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Characterization by ^{1}H NMR of a C32S,C35S double mutant of *Escherichia coli* thioredoxin confirms its resemblance to the reduced wild-type protein

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Abstract

A mutant of *Escherichia coli* thioredoxin containing serine residues in place of the two active-site cysteines, termed C32S,C35S, previously shown to be partially able to substitute for reduced thioredoxin in certain phage systems, has been characterized by ^{1}H NMR spectroscopy at pH values between 5.5 and 10. The ^{1}H NMR spectrum of the mutant at pH 5.5 is very similar to that of the wild-type protein in either the reduced or oxidized state. Chemical shift changes in the vicinity of the active site serines indicate that the nearby hydrophobic pocket is somewhat changed, probably as a result of the replacement of the cysteine thiols with the smaller, more hydrophilic hydroxyl side chains and a change in the preferred χ_1 angles of the side chains. Although the pattern of amide protons persistent in ^2H_2O differs only slightly between the two forms of the wild-type protein, the pattern observed for the C32S,C35S mutant shows characteristic features that correspond closely with those of the reduced wild-type protein rather than with the oxidized form. The pH dependence of the mutant protein shows a single group titrating close to the active site with a pK_a of 8.3, which we assign to the buried carboxyl group of Asp 26 by analogy with the behavior of wild-type thioredoxin. The pK_a is significantly higher for the mutant protein, consistent with an increase in the hydrophobicity of the pocket where the carboxyl is buried, probably due to repacking caused by the removal of the cysteine thiols and the placement of the serine hydroxyls in positions where they interact better with solvent. The results demonstrate that the solution behavior of the mutant protein is similar in many ways to that of reduced wild-type thioredoxin, explaining its partial activity in the two essential roles of reduced thioredoxin as a subunit of phage T7 DNA polymerase and in the assembly of filamentous phage.

Key words: NMR; Thioredoxin; Mutant; T7 DNA polymerase; Filamentous phage

1. Introduction

Thioredoxin is a small protein that occurs in all living systems and is also an important component of host cell-virus systems [1,2]. The numerous functions of thioredoxin have not yet been completely elucidated; recent work implicates it in cellular redox control mechanisms and also in mammalian viral systems, with possible applications in therapeutics [3]. The conserved active site of thioredoxin has the sequence Cys-Gly-Pro-Cys and contains a dithiol in the reduced state, and a disulfide in the oxidized state. Among its many functions it is capable of catalysing thiol-disulfide exchange reactions and protein disulfide isomerization.

Thioredoxin from *Escherichia coli* (108 residues, M_r 11500) has been the subject of a large amount of structural study. The X-ray crystal structure of oxidized thioredoxin (Trx-S_2) has been solved as the Cu^{2+} complex [4] and refined to 1.7Å resolution [5]. No crystals are available for reduced thioredoxin (Trx-$(SH)_2$) but the solution structure of this form has been calculated from NMR data [6]. The calculation of high-resolution solution structures for both Trx-S_2 and Trx-$(SH)_2$ is in progress (M.-F. Jeng and H.J. Dyson, unpublished). The two forms of the protein show identical secondary structures and tertiary folds, consisting of a 5-stranded β-sheet surrounded by 3 α-helices and one 3_{10} or irregular helix. The active-site residues, Cys-32 and Cys-35, are located on a turn or short loop between the second β-strand and the second α-helix, and are in contact with two other loops, residues 73–76 and 90–93 which, together with the active site residues, form a hydrophobic surface thought to be important in binding to other proteins [7]. Structural differences between Trx-S_2 and Trx-$(SH)_2$ are very subtle [6], and differences in the NMR spectrum are seen mainly in the active site region and the loops that contact it [8–11]. Subtle differences are also seen in the backbone dynamics measured by ^{15}N NMR relaxation [12] and in the hydrogen exchange behavior (M.-F. Jeng and H.J. Dyson, unpublished).

