.96	12(Q)			$H^{\beta3}$		1.95	13			$H^{\beta3}$
Phe[81]*	9.7	2.87	abs	xpk	−60	$H^{\beta3}$	9.1	2.85	5	xpk	−60	$H^{\beta3}$
		2.95	12(Q)			$H^{\beta2}$		2.95	10			$H^{\beta2}$
Lys[82]*	8.9	1.61	10(Q)		−60	$H^{\beta2}$	7.2	1.64	9		−60	$H^{\beta2}$
		1.80	3(Q)	xpk		$H^{\beta3}$		1.80	5	xpk		$H^{\beta3}$
Asn[83]	6.9	2.85	11(Q)		−60	$H^{\beta2}$	6.8	2.85	9		−60	$H^{\beta2}$
		3.11	5(Q)	xpk		$H^{\beta3}$		3.11	5	xpk		$H^{\beta3}$
Glu[85]	10.4	1.95	9		−60	$H^{\beta2}$	9.3	1.95	9		−60	$H^{\beta2}$
		2.11	5	xpk		$H^{\beta3}$		2.09	5	xpk?		$H^{\beta3}$
Val[86]	5.7	1.87	11(Q)		180		5.2	1.87	10		180	
		0.56				$Me2^i$		0.56				$Me2^i$
		0.60				$Me1^i$		0.60				$Me1^i$
Ala[87]	9.7						8.8					
Ala[88]	5.9						5.7					
Thr[89]	9.7	3.82	9(Q)		−60		9.6	3.81	8	?	−60	
Lys[90]	9.0	0.68	6		180	$H^{\beta2}$	8.2	0.70	6		180	$H^{\beta2}$
		1.20	9			$H^{\beta3}$		1.23	9			$H^{\beta3}$

TABLE 2
(continued)

Residue	Reduced						Oxidized					
	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo	$^3J_{HN\alpha}$ (Hz)	$\delta(C^\beta H)$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	Nβ (xpk)[a]	χ^1	Stereo
Val[91]	10.4	1.91	10(Q)		180		9.5	1.93	10		180	
		0.82				Me1[i]		0.86				Me1[i]
		0.90				Me2[i]		0.91				Me2[i]
Ala[93]	6.2						5.8					
Leu[94]	7.8	1.55[c]					7.6	1.58[c]				
Ser[95]	O/L	3.91	abs		+60	$H^{\beta 3}$	O/L	3.93	abs		+60	$H^{\beta 3}$
		4.31	small(N)	xpk		$H^{\beta 2}$		4.33	abs	xpk		$H^{\beta 2}$
Lys[96]	<1	1.86	small(N)		180	$H^{\beta 2}$	~3 (O/L)	1.87	small(N)		180	$H^{\beta 2}$
		1.95	large(N)			$H^{\beta 3}$		1.95	large(N)			$H^{\beta 3}$
Gln[98]	5.5	1.93	4	xpk	−60	$H^{\beta 3}$	5.1	1.94	5	xpk	−60	$H^{\beta 3}$
		2.27	11			$H^{\beta 2}$		2.29	11			$H^{\beta 2}$
Leu[99]	5.9	1.45	abs		180	$H^{\beta 2}$	4.4	1.50	4		180	$H^{\beta 2}$
		1.90	12(Q)			$H^{\beta 3}$		1.90	12			$H^{\beta 3}$
Lys[100]*	3.8	1.83	9		−60	$H^{\beta 2}$	3.6	1.82	11		−60	$H^{\beta 2}$
		1.99	6	xpk		$H^{\beta 3}$		2.00	5	xpk		$H^{\beta 3}$
Glu[101]*	2.3	2.10[c]					2.8	2.10[c]				
Phe[102]	3.9	3.27	O/L		180	$H^{\beta 2}$	3.8	3.27	abs		180	$H^{\beta 2}$
		3.33	12(Q)			$H^{\beta 3}$		3.33	12(Q)			$H^{\beta 3}$
Leu[103]*	3.8	0.92	5	xpk	−60	$H^{\beta 3}$	4.4	0.94	3	xpk	−60	$H^{\beta 3}$
		1.83	12			$H^{\beta 2}$		1.83	12			$H^{\beta 2}$
Asp[104]*	2.4	2.66	3	xpk	−60	$H^{\beta 3}$	1.9	2.66	4	xpk	−60	$H^{\beta 3}$
		2.70	11			$H^{\beta 2}$		2.73	10			$H^{\beta 2}$
Ala[105]	5.2						5.0					
Asn[106]	9.9	1.88	11		−60	$H^{\beta 2}$	9.6	1.88	10(Q)		−60	$H^{\beta 2}$
		2.23	2	xpk		$H^{\beta 3}$		2.23	abs	xpk		$H^{\beta 3}$
Leu[107]	7.7	1.65	abs	xpk	−60	$H^{\beta 3}$	7.9	1.61	5	xpk	−60	$H^{\beta 3}$
		1.73	11(Q)			$H^{\beta 2}$		1.73	10			$H^{\beta 2}$
Ala[108]	6.8						6.6					

