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^{1}H and ^{15}N resonance assignments and secondary structure of the human thioredoxin C62A, C69A, C73A mutant

Julie D. Forman-Kay[a,*], G. Marius Clore[a,**], Stephen J. Stahl[b] and Angela M. Gronenborn[a,**]

[a]*Laboratory of Chemical Physics, Building 2, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.*
[b]*Protein Expression Laboratory, Building 6B, Office of the Director, National Institutes of Health, Bethesda, MD 20892, U.S.A.*

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SUMMARY

The complete assignment of ^{1}H and ^{15}N backbone resonances and near-complete ^{1}H side-chain resonance assignments have been obtained for the reduced form of a mutant of human thioredoxin (105 residues) in which the three non-active site cysteines have been substituted by alanines: C62A, C69A, C73A. The assignments were made primarily on the basis of three-dimensional ^{15}N-separated nuclear Overhauser and Hartmann-Hahn spectroscopy, in conjunction with two-dimensional homonuclear and heteronuclear correlation experiments. Based on comparisons of short-range and interstrand nuclear Overhauser effects, patterns of amide exchange, and chemical-shift differences, the structure appears essentially unchanged from that of the previously determined solution structure of the native protein [Forman-Kay, J.D. et al. (1991) *Biochemistry*, **30**, 2685-2698]. An assay for thioredoxin shows that the C62A, C69A, C73A mutant retains activity. The assignment of the spectrum for this mutant of human thioredoxin constitutes the basis for future studies aimed at comparing the details of the active-site conformation in the reduced and oxidized forms of the protein.

INTRODUCTION

Thioredoxin is a ubiquitous protein which is involved in many aspects of thiol biochemistry in

*Present address: The Hospital for Sick Children, Biochemistry Research Division, Toronto, Ontario M5G 1X8, Canada.
**Authors to whom correspondence should be addressed.
Abbreviations: DTT, dithiothreitol; *E. coli*, *Escherichia coli*; PCR, polymerase chain reaction; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; PE-COSY, 2-dimensional primitive exclusive correlated spectroscopy; TPPI, time-proportional phase incrementation; 2D, 2-dimensional; 3D, 3-dimensional.

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the cell, acting as a general catalyst for dithiol–disulfide interchange, as well as a specific disulfide reductant for a number of proteins (Holmgren, 1989). The structures of a number of different thioredoxins have been determined, with the aim of shedding light on the catalytic mechanism of thioredoxin. In particular, the structures of oxidized *E. coli* (Holmgren et al., 1975; Katti et al., 1990) and T4 (Söderberg et al., 1978) thioredoxin have been determined by X-ray crystallography, and the solution structures of the reduced forms of *E. coli* (Dyson et al., 1990) and human (Forman-Kay et al., 1991) thioredoxin have been determined by NMR. At the present time, however, there is no high-resolution structure of an oxidized and reduced thioredoxin of the same species determined under the same conditions.

Based on the results of early biochemical and spectroscopic experiments on *E. coli* thioredoxin, a mechanism of thioredoxin action was proposed (Kallis and Holmgren, 1980). This involves nucleophilic attack by the Cys^{32} thiolate anion on a disulfide-containing substrate, producing a mixed-disulfide intermediate, which is then attacked by the thiolate of Cys^{35} to yield a reduced substrate and free, oxidized thioredoxin. No clear structural argument for the stabilization of the Cys^{32} thiolate anion, a crucial aspect of the proposed catalytic mechanism, has emerged from the studies of *E. coli* or T4 thioredoxin. The high-resolution NMR solution structure of the reduced form of human thioredoxin, however, reveals a hydrogen bond between the amide proton of Cys^{35} located at the N-terminus of helix α_2 and the Cys^{32} sulfur atom (Forman-Kay et al., 1991), yielding compelling evidence for the stabilization of the Cys^{32} thiolate anion at physiological pH values and accounting for the anomalously low pK_a value of 6.3 for Cys^{32} (Forman-Kay et al., 1992).

Clearly, a structural comparison of the oxidized and reduced forms of human thioredoxin would yield more insight into the interactions which control the catalytic thiol chemistry of thioredoxin. Unfortunately, human thioredoxin contains five cysteines, two in the active site (Cys^{32} and Cys^{35}) and three others, at positions 62, 69 and 73, which are only found in mammalian thioredoxins. Although Cys^{62} is totally buried, both Cys^{69} and Cys^{73} are exposed with surface accessibilities of 20 and 60%, respectively, relative to a Gly-Cys-Gly tripeptide. As a result it has not proven experimentally feasible to produce a sample of oxidized human thioredoxin without concomitant intermolecular disulfide bridge formation and consequent aggregation. To circumvent this problem we have constructed and expressed a mutant of human thioredoxin in which the three additional cysteines have been substituted by alanines. In this paper, we present the ^1H and ^{15}N resonance assignments and secondary-structure determination of the reduced form of the C62A, C69A, C73A mutant. We show that the structure of the mutant appears to be unchanged from that of the native protein and that the mutant retains enzymatic activity. Hence, this study shows that the C62A, C69A, C73A mutant can be used in future work aimed at a detailed structural comparison of the reduced and oxidized forms of the human enzyme.

MATERIALS AND METHODS

Materials

Calf thymus thioredoxin reductase was the kind gift of Dr. A. Holmgren of the Karolinska Institute in Stockholm. Bovine insulin (25 units/mg) and NADPH were products of Sigma, St. Louis, MO. All other reagents were analytical grade and obtained from commercial sources.

Sample preparation

The C62A, C69A, C73A mutant of human thioredoxin was constructed using mutagenic oligonucleotides in conjunction with PCR (Saiki et al., 1988) technology. All cloning procedures followed standard protocols (Maniatis et al., 1982). The mutant gene was expressed under the control of the λp_L promoter using the Mu ner ribosome binding site from a plasmid containing the tetracycline resistance gene and the cIts repressor gene (Allet et al., 1988). The coding sequence resides on a 320-bp *NcoI*/*XbaI* fragment and was verified by DNA sequencing (Sanger et al., 1977).

The ^{15}N-labeled mutant protein was produced by growing bacteria in minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source and purified essentially as described in Wollman et al. (1988). Amino acid analysis confirmed the mutant composition and N-terminal sequencing revealed two species of protein, differing in the presence or absence of the N-terminal methionine. The ratio of these two forms, N-Met and N-Val, was close to 1:1, which was in agreement with the relative peak intensities in the NMR spectra, with slightly more of the N-Val form.