The reaction catalysed by thioredoxin, dithiol-disul-

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fide exchange, may be characterized as a simultaneous electron and proton transfer. The mechanism of reduction by thoredoxin is thought to proceed via nucleophilic attack by a thiolate belonging to Cys-32 with formation of a mixed disulfide [13]. We therefore reasoned that pH control would be an important aspect of the mechanism of thioredoxin. NMR titration studies of Trx-$(SH)_2$ revealed that the thiol groups titrated with different pK_as ~7.0 and ~8.4 [14], consistent with values previously determined from chemical modification studies [13]. In addition, a group with pK_a ~7.5 titrated in the vicinity of the active site in Trx-S_2 and by implication also in Trx-$(SH)_2$. We assigned this titration to the buried carboxyl side chain of Asp-26 [14]; a similar conclusion was reached from denaturation and mutant studies [15–17].

One of the major functional differences between Trx-S_2 and Trx-$(SH)_2$ is in their behavior in the assembly of filamentous bacteriophages and in the replication of bacteriophage T7 DNA. Filamentous phages require thioredoxin for assembly [18] and are also partially dependent on functional thioredoxin reductase [19]. Replacement of one or both cysteine residues by serine or alanine renders phage assembly independent of thioredoxin reductase, suggesting that phage assembly requires the reduced conformation [19]. In *Escherichia coli* infected by phage T7, Trx-$(SH)_2$ is required for virus replication since it is an essential subunit of the T7-induced DNA polymerase, which consists of a strong 1:1 complex (K_d 5 nM) with T7 gene 5 protein. Trx-S_2 cannot bind to gene 5 protein, and only Trx-$(SH)_2$ will give activity [20–22]. Mutants where one or both of the active site cysteines were replaced with serine or alanine were active, demonstrating that the sulfhydryl groups of Trx-$(SH)_2$ are not involved in the T7 DNA polymerase mechanism [21]. The results in the T7 DNA replication and filamentous phage assembly systems strongly suggest that these cysteine mutants should resemble the reduced form of the wild-type protein. In this paper we present evidence from the NMR spectrum that this is indeed the case.

2. Materials and methods

A site-directed double mutant thioredoxin C32S,C35S was prepared as previously described [19]. An NMR sample was prepared in 0.1 M potassium phosphate, pH 5.5, in 90% ^1H_2O/10% ^2H_2O to obtain spectra for resonance assignment. The buffer was then exchanged to 0.1 M potassium phosphate in 99% ^2H_2O for the pH-dependence study. pH values in ^2H_2O are uncorrected meter readings, and the values quoted are those obtained after the completion of the NMR experiment. Spectra were referenced to an internal standard of dioxane (3.75 ppm). The probe temperature was 298K for all experiments.

NMR spectra were obtained using Bruker AMX500 and AMX600 spectrometers equipped with digital phase-shifting hardware. In order to obtain sequential assignments of the C32S,C35S mutant by comparison with the spectra of wild-type thioredoxin, three spectra were acquired for the sample in 90% ^1H_2O/10% ^2H_2O, a double-quantum filtered COSY spectrum (2QF COSY; [23]), a NOESY spectrum [24] and a double-quantum spectrum (2Q; [25]). A series of 2QF COSY spectra in ^2H_2O were acquired at a number of pH values as previously described [14]. Spectral widths were in the range 5500–8000 Hz for the experiments in 90% ^1H_2O/10% ^2H_2O and 6000 Hz for the experiments in ^2H_2O, with 4K complex points in ω_2 and 400–600 points in ω_1. The NOESY mixing time was 100 ms.

Spectra were Fourier transformed using the program FTNMR (Hare Research) on a Sun workstation. Shifted sine bell window functions were applied in both dimensions, and a linear baseline correction was applied in ω_2 of the 2Q and NOESY spectra.