[a] xpk: Cross peak present in the ^{15}N-edited spectrum (Chary et al., 1991).
[b] amb: Stereospecific assignment ambiguous due to resonance overlaps.
[c] $C^\beta H$ chemical shifts could not be distinguished for these residues.
[d] av: Coupling constants were contradictory, indicating more than one rotamer present.
[e] (Q): Value estimated from the 2QF-COSY spectrum; abs: data unobtainable from the E.COSY spectrum and cross peak absent from the 2QF-COSY and ^{15}N HSQC-TOCSY spectra.
[f] O/L: Data unobtainable due to cross-peak overlap.
[g] (N): Relative size of couplings estimated from ^{15}N HSQC-TOCSY spectrum.
[h] * Denotes residues with slowly exchanging amide protons (Dyson et al., 1989). The additional couplings in general cause the E.COSY cross peaks to be weakened and the $^3J_{\alpha\beta}$ coupling constants measured from the E.COSY and 2QF-COSY spectra appear artificially large.
[i] Stereospecific assignments made on the basis of intraresidue NOEs.
[j] For Lys[52], the NOEs were badly overlapped, so that stereospecific assignments remained ambiguous. For Lys[57], the NOEs were well resolved, allowing stereo assignments to be made.

those of the C^{β} carbons, but in the opposite sense. This phenomenon has been mentioned previously (Spera and Bax, 1991; Wishart et al., 1991) but this is the first time that the effect has been quantitated in this way. In many cases, the absolute numerical values of the shift differences are strikingly similar.

Proline conformation in thioredoxin

Thioredoxin contains five proline residues with distinctive characteristics, three of which are highly conserved. Pro^{34} forms part of the active-site loop, Pro^{40} makes a kink in the α-helix following the active site and Pro^{76} is a *cis*-proline that maintains the configuration of an important loop in contact with the active site. Only Pro^{64} shows ^{13}C chemical shifts typical of a 'random' proline (Howarth and Lilley, 1978). The C^{α} resonances of Pro^{40} and Pro^{68} are shifted downfield, and the C^{β} upfield, consistent with their presence in a helix. The C^{β} shift of the active-site Pro^{34} is closer to that typical of a helix in oxidized thioredoxin (Table 1); for the reduced protein, the C^{β} shift is close to the 'random' value.

Proline ^{13}C chemical shifts give information not only on the secondary structure at these sites, but also on the configuration of the X-Pro peptide bond, whether cis or trans (Wüthrich et al., 1972; Dorman and Bovey, 1973). A number of studies have elucidated the behavior of the X-Pro peptide bond under various solution conditions, utilizing this difference to quantitate the relative concentrations of cis and trans forms and the kinetics of interconversion (Wüthrich and Grath-