For NMR samples, 0.5 ml of a 1.17-mM solution of ^{15}N-labeled protein was reduced in excess dithiothreitol (DTT), dialyzed against 100 mM sodium phosphate buffer with 0.2 mM deuterated DTT, pH 5.5, lyophilized and redissolved in argon-purged 90% H_2O/10% D_2O or 99.996% D_2O, sealed in tubes with airtight rubber septa, and blanketed with argon for 30 min (Forman-Kay et al., 1989). For pH titrations, the pH was adjusted by the addition of small amounts of concentrated HCl or NaOH. Measurements of the pH of the sample were not corrected for deuterium isotope effects and were taken before and immediately after the NMR experiment, with the latter measurement considered the most accurate. These values differed by less than 0.1 pH units in most cases. Although the C62A, C69A, C73A mutant can be easily oxidized by stirring in air at 10 μM concentration to yield a disulfide between the two active-site cysteines, the protein was fully reduced for all studies reported here, with both active-site cysteines existing as free sulfhydryls.

NMR spectroscopy

All experiments were recorded on a Bruker AM600 spectrometer at 25°C. Quadrature detection in the t_1 dimension was carried out using the TPPI method (Marion and Wüthrich, 1983) for 2D spectra and the States-TPPI method (Marion et al., 1989a) for 3D spectra.

NH and ^{15}N chemical shifts for pH titration curves were measured from 2D ^1H-^{15}N Overbodenhausen correlation experiments (Bodenhausen and Ruben, 1980; Bax et al., 1990) recorded in H_2O at multiple pH values. Figure 1 shows the ^1H-^{15}N correlation spectrum of the reduced form of the C62A, C69A, C73A mutant of thioredoxin at pH 5.5.

All other spectra were recorded on a sample with a pH value of 5.5. A HOHAHA (Braunschweiler and Ernst, 1983; Davis and Bax, 1985) spectrum, utilizing a WALTZ17 mixing sequence (Bax, 1989) sandwiched between 1.5-ms time pulses, was recorded in D_2O, with a mixing time of 38 ms. A PE-COSY (Mueller, 1987) experiment with a 35° mixing pulse was also recorded in D_2O, as described by Marion and Bax (1988). In addition, a 150-ms NOESY experiment (Jeener et al., 1979; Macura et al., 1981) was recorded in D_2O.

A 3D ^{15}N-separated HOHAHA experiment with a mixing time of 32 ms and a 3D ^{15}N-separated NOESY experiment with a mixing time of 150 ms were recorded as described by Marion et al. (1989b). These spectra were recorded with 128 complex points in F_1 (^1H), 32 complex points in F_2 (^{15}N) and 1024 real points in F_3 (^1H) with spectral widths of 11.41 ppm, 34.99 ppm and 13.89 ppm,

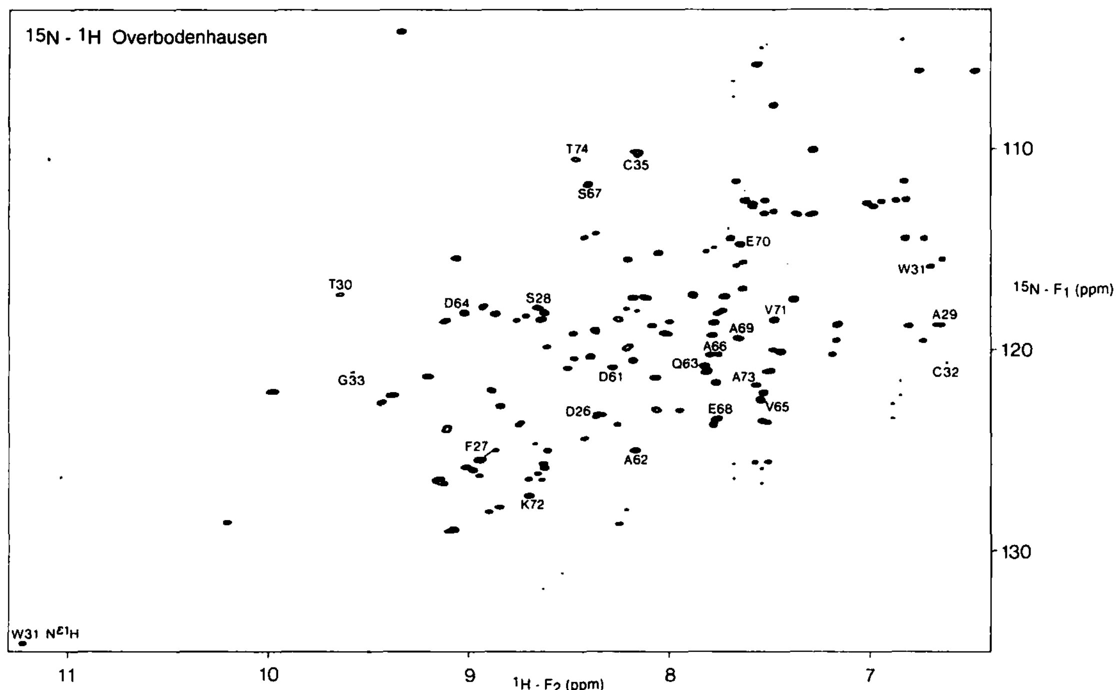

Fig. 1. ^{15}N (F_1 axis)-NH ^1H (F_2 axis) region of the ^{15}N-^1H Overbodenhausen correlation spectrum of the reduced form of the C62A, C69A, C73A mutant of human thioredoxin in H_2O at 25°C and pH 5.5. ^{15}N-^1H correlation peaks of the active-site region from Asp^{26} to Cys^{35} and of the region of the mutations from Asp^{61} to Thr^{74} have been labeled.

respectively. After zero-filling, the real portion of the final processed 3D data matrix comprised 256 × 64 × 1024 points.

3D spectra were processed on a Sun Sparc workstation using in-house routines for Fourier transformation (Kay et al., 1989), together with the commercially available software NMR2 (New Methods Research, Inc., Syracuse, NY). Analysis of the 3D spectra was aided by the use of the peak-picking and display programs CAPP and PIPP (Garrett et al., 1991).

pH titrations

Heteronuclear ^{15}N-^1H Overbodenhausen correlation experiments were recorded at six pH values: 5.5, 5.7, 6.0, 6.3, 6.6 and 7.1. ^1H chemical shifts were calibrated with respect to the internal ^1H_2O resonance and corrected for the small pH dependence (~0.02 ppm) of water, as described by Forman-Kay et al. (1992).

Thioredoxin assay

A modified spectrophotometric insulin reduction assay, similar to that described by Luthman and Holmgren (1982), was used to determine whether the C62A, C69A, C73A mutant retains redox activity. The assay mixture contained 50 mM potassium phosphate buffer (pH 7), 80 μM bovine insulin and 0.1 mM NADPH. The solution was protected from light and used immediately in the assay at room temperature. Thioredoxin (1–3 μM final concentration) and calf thymus thioredoxin reductase (1 μg) were added to 0.5 ml of the assay mixture in a cuvette and the reaction

rate was followed from the oxidation of NADPH at 340 nm. In a second assay (Holmgren, 1979), the catalytic activity of the different thioredoxins on DTT-dependent insulin reduction was investigated. In a total volume of 1 ml, the reaction mixture contained 100 mM potassium phosphate buffer, pH 7, 0.16 mM bovine insulin and thioredoxin at a concentration in the range of 0.1–0.7 μM. The reaction was initiated by the addition of 0.5 mM DTT and the absorbance at 650 nm was followed over 40 min.