Chemical shift data as a function of pH were fitted by the method of least squares to a titration curve of the form

$$\delta = \delta_{HA} - [(\delta_{HA} - \delta_A)/(1 + 10^{n(pK_a - pH)})] \quad (1)$$

as previously described [14]. Here, δ is the observed chemical shift of

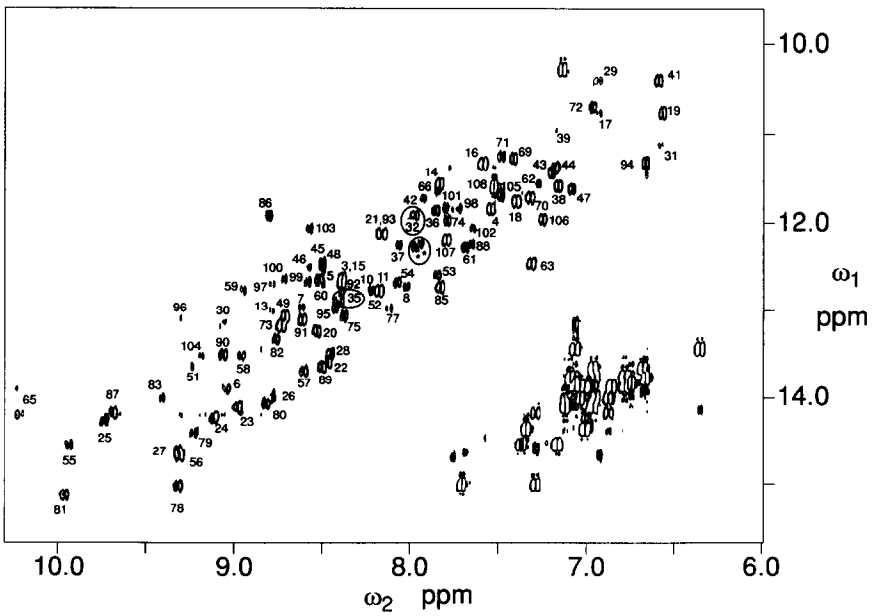

Fig. 1. Fingerprint region of a 500 MHz 2Q spectrum of the C32S, C35S mutant thioredoxin in 90% ^1H_2O/10% ^2H_2O, pH 5.5, 298K. The NH-C$^\alpha$H cross peaks of Ser-32 and Ser-35 are circled. The circled cross peaks with asterisks are unassigned (see text).

a resonance at a given pH, δ_{HA} and δ_A are the chemical shifts of the resonance at low and high pH respectively, n is the number of protons transferred (kept fixed at 1) and pK_a is the acid dissociation constant of the titrating group.

3. Results and discussion

3.1. Resonance assignments

Backbone and limited side chain assignments were obtained for the C32S,C35S mutant thioredoxin from the spectra in 90% ^1H_2O/10% ^2H_2O, by analogy with the very similar spectra of the wild-type protein [9]. Most of the proton resonances of the mutant protein are within 0.05 ppm of those of the corresponding protons in wild-type thioredoxin, as expected in view of the similarity of the behavior of the mutant and wild-type proteins in the T7 system. Assignments for most residues could readily be made from the three spectra in 90% ^1H_2O/10% ^2H_2O, at least as far along the amino acid side chain as the $C^\beta H$ and in some cases further. The fingerprint region of the 2Q spectrum is shown in Fig. 1, and resonance assignments of residues in the vicinity of the active site are given in Table 1. There are several unidentified cross peaks in Fig. 1, shown labeled with an asterisk. While the number of extra peaks is not sufficiently high to indicate the presence of major impurities or unfolded protein, they may indicate some local conformational heterogeneity. It is possible that this may be due to isomerization of the *cis*-Pro-76 [26], although the effect on the NMR spectrum appears more localized than might be expected for such a change in backbone configuration. Assignment of the resonances of the two serines at positions 32 and 35 was made on the basis of sequential connectivities in NOESY spectra, but these are tentative as the connec-