TABLE 3
NOE CONNECTIVITIES IN REDUCED AND OXIDIZED THIOREDOXIN

Atoms		Reduced	Oxidized	Atoms		Reduced	Oxidized
Asp^{26} HB1	Leu^{58} HN		●	Gly^{33} HN	Cys^{35} HN		●
Asp^{26} HN	Leu^{58} HB2	●		Cys^{35} HA	Pro^{76} HG−	●	
Asp^{26} HA	Thr^{77} HN		●	Ile^{38} HG+	Thr^{77} HN		●
Trp^{28} HH	Glu^{30} HN	●		Ile^{38} MD	Thr^{77} HN		●
Trp^{28} HH	Glu^{30} QPB	●		Ile^{38} HA	Ala^{93} HA		●
Trp^{28} HE	Cys^{32} HN	●		Ile^{38} HG−	Ala^{93} HN		●
Trp^{28} HN	Lys^{57} HB2		●	Ile^{38} MG	Ala^{93} HA	●	
Trp^{28} HN	Leu^{58} HN	●		Leu^{58} MD+	Ile^{60} HN	●	
Trp^{28} HN	Ile^{75} HA	●		Asn^{59} HA	Asp^{61} HB1	●	
Trp^{28} HN	Ile^{75} MD		●	Arg^{73} HN	Val^{91} MG2		●
Trp^{28} HN	Ile^{75} MG		●	Gly^{74} HN	Ile^{75} HN		●
Ala^{29} HA	Trp^{31} HN	●		Ile^{75} HA	Thr^{77} HN	●	
Ala^{29} HN	Ile^{75} HA	●		Ile^{75} HB	Gly^{92} HN	●	
Glu^{30} HB1	Asn^{59} HND+		●	Pro^{76} HB+	Ala^{93} HN		●
Glu^{30} HB1	Asn^{59} HND−		●	Pro^{76} HD+	Ala^{93} HA		●
Trp^{31} HZ2	Ile^{60} MD	●		Thr^{77} HA	Lys^{90} HA	●	
Trp^{31} HE	Ile^{75} MD	●		Thr^{77} MG	Lys^{90} HN	●	
Trp^{31} HH	Ile^{75} MD	●		Thr^{77} HA	Val^{91} MG1		●
Trp^{31} HZ2	Ile^{75} HG+	●		Thr^{77} HA	Val^{91} MG2		●
Trp^{31} HZ2	Ile^{75} MD	●		Thr^{77} MG	Val^{91} HA		●
Cys^{32} HB1	Ile^{75} MD	●		Thr^{77} HA	Ala^{93} HN		●
Gly^{33} HA+	Lys^{36} HN		●				

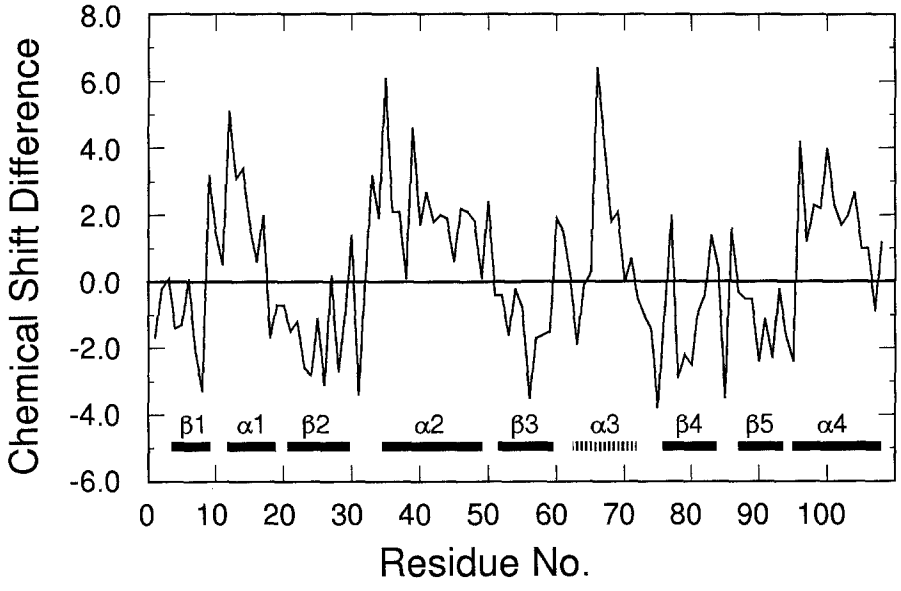

Fig. 4. Plot of the difference in $^{13}C^{\alpha}$ chemical shift between $^{13}C^{\alpha}$ resonances in reduced thioredoxin and random-coil values (Howarth and Lilley, 1978). (Values for random coil have been subtracted from the thioredoxin values, so that a downfield shift from the random-coil value appears as a positive difference.) Locations in the sequence of elements of secondary structure (Dyson et al., 1990) are indicated.

wohl, 1974; Grathwohl and Wüthrich, 1976a,b, 1981). While the 'random coil' chemical shift for proline C^{α} (61.7 ppm for trans and 61.2 ppm for cis) does not distinguish between the two forms, the C^{β} (30.4 ppm for trans and 33.0 ppm for cis) and C^{γ} (25.6 ppm for trans and 23.0 ppm for cis) shifts are significantly different (Howarth and Lilley, 1978). In both oxidized and reduced thioredoxin the Pro^{76} ^{13}C chemical shifts clearly show that it is cis (Table 1), with values for each ^{13}C in both forms of the protein consistent with the cis random-coil shifts. No evidence is seen for even small populations of trans isomers in this residue, as has been suggested on the basis of denaturation studies (Langsetmo et al., 1989). These observations provide another example, together with those seen for Staphylococcal nuclease (Torchia et al., 1989) and TGF-β1 (Archer et al., 1993), of the utility of the ^{13}C chemical shifts as a simple means of identifying *cis*-proline residues in proteins, in conjunction with more structural parameters such as the $d_{\alpha\alpha}$(X,Pro) NOE.