Calculation of the pK_a of Cys^{35}

The active-site pH titration curves were analyzed as described in Forman-Kay et al. (1992) by a nonlinear least-squares fit using the program FACSIMILE (Chance et al., 1977; Clore, 1983) to the following relationship derived from the Henderson–Hasselbalch equation:

$$\delta = [\delta_{acid} + \delta_{base} \cdot 10^{(pH-pK_a)}]/[1 + 10^{(pH-pK_a)}]$$

where δ is the chemical shift of a resonance as function of pH, and δ_{acid} and δ_{base} represent the chemical-shift values at the low and high extremes of pH, respectively.

RESULTS AND DISCUSSION

Resonance assignment

The assignment of the ^1H and ^{15}N NMR spectra of reduced C62A, C69A, C73A human thioredoxin was accomplished using 2D and 3D NMR. Backbone resonances were assigned using 3D ^{15}N-separated HOHAHA and NOESY spectra which provide through-bond and through-space connectivities, respectively, via conventional sequential assignment methods (Wüthrich, 1986; Clore and Gronenborn, 1987) as described by Driscoll et al. (1990). In this manner, amino acid spin systems identified in the 3D ^{15}N-separated HOHAHA spectrum can be linked via sequential through-space (< 5 Å) connectivities derived from the 3D ^{15}N-separated NOESY spectrum. The resolution afforded by the additional ^{15}N dimension permits the straightforward interpretation of the spectra and obviates the need for comparison with the native spectra to resolve ambiguities in the sequential assignment. Figures 2A and B show composites of narrow strips of 2D contour plots that are taken from different $^1H(F_1)$-$^1H(F_3)$ planes of the 3D ^{15}N-separated HOHAHA and NOESY spectra in the region of the mutations, from Asp^{61} to Thr^{74}, demonstrating the clarity of the assignment.

A minor complication in assignment arises from the duplication of resonances for approximately one-third of the residues of the protein due to the N-terminal heterogeneity as a result of the presence of an approximately 1:1 mixture of the N-Met and N-Val forms in the sample. In almost all cases, these duplications were paralleled by duplications in the spectrum of the native protein (Forman-Kay et al., 1989,1990). Knowledge of the spectrum of native human thioredoxin was of help in this regard, although not all duplicated resonances had a corresponding one in the native protein. For example, the duplication of the amide proton resonance of Ala^{66}, seen in Fig. 2, was not present in spectra of the native form of human thioredoxin.

Although some side-chain assignments could be made on the basis of the 3D ^{15}N-separated HOHAHA, most of the side-chain assignments were obtained using 2D ^1H-^1H PE-COSY and HOHAHA spectra recorded in D_2O to demonstrate direct and multiple relayed through-bond

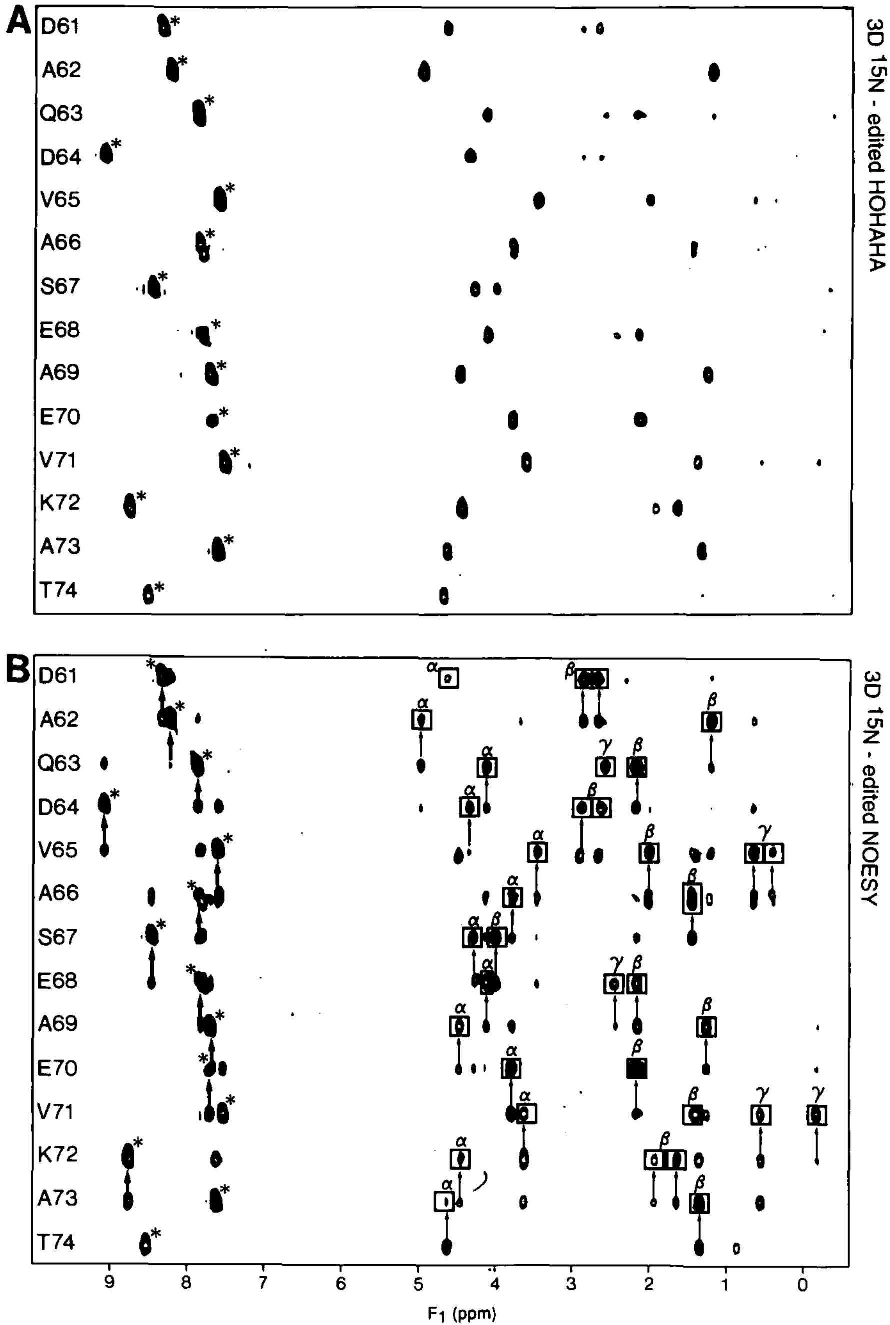

Fig. 2. Amide strips extending from Asp^{61} to Thr^{74}, covering the region of the three mutations, taken from the 3D ^{15}N-separated (A) HOHAHA and (B) NOESY spectra of the reduced form of the C62A, C69A, C73A mutant of human thioredoxin in H_2O at 25°C and pH 5.5. The figure is composed of narrow strips from different $^1H(F_1)$-$NH(F_3)$ planes of the 3D spectra, as described by Driscoll et al. (1990). Asterisks indicate the position of the diagonal peak for each residue. The mixing time for the HOHAHA was 32 ms, while that for the NOESY was 150 ms. In (B), boxes enclose intraresidue NH-$C^{\alpha}H$, NH-$C^{\beta}H$ and NH-$C^{\gamma}H$ cross peaks clearly observed in the NOESY spectrum. Sequential NH-NH(i+1) NOEs are indicated by thick arrows and sequential $C^{\alpha}H$-NH(i+1), $C^{\beta}H$-NH(i+1) and $C^{\gamma}H$-NH(i+1) NOEs by thin arrows.

