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Assignment of the ^{15}N NMR spectra of reduced and oxidized *Escherichia coli* thioredoxin

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As a necessary first step in the use of heteronuclear correlated spectra to obtain high resolution solution structures of the protein, assignment of the ^{15}N NMR spectra of reduced and oxidized *Escherichia coli* thioredoxin (M_r 12 000) uniformly labeled with ^{15}N has been performed. The ^{15}N chemical shifts of backbone amide nitrogen atoms have been determined for both oxidation states of thioredoxin using ^{15}N-^1H correlated and two-dimensional heteronuclear single-quantum coherence (HSQC) TOCSY and NOESY spectra. The backbone assignments are complete, except for the proline imide nitrogen resonances and include Gly^{33}, whose amide proton resonance is difficult to observe in homonuclear ^1H spectra. The differences in the ^{15}N chemical shift between oxidized and reduced thioredoxin, which occur mainly in the vicinity of the two active site cysteines, including residues distant in the amino acid sequence which form a hydrophobic surface close to the active site, are consistent with the differences observed for proton chemical shifts in earlier work on thioredoxin.

Thioredoxin; Heteronuclear NMR; Redox protein; Protein conformation

1. INTRODUCTION

Thioredoxin is a 12 kDa protein (108 amino acid residues) [1]. The reduced form of thioredoxin is a hydrogen donor for ribonucleotide reductase and other reductive enzymes [2,3] and also an effective protein disulfide reductase. The active site of *Escherichia coli* thioredoxin consists of -Trp^{31}-Cys^{32}-Gly^{33}-Pro^{34}-Cys^{35}-, a sequence which is conserved in all prokaryotic and eukaryotic thioredoxins [4]. Oxidized thioredoxin contains a disulfide bond between Cys^{32} and Cys^{35}; the reduced form contains a dithiol.

The crystal structure of oxidized *E. coli* thioredoxin has been available for some years [5] and has recently been refined to a resolution of 1.7 Å [6]. No crystals of reduced thioredoxin have been obtained [2]. The solution structure of reduced thioredoxin has recently been obtained by the use of two-dimensional proton NMR methods [7,8]. The differences observed between the ^1H NMR spectra of the two forms [7,9] are concentrated in the region of the active site, including the active-site sequence itself and residues from other parts of the sequence which are in close proximity to the active site and constitute a hydrophobic surface thought to be important in binding of thioredoxin to substrate proteins [10].

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Heteronuclear correlation methods viz. HSQC, HSQC-TOCSY and HSQC-NOESY [11,12] help in resolving ambiguities due to overlap of proton resonances by utilizing the favorable dispersion of the ^{15}N chemical shifts. This allows maximum use to be made of distance information derived from NOE connectivities: additional distance constraints can be used in structure calculations resulting in higher resolution structures.

In this communication, we report complete assignments for the ^{15}N spectrum of *E. coli* thioredoxin in both oxidation states, obtained by using heteronuclear-correlated methods. LeMaster and Richards [13,14] have utilized selective isotope labelling methods to obtain some ^{15}N assignments for oxidized *E. coli* thioredoxin by residue type. In contrast, our approach involves uniform labelling and the reported assignments for both oxidized and reduced thioredoxin are sequence-specific and complete except for proline. ^{15}N assignments have also been reported for human thioredoxin [15].

2. MATERIALS AND METHODS

2.1. *Preparation of uniformly labeled thioredoxin*

To obtain uniformly labelled recombinant thioredoxin, the *trxA* plasmid pBHK8 [8] was isolated from strain SK3981 and used to transform a prototrophic *Escherichia coli* C strain (C1a) selecting for ampicillin resistance. The *E. coli* C1a strain, which grows efficiently in minimal medium, was a gift of Dr Elisabeth Hagård-Ljungqvist, Department of Microbiological Genetics, Karolinska Institute. *E. coli* C1a/pBHK8 were grown on a preparative scale in an automatic