Fixed and averaged side-chain conformations

It is noticeable from Table 2 that a rather large proportion of the side chains of thioredoxin are present in both oxidation states as a single predominant rotamer. However, a number of residues show evidence of averaging of the rotamers at χ^1, as can be concluded from the presence of cross peaks characteristic of the larger αβ coupling constants at the positions of both of the $C^{\beta}H$ resonances in the 2D ^{15}N-edited TOCSY experiment (Fig. 2), and the presence of cross peaks at both $C^{\beta}H$ positions in the ^{15}N-edited experiment (Fig. 3); measured $^3J_{\alpha\beta}$ coupling constants reflect the averaging with intermediate values (~6–8 Hz). For these residues, the intraresidue NOEs are generally of very similar size, a further indication that averaging is occurring. The residues exhibiting rotational averaging include Ile^5, His^6, Asp^{13}, Asp^{20}, Ile^{45} and Glu^{48}. In addition,

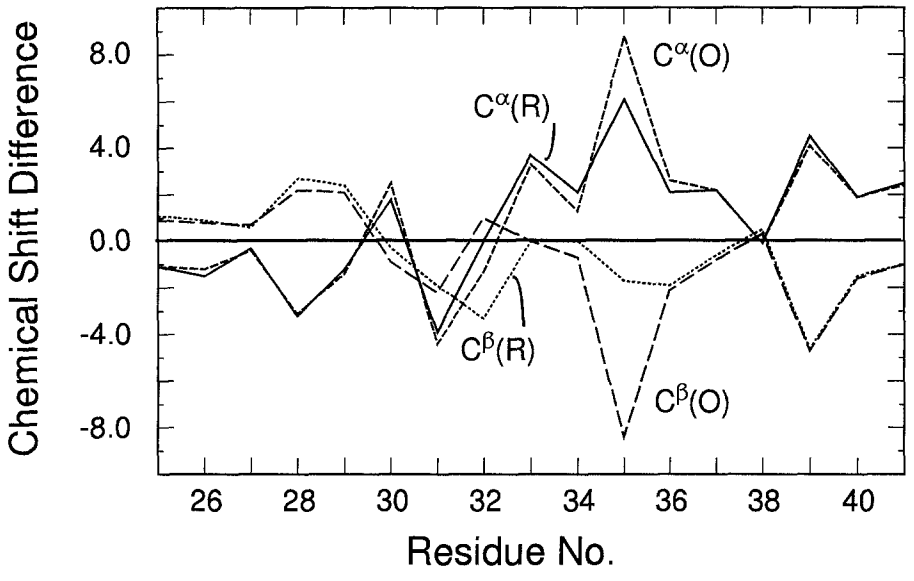

Fig. 5. C^{α} and C^{β} chemical-shift differences between resonances in oxidized and reduced thioredoxin and random-coil values (Howarth and Lilley, 1978) in the active-site region of the sequence. Solid line: C^{α} (reduced); dotted line: C^{β} (reduced); dashed line: C^{α} (oxidized); long-dashed line: C^{β} (oxidized). Since the NMR experiments were conducted at pH 5.7, the carboxyl of Asp^{26} in both forms of the protein, and of the two cysteine thiols in reduced thioredoxin are assumed to be fully protonated (Dyson et al., 1991; Langsetmo et al., 1991).

several residues, including His^{6}, Glu^{30}, Met^{37}, Asp^{47} and Leu^{94}, have coincident $C^{\beta}H$ resonances, which may also be indicative of averaging at χ^{1}.

Many of the averaged residues are hydrophilic, and are on the surface of the thioredoxin molecule. Only very small differences in the extent of averaging are seen between oxidized and reduced thioredoxin; most of those seen in Table 2 result rather from ambiguities arising in one of the spectra, but not in the other. On the other hand, a greater general mobility of the polypeptide chain in reduced thioredoxin would be consistent with a number of other experimental observations. Oxidized thioredoxin has a greater thermal stability, and the extent of the difference is rather larger than can be explained by the formation of a single disulfide bond between cysteines separated by only two residues (Hiraoki et al., 1988). The ^{15}N relaxation measurements show somewhat more flexibility in the reduced protein, though it appears to be localized around the active site (Stone et al., 1993). The rates of amide proton exchange also reflect greater overall flexibility in reduced thioredoxin (Dyson et al., 1989; M.-F. Jeng and H.J. Dyson, manuscript in preparation).