TABLE 1

^{1}H AND ^{15}N RESONANCE ASSIGNMENTS FOR THE REDUCED FORM OF THE C62A, C69A, C73A MUTANT OF HUMAN THIOREDOXIN AT 25°C AND pH 5.5[a]

Residue	NH	^{15}N	$C^{\alpha}H$	$C^{\beta}H$	Others
M1	b	b	4.02	2.18	$C^{\gamma}H$ 2.59
V2	8.68	124.6	4.38	1.67	$C^{\gamma}H$ 0.45, 0.86
K3*	8.26	128.6	4.59	1.85, 1.71	c
K3	8.22	127.9	4.56	1.83, 1.77	$C^{\gamma}H$ 1.35, 1.28; $C^{\delta}H$ 1.73, 1.63; $C^{\epsilon}H$ 2.99
Q4*	8.71	126.4	4.67	2.07, 2.02	$C^{\gamma}H$ 2.37, 2.25; $N^{\epsilon}H_2$ 7.63, 6.94; $^{15}N^{\epsilon}$ 112.5
Q4	8.65	126.4	4.77	c	c
I5*	8.66	126.1	4.35	2.06	$C^{\gamma m}H$ 0.90
I5	8.64	125.6	4.41	2.07	$C^{\gamma m}H$ 0.91
E6	9.12	123.9	4.56	2.00	$C^{\gamma}H$ 2.26
S7	7.29	110.2	5.06	4.27, 4.04	
K8	9.44	122.6	3.95	1.87, 1.80	$C^{\gamma}H$ 1.48; $C^{\delta}H$ 1.70; $C^{\epsilon}H$ 2.96
T9	8.22	115.5	4.02	4.06	$C^{\gamma}H$ 1.24
A10	7.78	123.7	4.22	1.59	
F11	8.19	120.7	4.11	3.31, 3.02	$C^{\delta}H$ 6.95; $C^{\epsilon}H$ 6.90; $C^{\zeta}H$ 6.78
Q12	8.13	117.4	3.59	2.20, 2.13	$C^{\gamma}H$ 2.44, 2.38; $N^{\epsilon}H_2$ 7.70, 6.83; $^{15}N^{\epsilon}$ 114.5
E13	8.19	117.4	4.00	2.08, 1.98	$C^{\gamma}H$ 2.49, 2.22
A14	7.77	121.6	4.08	1.35	
L15	7.73	117.4	3.73	1.60, 1.00	$C^{\gamma}H$ 0.90; $C^{\delta}H$ −0.09, −0.22
D16	8.22	119.9	4.32	2.71, 2.64	
A17	8.07	121.3	4.19	1.45	
A18	7.16	118.7	3.94	1.38	
G19	7.57	105.8	3.93, 3.77		
D20	9.08	128.9	4.66	2.77	
K21	7.79	119.3	4.31	1.93, 1.88	$C^{\gamma}H$ 1.55; $C^{\delta}H$ 1.75, 1.65; $C^{\epsilon}H$ 3.06
L22	8.85	122.8	4.44	1.99	$C^{\delta}H$ 1.04
V23	9.02	125.8	4.87	1.97	$C^{\gamma}H$ 0.83, 0.62
V24*	9.16	126.5	4.44	1.55	$C^{\gamma}H$ 0.57, 0.46
V24	9.13	126.6	4.49	1.47	$C^{\gamma}H$ 0.52, 0.43
V25	9.17	126.4	4.70	1.97	$C^{\gamma}H$ 0.90, 0.02
D26*	8.33	123.2	4.29	2.27, 1.34	
D26	8.36	123.3	4.25	2.23, 1.27	
F27*	8.94	125.5	4.98	2.97, 2.37	$C^{\delta}H$ 6.83; $C^{\epsilon}H$ 6.79; $C^{\zeta}H$ 6.70
F27	8.87	125.0	c	c	c
S28*	8.66	117.9	4.52	3.42	
S28	c	c	4.50	3.38	
A29	6.65	118.8	4.70	0.27	
T30	9.65	117.3	3.84	4.15	$C^{\gamma}H$ 1.23
W31	6.70	115.8	4.64	3.62, 3.19	$C^{\zeta 2}H$ 7.39; $C^{\epsilon 3}H$ 7.36; $C^{\eta 2}H$ 7.18; $C^{\zeta 3}H$ 7.09; $C^{\delta 1}H$ 7.28; $N^{\epsilon 1}H$ 11.23; $^{15}N^{\epsilon 1}$ 134.6
C32	6.61	120.6	4.65	3.18, 2.95	
G33	9.59	121.1	4.27, 4.00		
P34	–	b	4.46	2.64, 1.76	$C^{\gamma}H$ 2.33, 2.08; $C^{\delta}H$ 3.97, 3.87
C35	8.16	110.3	4.27	3.96, 3.16	
K36	7.82	121.2	4.01	2.08, 2.01	$C^{\gamma}H$ 1.51; $C^{\delta}H$ 1.76, 1.66; $C^{\epsilon}H$ 3.03
M37	7.64	116.9	4.29	2.30	$C^{\gamma}H$ 2.80, 2.64
I38	7.64	115.6	4.60	1.96	$C^{\gamma}H$ 2.09, 1.85; $C^{\gamma m}H$ 1.23; $C^{\delta}H$ 0.92
K39*	7.58	125.5	4.33	2.13, 2.03	$C^{\gamma}H$ 1.33; $C^{\delta}H$ 1.78; $C^{\epsilon}H$ 2.97

TABLE I (continued)