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fermentor (Microferm) at 37°C in 10 liter of medium containing 105 g K_2HPO_4, 45 g KH_2PO_4, 10 g $(^{15}NH_4)_2SO_4$ (99%; Isotech, OH, USA), 80 g glucose, 5 g sodium citrate, 5 g sodium pyruvate, 3 g $MgSO_4 \cdot 7H_2O$, 5 mg $FeCl_3$, 110 mg $CaCl_2$, 100 mg thiamine, 100 mg biotin and 500 mg of ampicillin. During growth the pH was kept constant at 7.0 by automatic titration with 10 M NaOH. After 20 h at 37°C, the cells were harvested with a Sharples centrifuge. The packed cells (63 g) were stored at −70°C before disruption of cell walls with an X-press at −20°C. The purification of thioredoxin followed previously-published procedures [8]. The protein obtained after the Sephadex G-50 step was sufficiently pure, as indicated by isoelectric focusing gels, that the final DE-32 column step could be omitted. A total of 75 mg of pure ^{15}N thioredoxin was obtained, a final yield of about 45%.

Samples for NMR spectroscopy were prepared by solvent exchange on a Sephadex G-25 column, equilibrated with phosphate buffer at pH 5.7 in 90% ^1H_2O/10% ^2H_2O. Protein concentration was typically 2–4 mM, measured by absorbance at 280 nm. Reduced thioredoxin was prepared as described previously [8] by the addition of a solution of DTT in the same buffer before passage through the Sephadex column.

2.2. *NMR Spectroscopy*

All NMR experiments were carried out at 380 K on a Bruker AM-600 spectrometer operating in the inverse detection mode. Standard pulse sequences and phase cycling schemes were used to acquire HSQC, HSQC-NOESY and HSQC-TOCSY spectra [11,12]. The resonance of the solvent water was suppressed by presaturation or by the application of spin lock purge pulses [16]. Spectra generally contained 512 t1 points with each free induction decay having 4096 complex points. Fourier transformation of the data was carried out on a SUN workstation utilizing software provided by Dr. Dennis Hare. Spectral widths were 12500 Hz in ω_2 and 4700 Hz in ω_1. Phase shifted sine bell window functions were used in both dimensions and,

Table I

^{15}N Chemical shifts for reduced thioredoxin, pH 5.7, 308 K

Residue	δ(ppm)	Residue	δ(ppm)	Residue	δ(ppm)
Ser 1		Met 37	117.2	Arg 73	128.1
Asp 2		Ile 38	110.4	Gly 74	108.5
Lys 3	119.8	Ala 39	125.3	Ile 75	114.3
Ile 4	120.7	Pro 40		Pro 76	
Ile 5	126.6	Ile 41	116.9	Thr 77	118.7
His 6	126.4	Leu 42	119.6	Leu 78	127.2
Leu 7	124.8	Asp 43	118.7	Leu 79	122.2
Thr 8	108.4	Glu 44	118.4	Leu 80	125.4
Asp 9	120.0	Ile 45	121.9	Phe 81	128.5
Asp 10	116.3	Ala 46	122.5	Lys 82	117.5
Ser 11	117.4	Asp 47	115.3	Asn 83	124.3
Phe 12	125.5	Glu 48	120.6	Gly 84	104.5
Asp 13	117.3	Tyr 49	115.5	Glu 85	118.8
Thr 14	112.5	Gln 50	121.5	Val 86	123.1
Asp 15	118.7	Gly 51	115.5	Ala 87	133.3
Val 16	113.4	Lys 52	118.2	Ala 88	117.4
Leu 17	116.4	Leu 53	119.5	Thr 89	115.4
Lys 18	115.4	Thr 54	123.5	Lys 90	126.3
Ala 19	122.6	Val 55	129.9	Val 91	125.5
Asp 20	121.2	Ala 56	129.4	Gly 92	113.7
Gly 21	108.7	Lys 57	118.4	Ala 93	118.5
Ala 22	123.6	Leu 58	123.6	Leu 94	119.8
Ile 23	123.8	Asn 59	126.8	Ser 95	120.7
Leu 24	130.7	Ile 60	122.9	Lys 96	121.7
Val 25	126.7	Asp 61	122.0	Gly 97	105.5
Asp 26	124.5	Gln 62	116.2	Gln 98	120.8
Phe 27	129.2	Asn 63	116.5	Leu 99	123.5
Trp 28	120.0	Pro 64		Lys 100	119.8
Ala 29	118.0	Gly 65	112.4	Glu 101	117.9
Glu 30	120.8	Thr 66	118.8	Phe 102	120.3
Trp 31	111.3	Ala 67	124.2	Leu 103	121.1
Cys 32	121.8	Pro 68		Asp 104	120.1
Gly 33	120.1	Lys 69	117.1	Ala 105	118.5
Pro 34		Tyr 70	114.7	Asn 106	113.3
Cys 35	113.5	Gly 71	108.1	Leu 107	121.1
Lys 36	121.1	Ile 72	119.2	Ala 108	128.8