Table 1
Selected resonance assignments (ppm) for thioredoxin mutant C32S,C35S

	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	Other
Leu-24	9.23	5.18	2.03	1.54	0.94,0.86	
Val-25	9.74	4.54	2.27	0.80,0.24		
Asp-26	8.79	5.22	2.89,2.28			
Phe-27	9.32	5.3	2.76,3.36	7.35(δ) 7.03(ε), 6.86(ζ)		
Trp-28	8.46	5.04	3.12,3.29		10.7(Nε1),7.20(δ)	
Ala-29	6.94	3.47	0.31			
Glu-30	9.06	4.09	2.05,2.05	2.4		
Trp-31	6.58	4.54	3.67,3.18		11.4(Nε1),7.47(δ)	
Ser-32	7.98	3.95	4.63,4.12			
Gly-33	9.56	4.01,4.26				
Pro-34		4.41	2.44,1.76	2.02	3.80,4.04	
Ser-35	8.40	4.50	4.06			
Lys-36	7.86	4.02	2.06,1.93			
Met-37	8.07	4.2	2.21,2.21			
Ile-38	7.17	4.42	1.93		1.08($C^\gamma H_3$)	
Ala-39	7.18	3.8	1.32			
Pro-40		4.5	2.34,1.91		3.63,3.88	
Ile-41	6.59	3.81	2.02	1.21,1.66	0.87	0.82($C^\gamma H_3$)
Leu-42	7.93	3.81	1.90,1.20			
Lys-57	8.61	5.09	1.64			
Leu-58	8.97	4.55	1.37			
Asn-59	8.96	3.82	2.64,2.26			
Ile-60	8.51	4.19	2.20			
Asp-61	7.69	4.59	2.82,2.70			
Gln-62	7.28	4.21	1.77,2.21			
Asn-63	7.32	5.16	2.68,2.75			
Tyr-70	7.33	4.39	3.41,2.13		7.08	6.75($C^\varepsilon H$)
Gly-71	7.49	3.91,3.77				
Ile-72	6.97	3.74	1.56	0.68,1.02	0.08	
Arg-73	8.74	4.45	1.68,1.89	1.58	3.16	
Gly-74	7.79	4.19,3.61				
Ile-75	8.38	4.71	1.97			
Pro-76		5.44	2.54			
Thr-77	8.13	4.88	3.9	1.11		
Leu-78	9.32	5.71	1.72			
Thr-89	8.51	5.14	3.79	1.05		
Lys-90	9.07	4.44	1.22,0.65			
Val-91	8.62	4.51	1.91	0.82,0.90		
Gly-92	8.40	3.59,4.36				
Ala-93	8.17	3.95	1.36			

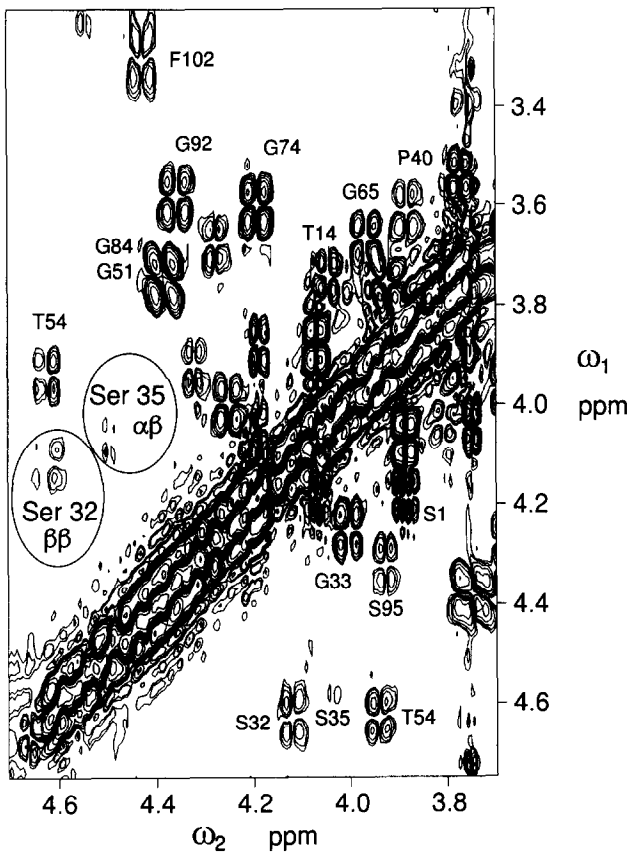

Fig. 2. $C^{\alpha}H$-$C^{\beta}H$ region of a 500 MHz 2QF COSY spectrum of the C32S,C35S mutant thioredoxin in 90% $^{1}H_2O$/10% $^{2}H_2O$, pH 6.0, 298K showing cross peaks for Ser-32 and Ser-35.