Residue	NH	^{15}N	$C^{\alpha}H$	$C^{\beta}H$	Others
K39	7.51	125.5	4.27	2.09, 2.00	c
P40	–	b	4.42	2.37	$C^{\gamma}H$ 2.18, 2.04; $C^{\delta}H$ 3.89, 3.76
F41*	7.49	120.0	4.49	3.50, 3.24	$C^{\delta}H$ 7.24; $C^{\epsilon/\zeta}H$ 7.52
F41	7.45	120.1	4.53	3.52, 3.27	$C^{\delta}H$ 7.26; $C^{\epsilon}H$ 7.49; $C^{\zeta}H$ 7.54
F42*	8.89	122.0	3.99	3.57, 3.15	$C^{\delta}H$ 7.37; $C^{\epsilon/\zeta}H$ 7.66
F42	c	c	4.01	3.60, 3.16	$C^{\delta}H$ 7.35; $C^{\epsilon/\zeta}H$ 7.68
H43*	8.43	114.5	3.80	3.28	$C^{\delta2}H$ 7.32
H43	8.38	114.2	3.71	3.27, 3.23	$C^{\delta2}H$ 7.42
S44*	8.49	119.1	4.18	3.94	
S44	8.62	119.8	4.11	3.94	
L45*	7.95	122.9	3.84	1.54	$C^{\gamma}H$ 1.11; $C^{\delta}H$ 0.68
L45	8.27	123.7	3.82	1.67	$C^{\gamma}H$ 1.11; $C^{\delta}H$ 0.69
S46*	6.73	114.5	3.32	2.87	
S46	6.64	115.5	3.04	3.18, 2.42	
E47*	6.81	118.8	4.06	1.96	$C^{\gamma}H$ 2.20, 2.09
E47	6.74	119.5	4.13	2.03, 1.88	$C^{\gamma}H$ 2.19
K48*	7.17	119.6	4.02	1.63, 1.56	$C^{\gamma}H$ 1.11, 0.50; $C^{\delta}H$ 1.49, 1.41; $C^{\epsilon}H$ 2.77, 2.71
K48	7.19	120.1	c	c	c
Y49*	8.09	118.8	4.80	3.05	$C^{\delta}H$ 7.33; $C^{\epsilon}H$ 6.89
Y49	8.00	118.5	c	c	c
S50*	7.82	115.1	4.44	4.03, 3.90	
S50	7.78	114.9	c	c	
N51	9.13	118.5	4.82	2.98, 2.88	$N^{\delta}H_2$ 7.63, 6.82; $^{15}N^{\delta}$ 112.5
V52*	7.51	121.1	4.47	2.13	$C^{\gamma}H$ 0.88, 0.22
V52	7.49	121.1	4.50	2.18	$C^{\gamma}H$ 0.89, 0.28
I53*	8.86	127.8	4.40	1.90	$C^{\gamma m}H$ 0.87
I53	8.91	128.0	4.36	1.93	$C^{\gamma m}H$ 0.86
F54*	8.63	125.9	5.21	2.81, 2.41	$C^{\delta}H$ 6.63; $C^{\epsilon}H$ 7.10; $C^{\zeta}H$ 6.74
F54	8.96	126.2	5.18	3.07, 2.38	$C^{\delta}H$ 6.56; $C^{\epsilon}H$ 7.07; $C^{\zeta}H$ 6.71
L55*	9.37	122.3	5.41	1.65, 1.29	$C^{\gamma}H$ 1.50; $C^{\delta}H$ 0.84, 0.57
L55	9.40	122.3	5.59	1.70, 1.31	c
E56*	8.49	120.5	4.92	1.83	$C^{\gamma}H$ 2.15, 2.03
E56	8.52	121.0	4.99	1.78	c
V57*	8.37	123.2	4.04	1.14	$C^{\gamma}H$ 0.62, 0.13
V57	8.43	124.4	4.06	1.15	$C^{\gamma}H$ 0.65, 0.15
D58	9.11	128.8	4.95	2.84, 2.30	
V59*	8.72	118.3	3.66	2.21	$C^{\gamma}H$ 0.96, 0.84
V59	8.77	118.4	c	c	c
D60	8.26	118.5	4.84	2.84, 2.76	
D61	8.29	120.9	4.63	2.86, 2.65	
A62	8.18	125.0	4.95	1.17	
Q63	7.83	120.7	4.11	2.16	$C^{\gamma}H$ 2.55, 2.37; $N^{\epsilon}H_2$ 7.67, 6.83; $^{15}N^{\epsilon}$ 111.7
D64	9.03	118.2	4.32	2.86, 2.61	
V65	7.55	122.4	3.44	1.99	$C^{\gamma}H$ 0.63, 0.37
A66*	7.80	120.2	3.76	1.43	
A66	7.76	120.2	c	c	
S67	8.41	111.8	4.26	3.97	
E68	7.77	123.4	4.10	2.13	$C^{\gamma}H$ 2.46, 2.38
A69	7.66	119.4	4.45	1.23	

TABLE 1 (continued)

Residue	NH	^{15}N	$C^{\alpha}H$	$C^{\beta}H$	Others
E70	7.65	114.7	3.76	2.13	$C^{\gamma}H$ 2.19
V71	7.48	118.5	3.60	1.37	$C^{\gamma}H$ 0.53, −0.20
K72	8.71	127.1	4.42	1.90, 1.62	$C^{\gamma}H$ 1.32; $C^{\epsilon}H$ 2.92
A73	7.57	121.7	4.62	1.31	
T74	8.48	110.6	4.67	3.92	$C^{\gamma}H$ 0.83
P75	–	b	5.10	2.26, 1.40	$C^{\gamma}H$ 1.85, 1.71; $C^{\delta}H$ 3.66, 3.42
T76*	8.23	118.0	4.60	3.75	$C^{\gamma}H$ 1.11
T76	8.17	118.1	c	c	c
F77*	8.96	125.4	5.67	2.23, 1.98	$C^{\delta}H$ 6.57; $C^{\epsilon}H$ 7.00; $C^{\zeta}H$ 6.87
F77	c	c	c	c	$C^{\delta}H$ 6.59; $C^{\epsilon}H$ 7.05; $C^{\zeta}H$ 6.88
Q78	8.64	118.5	5.04	2.19, 1.97	$C^{\gamma}H$ 2.35; $N^{\epsilon}H_2$ 6.76, 6.47; $^{15}N^{\epsilon}$ 106.0
F79	8.02	119.0	5.55	2.62, 2.47	$C^{\delta}H$ 6.95; $C^{\epsilon}H$ 7.14; $C^{\zeta}H$ 7.23
F80	9.99	122.1	5.62	2.72, 2.59	$C^{\delta}H$ 6.74; $C^{\epsilon}H$ 7.39; $C^{\zeta}H$ 7.79
K81	8.87	118.3	4.57	1.73, 1.65	$C^{\gamma}H$ 1.42; $C^{\delta}H$ 1.76; $C^{\epsilon}H$ 3.05
K82	10.21	128.5	3.99	2.11, 1.82	$C^{\gamma}H$ 1.48, 1.41; $C^{\delta}H$ 1.73; $C^{\epsilon}H$ 2.99
G83	9.35	104.2	4.19, 3.36		
Q84	7.78	118.5	4.75	2.13, 1.98	$C^{\gamma}H$ 2.37; $N^{\epsilon}H_2$ 7.53, 6.87; $^{15}N^{\epsilon}$ 112.5
K85	8.99	126.0	3.96	1.78	$C^{\gamma}H$ 1.08; $C^{\epsilon}H$ 2.92
V86	9.21	121.3	4.57	2.45	$C^{\gamma}H$ 0.96, 0.65
G87	7.48	107.9	4.31, 3.72		
E88	8.63	118.2	5.37	2.15	$C^{\gamma}H$ 2.37
F89	8.94	117.9	5.07	3.54, 3.42	$C^{\delta}H$ 7.32; $C^{\epsilon}H$ 7.39; $C^{\zeta}H$ 6.80
S90	9.07	115.4	5.39	3.88, 3.73	
G91	8.18	110.2	4.74, 3.76		
A92	8.75	123.7	4.47	1.38	
N93	7.54	122.1	4.83	2.89, 2.66	$N^{\delta}H_2$ 7.59, 6.98; $^{15}N^{\delta}$ 112.8
K94*	8.62	125.0	3.62	1.35, 1.24	$C^{\gamma}H$ 0.66; $C^{\delta}H$ 1.04; $C^{\epsilon}H$ 2.61
K94	c	c	3.65	1.38, 1.26	$C^{\gamma}H$ 0.68; $C^{\delta}H$ 1.06; $C^{\epsilon}H$ 2.59
E95	8.38	119.0	4.09	2.13, 1.96	$C^{\gamma}H$ 2.37, 2.25
K96	7.81	121.1	3.87	1.34, 0.95	$C^{\gamma}H$ 0.74, 0.27; $C^{\delta}H$ 1.12, 0.92; $C^{\epsilon}H$ 2.58, 2.47
L97	8.07	122.9	4.19	2.34	$C^{\gamma}H$ 1.54; $C^{\delta}H$ 0.87, 0.72
E98	7.88	117.3	3.88	2.28	$C^{\gamma}H$ 2.55
A99	8.40	120.4	4.14	1.41	
T100	8.06	115.2	3.68	3.95	$C^{\gamma}H$ 0.24
I101	7.45	120.1	3.24	1.71	$C^{\gamma}H$ 0.81, 0.75; $C^{\gamma m}H$ 0.16; $C^{\delta}H$ 0.86
N102*	7.53	113.3	4.32	2.77, 2.71	$N^{\delta}H_2$ 7.37, 7.29; $^{15}N^{\delta}$ 113.1
N102	7.48	113.1	c	c	c
E103	7.75	118.1	4.13	2.15, 2.04	$C^{\gamma}H$ 2.39, 2.25
L104	7.38	117.5	4.42	1.43	$C^{\gamma}H$ 1.65; $C^{\delta}H$ 0.72, 0.33
V105*	7.54	123.5	3.81	2.15	$C^{\gamma}H$ 1.02, 0.98
V105	7.52	123.6	c	c	c