Table II

^{15}N chemical shifts for oxidized thioredoxin, pH 5.7, 308 K

Residue	δ(ppm)	Residue	δ(ppm)	Residue	δ(ppm)
Ser 1		Met 37	117.7	Arg 73	128.2
Asp 2		Ile 38	109.8	Gly 74	108.4
Lys 3	119.8	Ala 39	125.4	Ile 75	113.3
Ile 4	120.8	Pro 40		Pro 76	
Ile 5	126.4	Ile 41	116.7	Thr 77	118.7
His 6	126.3	Leu 42	119.6	Leu 78	127.0
Leu 7	124.8	Asp 43	118.6	Leu 79	122.0
Thr 8	108.4	Glu 44	118.5	Leu 80	125.4
Asp 9	120.1	Ile 45	121.0	Phe 81	128.5
Asp 10	116.4	Ala 46	122.5	Lys 82	117.5
Ser 11	117.4	Asp 47	115.2	Asn 83	124.2
Phe 12	125.5	Glu 48	120.6	Gly 84	104.4
Asp 13	117.4	Tyr 49	115.5	Glu 85	118.7
Thr 14	112.5	Gln 50	121.5	Val 86	123.1
Asp 15	118.7	Gly 51	115.5	Ala 87	133.4
Val 16	113.3	Lys 52	118.2	Ala 88	117.4
Leu 17	116.5	Leu 53	119.5	Thr 89	115.4
Lys 18	115.3	Thr 54	123.5	Lys 90	126.2
Ala 19	122.5	Val 55	129.9	Val 91	125.6
Asp 20	121.1	Ala 56	129.4	Gly 92	113.3
Gly 21	108.7	Lys 57	118.4	Ala 93	118.3
Ala 22	123.6	Leu 58	123.5	Leu 94	119.4
Ile 23	123.8	Asn 59	126.7	Ser 95	120.7
Leu 24	130.6	Ile 60	122.4	Lys 96	121.6
Val 25	126.8	Asp 61	122.2	Gly 97	105.6
Asp 26	124.6	Gln 62	116.1	Gln 98	120.9
Phe 27	128.6	Asn 63	116.4	Leu 99	123.6
Trp 28	120.0	Pro 64		Lys 100	119.8
Ala 29	116.7	Gly 65	112.4	Glu 101	117.9
Glu 30	121.7	Thr 66	118.8	Phe 102	120.3
Trp 31	111.7	Ala 67	124.2	Leu 103	121.2
Cys 32	120.8	Pro 68		Asp 104	120.2
Gly 33	121.6	Lys 69	117.1	Ala 105	118.5
Pro 34		Tyr 70	114.7	Asn 106	113.2
Cys 35	111.1	Gly 71	108.1	Leu 107	121.0
Lys 36	121.7	Ile 72	119.3	Ala 108	128.9

wherever purge pulses had been used, a low pass filter was employed to suppress the H_2O resonance [17]. The final digital resolution was 6 Hz/pt in ω_2 and 2.3 Hz/pt in ω_1. Proton chemical shifts are referenced to internal dioxane (3.75 ppm from DSS) and ^{15}N chemical shifts are referenced by an indirect method using the ^1H frequency of the H_2O resonance, to the chemical shift of NH_3 at 0 ppm [18,19].