tivities are overlapped. Completion of the spin system assignments for these serine residues was made difficult by the absence of $C^{\alpha}H$-$C^{\beta}H$ cross peaks in 2QF COSY and 2Q spectra, probably due to small $^{3}J_{\alpha\beta}$ coupling constants, which are common for serine. The $C^{\alpha}H$–$C^{\beta}H$ region of a 2QF COSY spectrum is shown in Fig. 2. The $C^{\beta}H$-$C^{\beta}H$ cross peak was identifiable for Ser-32 (Fig. 2); this cross peak is not visible for Ser-35 because of overlap with the diagonal, but was identifiable from the 2Q spectrum.

A comparison of the chemical shifts for resonances of the mutant (omitting those of the two altered residues themselves) that were significantly different from those of the wild-type Trx-S_2 and Trx-$(SH)_2$ [9] is shown in Fig. 3. It is evident that a number of side-chain resonances have been affected, as well as 12 amide protons. An extensive effect is seen in the loop between residues 73 and 77, which is in close contact with the active site. In particular, residues Ile-75 and Pro-76, which are highly conserved among thioredoxins [27] and which are in van der Waals contact with the disulfide in Trx-S_2 and the thiol of Cys-35 in Trx-$(SH)_2$, are greatly affected, indicating that the packing of the loops in the vicinity of the active site is altered in the mutant. This alteration is probably quite subtle, since the number of resonances significantly affected is quite small, and the changes in chemical shift are also quite small, of the same order as those between Trx-$(SH)_2$ and Trx-S_2. Structural differences between the two forms of the wild-type protein are extremely subtle [6], and even the backbone dynamics are almost identical, with the exception of the loop from Arg-73 to Ile-75 [12]. The results for the C32S,C35S mutant indicate that it too has a close resemblance to the

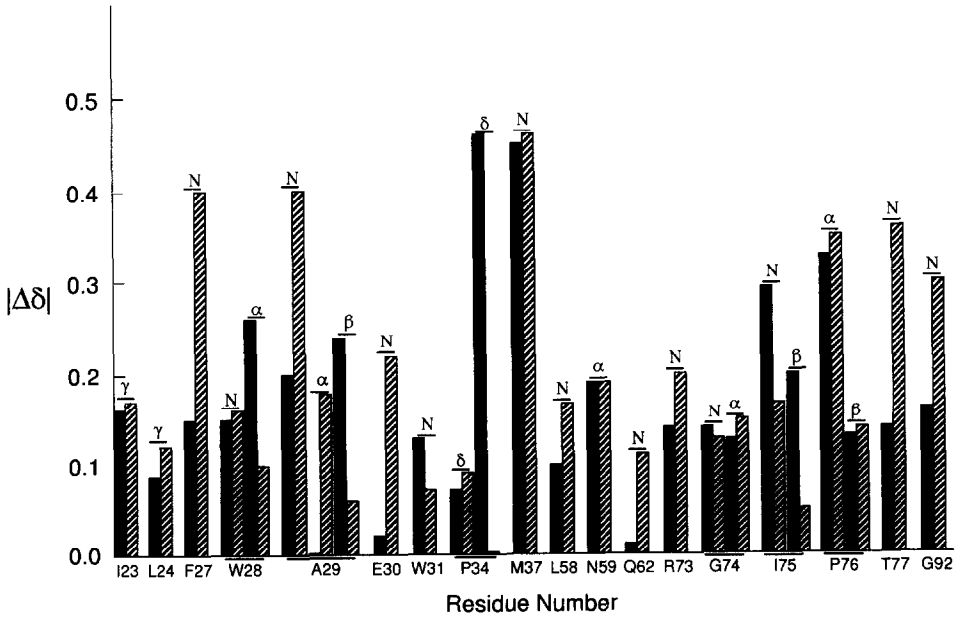

Fig. 3. Comparison of chemical shifts of selected resonances in the C32S,C35S mutant with those of the corresponding protons in Trx-$(SH)_2$ and Trx-S_2. Filled and hatched bars show the absolute value of the chemical shift difference $|\Delta\delta|$ in ppm between the chemical shifts for the mutant and wild-type Trx-$(SH)_2$ and Trx-S_2, respectively.

wild-type protein. Since there is no possibility of disulfide bond formation in the mutant, we expect it to resemble most closely the reduced wild-type protein. Evidence for this is observed in the spectra in ^2H_2O.