[a] Residues which give different chemical shifts for the N-Met and N-Val forms of human thioredoxin are denoted with an asterisk (*) at the amino acid name for the resonance arising from the slightly more abundant N-terminal Val species. ^{1}H chemical shifts are expressed relative to 4,4-dimethyl-4-silapentane-1-sulfonate and measured with respect to the water resonance which has a chemical shift of 4.76 ppm at 25°C. ^{15}N chemical shifts are reported with respect to external liquid NH_3.

[b] Unassigned or undetected resonance.

[c] The resonances of the N-Met and N-Val forms are indistinguishable or those of the N-Met form were undetected.

scalar connectivities, respectively. Comparison with the native spectrum was quite useful at this stage of the assignment process, due to the significant overlap in the aliphatic region of the spectrum.

The ^{1}H and ^{15}N assignments for the reduced C62A, C69A, C73A mutant thioredoxin at pH 5.5 and 25°C are provided in Table 1, and resonances of the N-terminal Val species which are distinct from those of the N-terminal Met species are indicated by an asterisk.

Structure of the mutant

A summary of the sequential and short-range ($|i-j| \leqslant 3$) NOE connectivities involving the NH, C$^{\alpha}$H and C$^{\beta}$H protons, together with the slowly exchanging amide protons, is shown in Fig. 3. No significant differences were observed in the pattern of the NOEs for the N-Met and N-Val forms of the enzyme. A comparison of these data for the mutant with the native human thioredoxin reveals that the secondary structural elements of the C62A, C69A, C73A mutant are the same as those in the native protein (Forman-Kay et al., 1989, 1991), as indicated below the NOE connectivities in Fig. 3. The presence of five β-strands [Val^{2} to Ile^{5} (β_1), Leu^{22} to Ser^{28} (β_2), Ile^{53} to Val^{59} (β_3), Pro^{75} to Lys^{81} (β_4) and Gln^{84} to Gly^{91} (β_5)], characterized by strong C$^{\alpha}$H(i)-NH(i+1) NOEs and four α-helices [Ser^{7} to Ala^{17} (α_1), Gly^{33} to Tyr^{49} (α_2), Ala^{62} to Glu^{70} (α_3) and Lys^{94} to Val^{105} (α_4), as well as a helical turn from Asp^{58} to Ala^{62}, characterized by a stretch of NH(i)-NH(i+1), C$^{\alpha}$H(i)-NH(i, i+2,3) and C$^{\alpha}$H(i)-C$^{\beta}$H(i+3) NOEs, is clearly supported by the NOE and NH exchange data.

A qualitative analysis of NOE peaks corresponding to connectivities across the β-strands confirms that the topology of the five-stranded β-sheet is also retained in the C62A, C69A, C73A mutant. This is illustrated in the schematic diagram presented in Fig. 4 which depicts the β-sheet of the mutant protein as deduced from the pattern of interstrand NOE connectivities and slowly exchanging amide protons.

Thus, as evidenced by both the preservation of secondary-structure elements and overall β-sheet topology, the global structure of the C62A, C69A, C73A mutant appears to be nearly identical to that of native human thioredoxin. Although Cys^{62} is totally buried in the native protein and the surface accessibility of Cys^{69} is only ~20% relative to that in a Gly-Cys-Gly tripeptide (Forman-Kay et al., 1991), the conservative substitutions for alanine have allowed the structure to be preserved with little change. (Note that the surface accessibility of Cys^{73}, located in an extended loop region, is much greater, at about 60%, while Cys^{62} and Cys^{69} are found in helical structures.)