3. RESULTS

Chemical shift assignments of the ^{15}N resonances in the spectrum of *E. coli* thioredoxin in both reduced and oxidized forms are summarized in Tables I and II respectively. Assignments were obtained from the

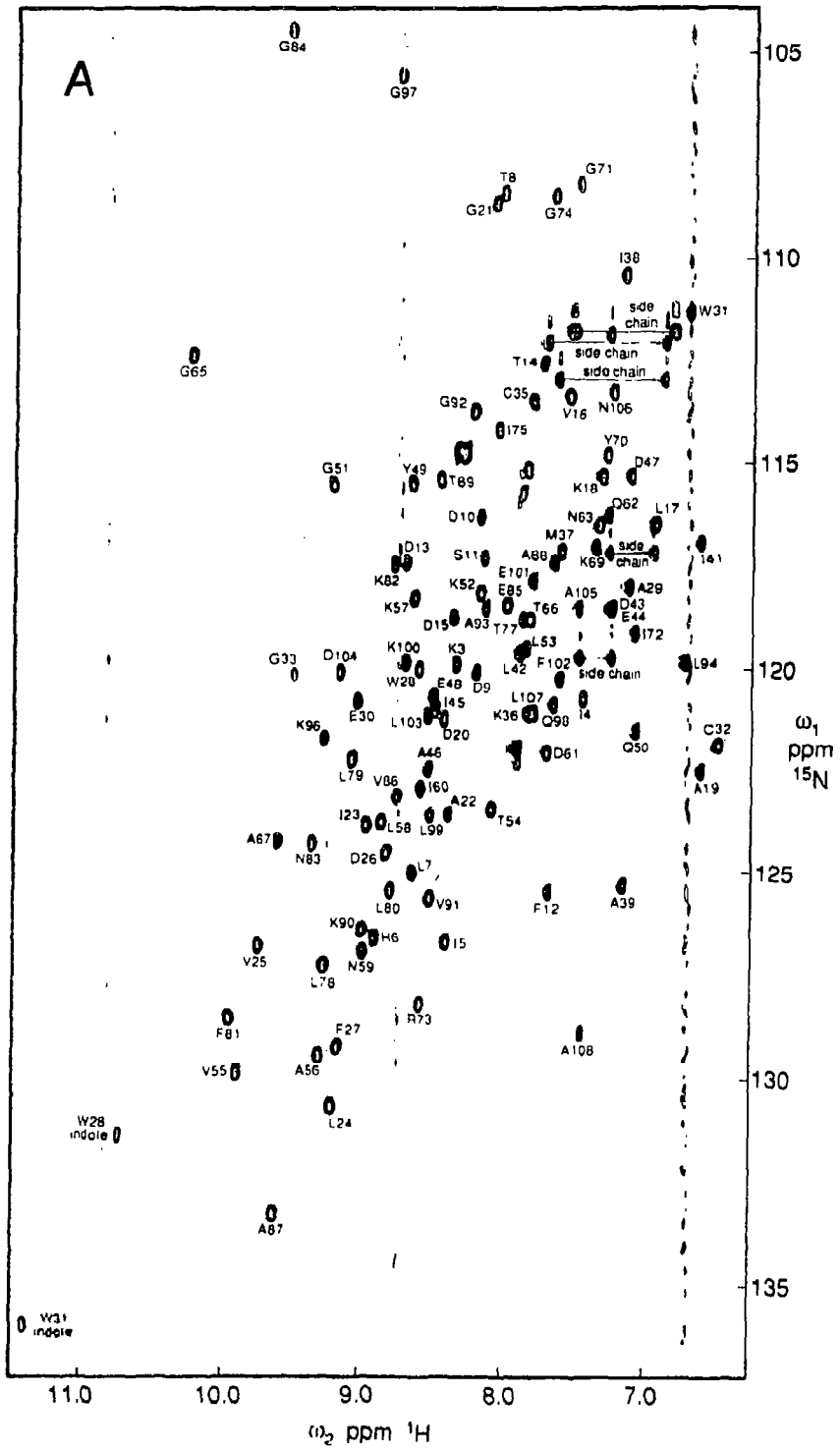

Fig. 1. Heteronuclear single-quantum coherence spectrum [11,12] obtained under identical conditions for (A) reduced (2 mM) and (B) oxidized (4 mM) thioredoxin. The water resonance was suppressed by the use of spin lock purge pulses [16].

^{15}N-^{1}H-correlated (HSQC) spectrum and confirmed using the HSQC-TOCSY and HSQC-NOESY spectra. Fig. 1 shows the HSQC spectra of reduced and oxidized thioredoxin. Using these methods, the nitrogen resonances are identified through their correlation to proton resonances. Assignments for the N-terminal serine residue and for all proline residues, which do not possess amide protons, and for Asp 2, whose amide proton is not observed in the NMR spectra, are not possible by these means.