A number of amide protons in the C32S,C35S mutant are persistent after buffer exchange into ^2H_2O. Exchange rates were not measured, but the persistent amide protons correspond almost exactly with the slowest-exchanging amide protons of reduced thioredoxin ([9], M.-F. Jeng and H.J. Dyson, unpublished). In particular, the amide proton of Leu 42, which is persistent in wild-type Trx-S_2 but has a significantly faster exchange rate in Trx-$(SH)_2$, was not observed in the spectra of the mutant in ^2H_2O. We infer from this that the structure and dynamics of the mutant resemble those of the wild-type reduced protein.

3.2. pH-dependence of the NMR spectrum

In the C32S,C35S mutant the conformation is unconstrained by the disulfide bond, similar to the reduced wild-type protein. However, the pH-dependence of resonances in the vicinity of the active site should resemble that of the oxidized protein, since the cysteine thiol groups are not available for titration. As for Trx-S_2, the influence of 3 titrating groups is seen in the spectrum of the mutant C32S,C35S as the pH is raised from 5.5 to 10. The N-terminal amine group (average pK_a 7.5) influences the resonances of Ser-1, Asp-2 and Ile-4, and the imidazole group of His-6 (average pK_a 6.4) influences His-6 and Ala-56. These results are identical to those obtained for Trx-S_2 and Trx-$(SH)_2$ [14]. A large number of resonances close to the active-site serine residues are influenced by the titration of a group with average pK_a 8.3; the values obtained for each proton are shown in Table 2. Plots of the variation in chemical shift with pH for the resonances of Asp-26 are shown in Fig. 4. It is noticeable that the proton resonances that are affected by the titration of this group are the same ones that are affected by changes in pH in the wild-type protein [14].

The extent of the chemical shift difference $\Delta\delta$ for a given resonance in the protonated and unprotonated forms of the protein is about the same in the mutant and wild-type, but the largest pH-dependent changes occur for different resonances. The pattern of pH-dependent behavior of the resonances of Asp-26 is almost identical in wild-type Trx-S_2 and the mutant: the $C^\alpha H$, $C^\beta H1$ and $C^\beta H2$ have a total chemical shift change over the pH range ($\Delta\delta$) of 0.21, 0.30 and 0.07 ppm respectively in Trx-S_2 [14], while the corresponding values for the C32S,C35S mutant are 0.20, 0.35 and 0.09 (Table 2). This similarity, together with the overall similarity in the set of protons influenced by the titration in the vicinity of the active site, indicates that the same group is probably titrating in the mutant as in wild-type Trx-S_2, that is, the carboxyl group of Asp-26.

3.3. Structure of the C32S,C35S mutant protein

The pK_a of the titrating group (8.3) is significantly shifted from the value observed in wild-type Trx-S_2 (7.5) and the value inferred for wild-type Trx-$(SH)_2$ (7.1) [14]. Solvation energy [16] and electrostatics (D. Bashford, unpublished) calculations of the expected pK_a for the buried carboxyl group in the environment seen in the 3-dimensional structures of Trx-S_2 and Trx-$(SH)_2$ give values close to 10, about 6 pH units higher than normal