Differences in the chemical shifts of spectral resonances of the native and mutant thioredoxins

The chemical shifts of the resonances of the C62A, C69A, C73A mutant human thioredoxin did not differ for the most part from those of the native protein, confirming the expectation that these substitutions would not change the structure of the protein significantly. Since chemical shifts are extremely sensitive to very subtle changes in structure, the observed small changes are a stronger confirmation of the retention of native structure than the NOE patterns. The differences in NH proton and ^{15}N chemical shifts between the mutant and native enzyme are also included in Fig. 3. The greatest chemical-shift differences are observed at the three positions where the substitutions were made (Ala^{62}, Ala^{69} and Ala^{73}) and at the near-by active-site residues Ala^{29} and Cys^{35}. A ribbon diagram of the structure of the native human thioredoxin with the locations of the three cysteine-to-alanine substitutions is shown in Fig. 5. Residues whose NH chemical shifts differ by

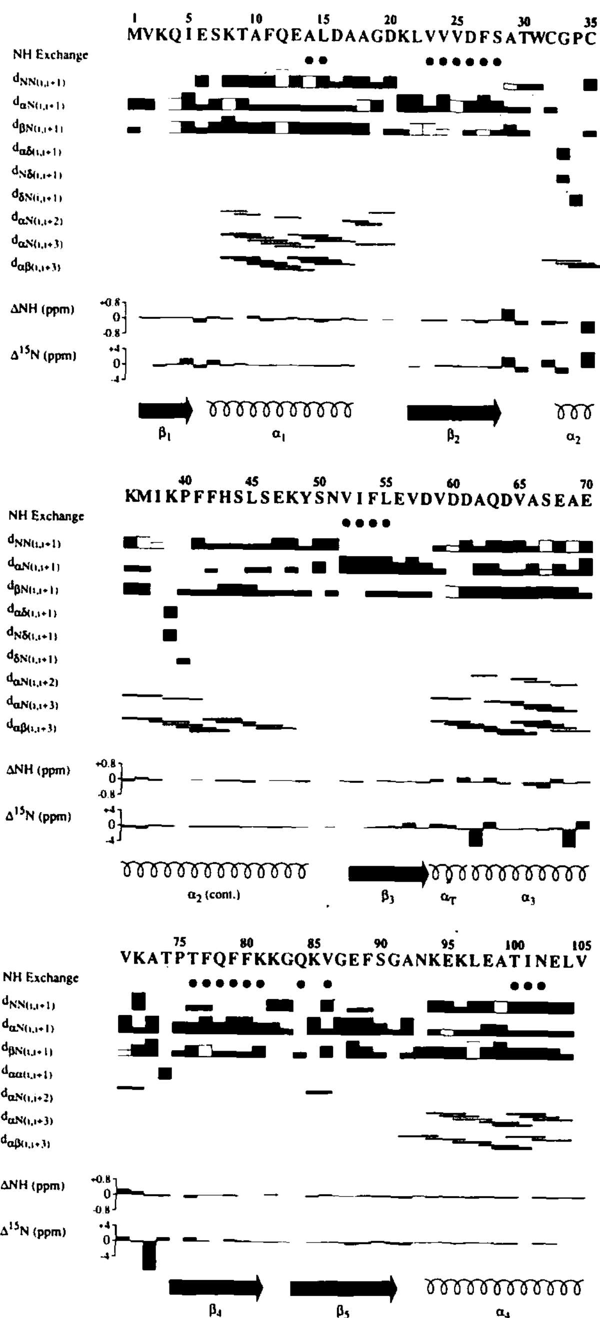

Fig. 3. Summary of the sequential and short-range ($|i-j| \leq 3$) NOEs involving the NH, $C^{\alpha}H$ and $C^{\beta}H$ protons, as well as the $C^{\delta}H$ protons of proline residues, the amide exchange data, and the NH and ^{15}N chemical-shift differences from the native spectrum observed for the reduced form of the C62A, C69A, C73A mutant of human thioredoxin, together with the secondary structure derived from the previously determined solution structure of native human thioredoxin (Forman-Kay et al., 1991). The thickness of the lines reflects the relative strength of the NOEs. Open boxes and dashed lines represent potential sequential connectivities obscured by resonance overlap. For residues having duplications arising from the N-terminal heterogeneity, intensities are taken from peaks of the N-Val form. Amide protons which were present in the homonuclear HOHAHA spectrum recorded after redissolving a lyophilized sample in D_2O are indicated by filled circles.

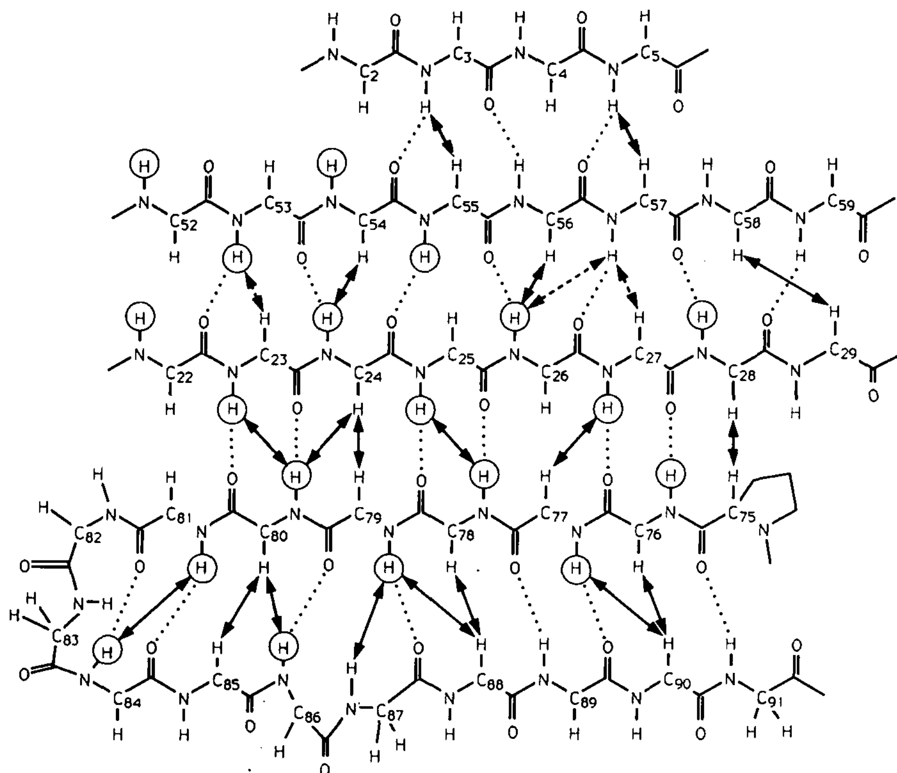

Fig. 4. Schematic representation of the β-sheet of the C62A, C69A, C73A mutant of human thioredoxin deduced from a qualitative analysis of the NMR data. Observed interstrand NOE connectivities between backbone $C^{\alpha}H$ or NH protons are indicated by solid arrows. In the case of resonance overlap, the potential NOE connectivity is indicated by a dashed arrow. Slowly exchanging amide protons are circled. Hydrogen bonds suggested by the NOE patterns and slowly exchanging NH protons are marked with dotted lines.

greater than or equal to 0.2 ppm from the corresponding resonance in the spectrum of the native protein at pH 5.5 and 25°C or whose amide ^{15}N chemical shift differs by 1.0 ppm, or more are indicated. These residues lie in the regions of Ile^{5} to Ser^{7}, Ala^{29} to Cys^{35}, Val^{57} to Ala^{62}, Ser^{67} to Val^{71}, Ala^{73} and Thr^{76}, all of which are in close spatial proximity to the sites of mutation.