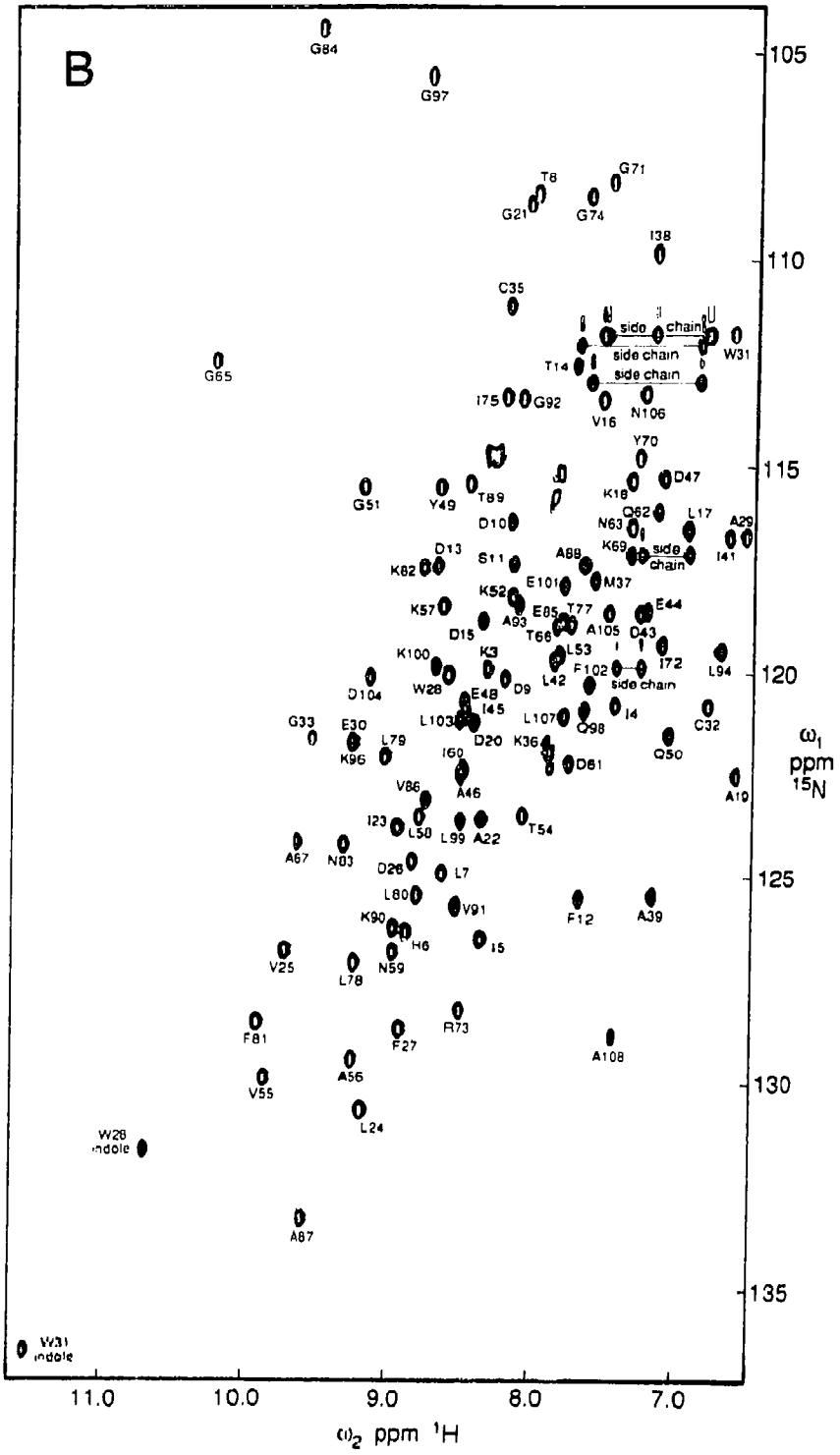

Fig. 1 (continued).

4. DISCUSSION

For the majority of the resonances of both oxidized and reduced *E. coli* thioredoxin, the assignment of the backbone ^{15}N resonances was straightforward using the HSQC spectrum, with confirmation from the HSQC-TOCSY and HSQC-NOESY spectra. The task was considerably simplified by the availability of complete proton assignments for both forms of the protein [8]. Our data for oxidized thioredoxin and the assignments by residue type reported by LeMaster and Richards [13] appear to be consistent, given differences in spectrum presentation and referencing between the two reports.

Difficulties were experienced with the assignments of several of the ^{15}N resonances. The cross peak assigned to Gly^{33} was not observed in spectra where the solvent water resonance was suppressed by presaturation, but a weak cross peak assigned by elimination to Gly^{33} was present when presaturation was not used. The amide proton of Gly^{33} is particularly difficult to observe: in proton spectra, the Gly^{33} amide proton could only be seen at 298K when no presaturation was applied to the solvent resonance [8]. Other residues for which difficulties were experienced in observation of ^{15}N-^{1}H connectivities include Phe^{12}, Ile^{75}, Thr^{8} and Ser^{95}. For Ser^{95}, no cross peak was observed in the ^{15}N-^{1}H HSQC spectrum for either form of the protein (Fig. 1), and no cross peaks were observed in the HSQC-TOCSY spectra corresponding to the