Table 2
Titration data for thioredoxin double mutant C32S,C35S

Residue	Proton	pK_a	$\Delta\delta^1$
Val-25	$C^\alpha H$	7.8	0.10
	$C^\beta H$	8.5	0.07
Asp-26	$C^\alpha H$	8.3	0.20
	$C^\beta H$	8.3	0.35
	$C^\beta H$	8.4	0.09
Phe-27	$C^\alpha H$	8.2	0.05
	$C^\beta H$	8.6	0.04
	$C^\delta H$	8.0	0.07
	$C^\zeta H$	7.8	0.03
Trp-28	$C^\varepsilon H$	8.0	0.06
Ala-29	$C^\alpha H$	8.2	0.06
	$C^\beta H$	8.3	0.04
Glu-30	$C^\alpha H$	7.8	0.05
	$C^\beta H$	8.0	0.07
Ser-32			
Gly-33	$C^\alpha H$	8.6	0.08
Pro-34	$C^\alpha H$	8.7	0.05
	$C^\beta H$	8.4	0.06
Ser-35			
Ile-38	$C^\alpha H$	8.8	0.09
	$C^\beta H$	8.8	0.05
Ala-39	$C^\alpha H$	8.1	0.13
	$C^\beta H$	7.8	0.06
Ile-41	$C^\beta H$	8.3	0.08
	$C^\gamma H$	8.5	0.04
Leu-42	$C^\beta H$	8.5	0.05
Lys-57	$C^\alpha H$	8.3	0.05
	$C^\beta H$	8.2	0.14
	$C^\delta H$	8.1	0.17
Leu-58	$C^\alpha H$	7.4	0.09
	$C^\beta H$	8.3	0.11
	$C^\gamma H$	7.4	0.11
	$C^\delta H$	8.2	0.04
Asn-63	$C^\beta H$	7.9	0.08
Tyr-70	$C^\beta H$	8.1	0.06
Gly-74	$C^\alpha H$	8.2	0.19
Leu-78	$C^\beta H$	8.3	0.13
Thr-89	$C^\alpha H$	9.2	0.07
Lys-90	$C^\alpha H$	7.8	0.03
	$C^\beta H$	8.8	0.21
Val-91	$C^\beta H$	8.3	0.08
	$C^\gamma H$	8.1	0.08
Gly-92	$C^\alpha H$	9.3	0.06
	$C^\alpha H$	8.0	0.05
Ala-93	$C^\alpha H$	8.3	0.05
	$C^\beta H$	8.9	0.04

[1] $\Delta\delta$ is the total change in chemical shift of a given resonance between the protonated and unprotonated states of the protein, calculated as the difference between the extrema δ_{HA} and δ_A obtained from the least squares fit to Eqn. (1).

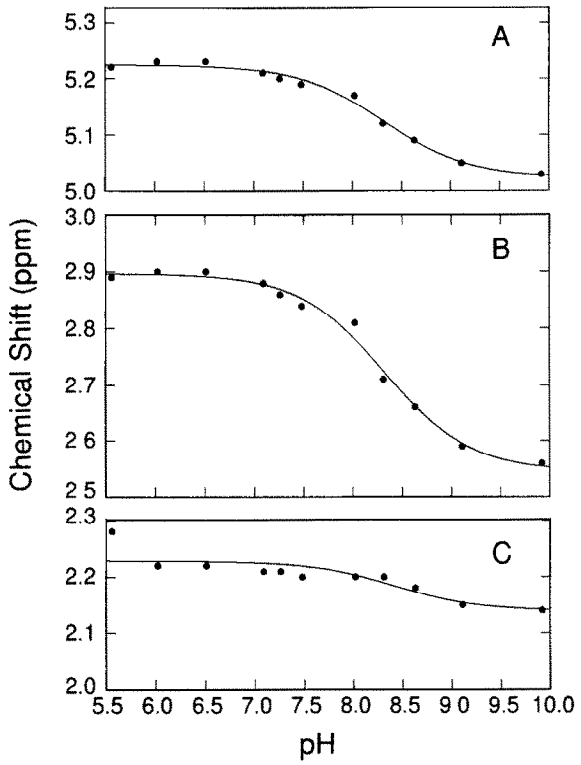

Fig. 4. Plot of the chemical shifts of the (A) $C^{\alpha}H$ and (B,C) $C^{\beta}H$ resonances of Asp-26 as a function of pH for the C32S,C35S mutant. Solid curves were computed by a least-squares fit to the data according to Equation 1.

values for solvent exposed carboxyl groups. The pK_as observed in wild-type thioredoxin are lower by about 3 pH units than these calculated values; this may indicate that the hydrophobicity of the environment of the carboxyl group is lower than would appear likely from the structures, possibly due to the presence of a salt bridge between the charged side chains of Asp-26 and Lys-57 [16] and/or the presence of a water molecule, as observed for reduced human thioredoxin [28]. The pKa value observed for the mutant is higher than for the wild-type, an indication that the carboxyl group is buried in a more hydrophobic environment.