Thioredoxin activity

The activities of *E. coli* thioredoxin, native human thioredoxin and the C62A, C69A, C73A mutant of human thioredoxin were assayed using a modified spectrophotometric insulin reduction assay, as described in Holmgren (1979) and Luthman and Holmgren (1982). Figure 6 shows the results for the reduction of insulin disulfide bridges by DTT catalyzed by different concentrations of human wild-type and mutant thioredoxins as well as, for comparison, by *E. coli* thioredoxin. All three thioredoxins assayed exhibited activities within the same order of magnitude, indicating that the mutation had not affected the catalytic activity adversely.

pK_a of Cys^{32}

The ^{1}H and ^{15}N chemical shifts for backbone amide groups of residues near the active site of the

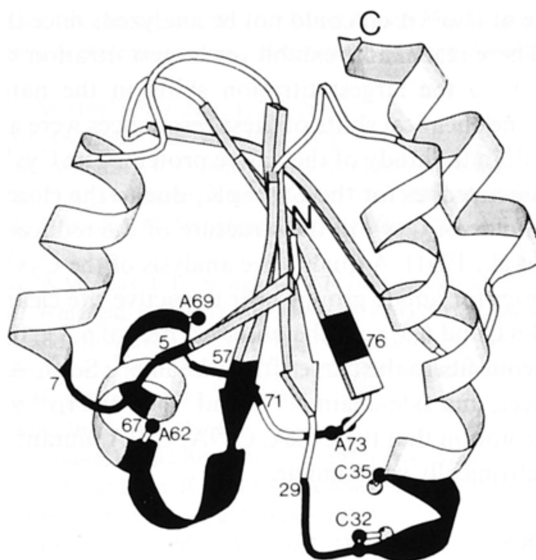

Fig. 5. Ribbon diagram based on the coordinates of the previously determined solution structure of the native human thioredoxin (Forman-Kay et al., 1991) indicating the position of the three cysteine-to-alanine substitutions and the two active-site cysteines. The residues having amide chemical-shift differences greater than or equal to 0.2 ppm for ^{1}H or 1.0 ppm for ^{15}N, are indicated in black. These include residues Ile^{5} to Ser^{7}, Ala^{29} to Cys^{35}, Val^{57} to Ala^{62}, Ser^{67} to Val^{71}, Ala^{73} and Thr^{76}. This ribbon drawing was produced with the program MOLSCRIPT (Kraulis, 1991).

C62A, C69A, C73A mutant of human thioredoxin were measured from ^{1}H-^{15}N Overbodenhausen correlation experiments performed at six different values from pH 5.5 to 7.1. Titration curves were derived for Asp^{26}, Phe^{27}*, Phe^{27}, Ser^{28}, Ala^{29}, Thr^{30}, Trp^{31}, Cys^{35}, Asp^{61}, Ala^{62} and Gln^{63} amide NH and ^{15}N resonances, as well as the side-chain $N^{\varepsilon 1}H$ and $^{15}N^{\varepsilon 1}$ of Trp^{31}. (Note that the

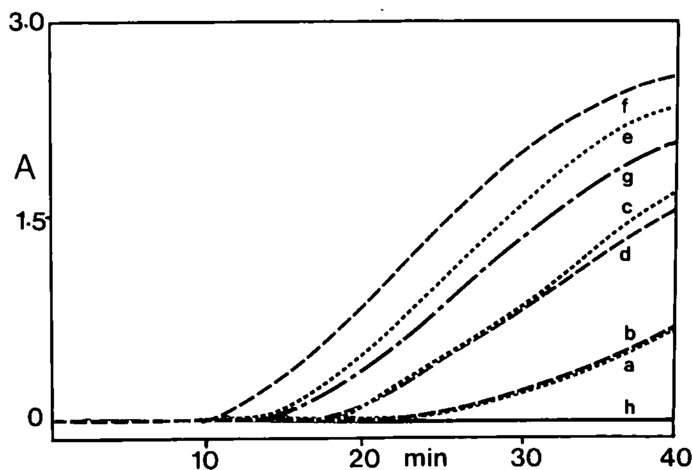

Fig. 6. Thioredoxin-catalyzed reduction of insulin by dithiothreitol. The absorbance at 650 nm is plotted as a function of time. (a), (c), (e): 0.13 μM, 0.35 μM, 0.65 μM C62A, C69A, C73A mutant human thioredoxin, respectively; (b), (d), (f): 0.13 μM, 0.35 μM, 0.65 μM wild-type human thioredoxin, respectively; (g) 1 μM *E. coli* thioredoxin; (h) without thioredoxin.

titration curve for the amide of Cys^{32} itself could not be analyzed, since the amide proton rapidly exchanged above pH 5.5.) These resonances exhibit the largest titration shifts over this pH range and are identical to those with the largest titration shifts in the native human thioredoxin (Forman-Kay et al., 1992). The chemical shifts of these resonances were also the most sensitive to the protonation state of Cys^{32} in the study of the native protein. The Cys^{35} amide NH and ^{15}N titration curves are almost direct probes for the Cys^{32} pK_a due to the close interaction of the side-chain S^{γ} of Cys^{32} with the amide of Cys^{35} in the structure of the reduced form of native human thioredoxin (Forman-Kay et al., 1991). A qualitative analysis of the Cys^{35} amide NH and ^{15}N titration curves and other curves for amide groups near the active site clearly demonstrates a titration with a pK_a between pH 6.0 and 6.3, near the observed pK_a of 6.3 ± 0.1 found for Cys^{32} in the wild-type protein. Simultaneous fits to the curves from the Phe^{27}, Ser^{28}, Ala^{29}, Thr^{30}, Trp^{31}, Cys^{35} amide NH and ^{15}N resonances, and side-chain $N^{\varepsilon 1}H$ and $^{15}N^{\varepsilon 1}$ of Trp^{31} yielded pK_a values in the range 5.5–6.3 confirming the notion that the C62A, C69A, C73A mutant and wild-type thioredoxins are structurally and electronically very similar.

CONCLUDING REMARKS

The assignment of the ^{1}H and ^{15}N NMR spectra of the reduced form of the C62A, C69A, C73A mutant of human thioredoxin has been accomplished. Based on comparisons of short-range ($|i-j| \leqslant 3$) and interstrand NOEs and amide exchange rates, the secondary structure and topology appear to be nearly identical to those found in the previously determined solution structure of the native protein (Forman-Kay et al., 1991). In addition, the observation of only minor differences in chemical shifts between the native and mutant thioredoxins also supports the conclusion that the mutant retains native structure. Finally, the mutant retains enzymatic activity and the active-site Cys^{32} appears to have a similar pK_a (~ 6) to that of the native protein. Since the mutant retains both native structure and biological activity, and can be oxidized without intermolecular disulfide bridge formation and aggregation, it will facilitate comparative studies of the reduced and oxidized forms of human thioredoxin. Thus, the ^{1}H and ^{15}N resonance assignments of the mutant reported in this paper provide a foundation for future work probing the structural basis of the catalytic mechanism of the thiol chemistry in the active site of thioredoxin.

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