Differences in backbone and side-chain conformation

The majority of the $^{3}J_{HN\alpha}$ coupling constants agree between the two forms of thioredoxin to within 1 Hz. Significant differences between the two are observed for Thr^{77} (3.4 Hz), Ile^{60} (2.8 Hz), Leu^{58} (2.7 Hz), Thr^{66} (2.6 Hz) and Lys^{52} (2.3 Hz), most of which are in the vicinity of the active site. The relative sizes of these coupling constants (Table 2) would appear to indicate that the β-strands of which Thr^{77}, Leu^{58} and Leu^{52} form terminal residues are extended in the reduced form, compared to the oxidized form. In these cases it will be of interest to observe the differences

to be seen in the high-resolution structures, since the conformations calculated will be less dependent on the dihedral angle constraints obtained from these coupling constants than on the many NOE distance constraints. Differences of about 1 Hz occur for the $^3J_{HN\alpha}$ values of Cys^{35} and Met^{37}, in a direction that suggests that the region is more helical in oxidized thioredoxin. Alternatively, the higher values for reduced thioredoxin could indicate that the region is more flexible and samples conformations other than helix.

Interestingly, there is no difference between the two forms of the protein in the preferred χ^1 angle. It is unfortunate that a number of the active-site residues, including Ala^{29}, Gly^{33} (no χ^1), Glu^{30}, Lys^{36}, Met^{37} (overlapped) and Pro^{34}, are inaccessible to such measurements, so that even an estimate of the relative flexibility of the active site, reflected in averaging of χ^1, is impossible by these means. Other, better determined parts of the molecule reflect a high degree of similarity in the preferred rotameric forms, consistent with the similarity of the backbone conformation and the near-identity of the chemical shifts between oxidized and reduced thioredoxin.

Effect of a 1 → 4 disulfide bond on the NMR spectrum of thioredoxin

Plainly, the presence of a disulfide or dithiol bond in the active site of thioredoxin has little effect on the structure of the molecule. This has already been seen in the comparison of the X-ray crystal structure of the oxidized protein (Katti et al., 1990) and the NMR structure of the reduced protein (Dyson et al., 1990). Local perturbations close to the active site apparently occur, since the ^1H, ^{15}N and ^{13}C chemical shifts show significant differences between the two forms in this region. Possible sources for these differences include ring current effects from the two tryptophan rings, which would affect the ^1H spectrum most, changes in hydrogen bonding, which would affect the ^{15}N spectrum and the amide protons most, and changes in the 3D structure of the polypeptide backbone, which would affect the ^{13}C spectrum most.

Some effect is seen on the ^{13}C chemical shifts of the cysteine residues in the two proteins, independent of the effects of the disulfide bond itself on the random-coil shifts (Fig. 5). The C^β shifts of Cys^{32} and Cys^{35}, corrected for the random-coil shifts, differ significantly between the two proteins, and the C^α shifts of Cys^{35} to a lesser extent. Structural interpretation of such quantities, corrected using data derived from other systems that may contain cysteines in very different electronic states, should be approached with caution. We offer the following interpretation in this spirit. If the changes in C^α shift can be directly correlated with the secondary structure in this case, then the oxidized form of the protein should have a significantly greater helical character at Cys^{35}; this is consistent with the $^3J_{HN\alpha}$ coupling constants (5.7 Hz for reduced and 4.6 Hz for oxidized thioredoxin) (Table 2). As mentioned above, the C^β shifts of Pro^{34} also indicate a stronger helical character in oxidized thioredoxin. In principle, the NOE connectivities should also reflect a change in helicity, but such a comparison is impossible at the level of the NOE data, because of ambiguities in the spectrum of reduced thioredoxin, arising from the overlap of both the $C^\alpha H$ and NH of Cys^{35} and Lys^{36}. The C^α chemical-shift values for Cys^{32} are less obviously correlated with secondary structure — for both oxidized and reduced thioredoxin the values, corrected for random-coil shifts, are close to 0 ppm (Fig. 5).