Ser^{95} proton spin system. The Ser^{95} ^{15}N resonance was assigned from a weak cross peak observed in the HSQC-NOESY spectrum at the frequency to be expected for the Ser^{95} amide proton [8]. Confirmation of the assignment was provided by further weak cross peaks at the same ^{15}N frequency corresponding to the ^{1}H frequencies of the $C^{\beta}H$ resonances of Ser^{95}; the $C^{\alpha}H$ frequency (4.60 ppm) is masked by its close proximity to the water resonance. Cross peaks were observed in the HSQC spectra corresponding to the amide proton frequencies of Phe^{12}, Ile^{75} and Thr^{8}, but cross peaks at the $C^{\alpha}H$ and $C^{\beta}H$ frequencies were not visible in the HSQC-TOCSY spectrum. For Phe^{12}, the ^{15}N assignment was confirmed by the observation of strong cross peaks in the HSQC-NOESY spectrum at the ^{15}N frequency assigned to Phe^{12}, corresponding to the $C^{\alpha}H$ and both $C^{\beta}H$ resonances of Phe^{12}. For both Ile^{75} and Thr^{8}, the $C^{\alpha}H$ (and the $C^{\beta}H$ of Thr^{8}) lies under the H_2O resonance, making observation of scalar connectivities difficult in spectra where presaturation of the water resonance is used (as in the case of the HSQC-TOCSY). These ^{15}N assignments were confirmed by the observation of cross peaks in the HSQC-NOESY spectrum at the assigned ^{15}N frequencies corresponding to the proton frequencies of Thr^{8} $C^{\gamma}H_3$ and Leu^{7} $C^{\alpha}H$ (for Thr^{8} and Ile^{75} $C^{\gamma}H_3$ and both Gly^{74} $C^{\alpha}H$ (for Ile^{75}).