The other ^{13}C shifts in the active-site region show very little change upon changing the oxidation state, except for the methylene C^γ and C^δ of Ile^{75}. Table 3 shows that a number of the NOE connectivities that differ between oxidized and reduced thioredoxin involve Ile^{75}, particularly in its relationship to Trp^{28}, and the ^1H chemical shifts for Ile^{75} also differ between the two forms

(Dyson et al., 1989). In addition, Ile^{75} shows increased mobility in polypeptide backbone dynamics in reduced thioredoxin (Stone et al., 1993). The differences observed for Ile^{75} may reflect functional differences, since this residue is highly conserved among bacterial thioredoxins, but is substituted by methionine in the thioredoxins of animals (Eklund et al., 1991); the *cis*-Pro^{76} adjacent to Ile^{75} is universally conserved. The differences in the ^{13}C spectrum between oxidized and reduced thioredoxin do appear to reflect structural differences in the active-site region — the general similarity of the ^{13}C chemical shifts gives a measure of their subtle nature.

The proton NMR spectrum is much more sensitive to ring current effects and, upon changing the oxidation state, large changes are seen in ^1H chemical shifts in the vicinity of the active site (Dyson et al., 1988,1989). While amide protons might be affected both by ring current effects and by changes in hydrogen bonding, the $C^{\alpha}H$ and the aliphatic side-chain protons should reflect only local changes in ring currents or the proximity of carbonyl groups (Ösapay and Case, 1991). Major differences (> 0.1 ppm) in $C^{\alpha}H$ chemical shifts occur only for Trp^{28}, Ala^{29}, Cys^{32} and Cys^{35}. While the latter two values could conceivably be affected by the chemical difference in the formation of the disulfide bond as well as, perhaps, proximity to the Trp^{28} and Trp^{31} rings, the values for Trp^{28} and Ala^{29} clearly reflect changes in local electronic structure, including ring currents. For side-chain protons, the $C^{\beta}H$ of Trp^{28}, Ala^{29}, Cys^{32}, Pro^{34}, Cys^{35} and Ile^{75}, and the $C^{\delta}H$ of Pro^{34} differ significantly between the two forms of thioredoxin, once again probably due to the ring current effects of the two tryptophan residues or to changes in interactions of neighboring carbonyl groups.

In spite of the general evidence presented above, the involvement of the tryptophan rings may not be the major contributing factor in the spectroscopic (and reactivity) differences between oxidized and reduced thioredoxin. Significant differences are observed between the chemical shifts in the two oxidation states of a closely related protein, glutaredoxin from *E. coli* (Sodano et al., 1991a,b). The active-site sequence is Cys^{11}-Pro-Tyr-Cys^{14}, and the only tryptophan residue is distant from the active site (Sodano et al., 1991b; Xia et al., 1992). The structures of the oxidized and reduced forms of the protein are quite similar (Xia et al., 1992), but the aliphatic proton chemical shifts, particularly in the region preceding the active-site sequence and the active site itself, differ by as much as 0.7 ppm (for the Cys^{11} $C^{\beta}H$, which corresponds to Cys^{32} in thioredoxin). The ^1H chemical-shift differences observed for thioredoxin are a little larger (up to 1.2 ppm for the Cys^{32} $C^{\beta}H$). The two proteins show a striking similarity in the patterns of the largest chemical-shift differences: as well as the cysteine $C^{\beta}H$ noted above, the NH resonances of Ala^{29} of thioredoxin and the corresponding residue Arg^{8} of glutaredoxin have almost identical large differences between the oxidized and reduced forms (~0.6 ppm), possibly due in each case to a change in hydrogen bonding. These similarities serve to underline the similarity of structure and function in these two proteins, and call into question the role of the tryptophan rings in determining the relative chemical shifts in thioredoxin. The data presented in this paper suggest that they may be involved, but a summation of the available data, obtained in the calculation of structures from the data, will be necessary to explore fully the implications of these findings. Such calculations are in progress.

CONCLUSIONS

The structural differences between oxidized and reduced thioredoxin remain extremely subtle,

consistent with its major physiological function as a dithiol-disulfide oxidoreductase. The rate of reaction in thiol-disulfide exchange are rapid, which implies that only small or negligible conformational changes occur when oxidized thioredoxin is reduced, or when reduced thioredoxin is oxidized. The source of the selectivity for the reduced form of the protein in the T7 and filamentous phage systems does not appear from this work to arise primarily from structural changes. As we have suggested previously (Stone et al., 1993), it appears to be a consequence of the increased accessibility in the reduced form of conformations other than the ground or native state.

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