It is evident from Fig. 1 and the tables that most of the ^{15}N chemical shifts are unchanged in the two forms. There are significant changes in the active site region, consistent with our earlier observations of proton chemical shifts in the two forms [9]. The absolute differences in ^{15}N chemical shift between the two forms of thioredoxin are plotted in Fig. 2 as a function of residue position. As for the proton chemical shifts [9], significant differences are observed in the active site sequence, residues 26–37 and in the loops in the vicinity of residues 60, 75 and 92. The observed differences between oxidized and reduced thioredoxin do not occur for exactly the same residues in the proton and ^{15}N spectra; for example, only Ile^{75} appears to differ in ^{15}N chemical shift between oxidized and reduced thioredoxin, whereas the proton spectra show differences for residues 73, 74 and 77. In addition, small changes are observed in the helix immediately following the active site (residues 37, 38 and 41).

Differences in ^{15}N frequency for the same residues in the two forms of thioredoxin may be a significant indicator of the real nature of the differences between them. Little obvious structural change appears to accompany the conversion of thioredoxin from one oxidation state to the other. Subtle differences were observed in the active site region between the X-ray structure of oxidized thioredoxin [6] and the NMR structure of reduced thioredoxin [7], including a change in the χ_1 dihedral angle of Cys^{35} [7]. It is probable that these differences are due, at least in part, to differences between crystal and solution. In addition, the changes are sufficiently subtle as to be difficult to reconcile with the significant differences in reactivity of reduced and oxidized thioredoxin, for example, in the binding to the gene 5 protein of phage T7 DNA polymerase [20–22]. However, changes in ^{15}N frequency have been thought to indicate changes in local hydrogen-bonding interactions [18]. We report differences in the ^{15}N frequencies between the two forms of thioredoxin for residues which are not actual participants in the disulfide-dithiol reaction. At least some of these differences may reflect changes in hydrogen-bonding patterns, for example,

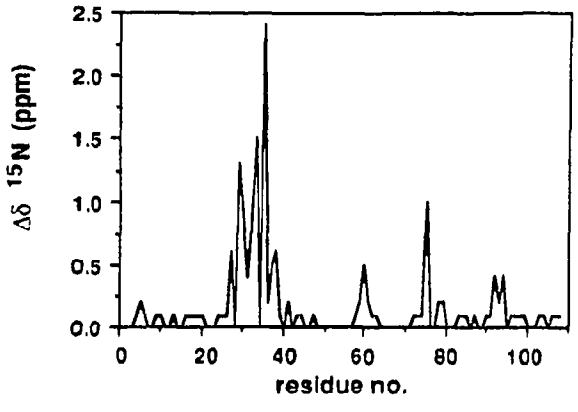

Fig. 2. Plot of the absolute value of the difference in ^{15}N chemical shift between reduced and oxidized thioredoxin as a function of residue number.

residues 37, 38 and 41, which are present in the helix immediately following the active site, could well undergo hydrogen-bonding changes which are not reflected in structural changes visible at the level of resolution so far obtained for thioredoxin. The studies reported herein are preliminary to a major effort to identify actual structural and other differences between reduced and oxidized thioredoxin. The ^{15}N assignments will be utilized in multi-dimensional NMR spectra to identify additional distance and dihedral angle constraints for use in the calculation of high resolution solution structures of thioredoxin. Such structures are needed for both oxidation states of thioredoxin before meaningful correlations can be made to the observed functional diversity between the two forms.

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