At first sight, a more hydrophobic environment would not be expected in this region in the mutant. The cysteine thiols in Trx-$(SH)_2$ and the disulfide in Trx-S_2 are close to the Asp-26 carboxyl group [5,6], and the ^1H resonances of both cysteines show the influence of the titration of this group [14]. The substitution of serine for the two cysteine residues assumes that the CH_2-OH side chain is the most similar electronically and conformationally to the CH_2-SH side chain of the cysteine. In practice, these residues often behave very differently due to the small size and electronegativity of the hydroxyl oxygen, which makes the serine residue considerably more hydrophilic than cysteine (although not more acidic, especially in thioredoxin). The difference in general behavior found for these two amino acids is illustrated by the preferred χ_1 dihedral angle most commonly found, for serine, +60°, and for cysteine −60° or 180° [29]. It is therefore not perhaps surprising that the hydrophobic packing in the vicinity of the active site appears to be altered as a result of the double mutation of the cysteine residues to serine. However, the apparent direction of the alteration, towards greater burial of the Asp-26 carboxyl is at first sight puzzling. The explanation may well lie in a change in the side chain configuration of the serines compared with that of the cysteines in the wild-type structure. The 2QF COSY spectrum (Fig. 2) gives a strong indication that the two serine residues have preferred χ_1 dihedral angles of +60°, (shown by the absence or near-absence of $C^{\alpha}H$-$C^{\beta}H$ cross peaks in the 2QF COSY spectrum due to small $^3J_{\alpha\beta}$ coupling constants). This immediately shows that the side-chain packing must be different in the active site region in the mutant, since the χ_1 dihedral angles for Cys-32 and Cys-35 are 180° and −60°, respectively, in both Trx-S_2 and Trx-$(SH)_2$ [5,11]. Significant pH dependences occur for the resonances of Phe-27, Ile-41, Leu-42 and Thr-89 in the mutant but not in Trx-S_2 or Trx-$(SH)_2$, another indication that the environment of the Asp-26 carboxyl has changed in the mutant. Thus it appears likely that the substitution of serine for cysteine at positions 32 and 35 of thioredoxin has resulted in altered side-chain packing in the vicinity of the active site, probably involving the exposure of the serine hydroxyl groups to solvent, with consequent tighter packing of the hydrophobic pocket containing the Asp-26 carboxyl group, resulting in its higher pK_a and the more widespread effect of the titration of this group in the mutant protein.

4. Conclusions

We have shown that the behavior of the mutant C32S,C35S thioredoxin in solution strongly resembles that of reduced wild-type thioredoxin, consistent with its behavior in the T7 DNA polymerase and filamentous phage assembly systems. This work also confirms that the titrating group near the active site in thioredoxin is most likely the Asp-26 carboxyl group. The majority of the protein is unaffected by the mutation; the overall structure of the molecule remains identical with that of the wild-type protein, as shown by the close correspondence of the NMR spectra of the mutant and wild-type proteins. The observed increase in the pK_a of the Asp-26 carboxyl group, together with the slight differences in the local effects of this titrating group indicate that the side-chain packing in the active-site region is altered as a result of the substitution of the more hydrophilic hydroxyl groups for the cysteine thiols at positions 32 and 35 and the increased solvent exposure of these side chains.

In the active site sequence itself, the mutant protein

apparently retains a structure highly similar to that of the reduced protein. Subtle increases in the polypeptide backbone dynamics were observed for Trx-$(SH)_2$ compared to Trx-S_2 [12]; these were interpreted as an increased flexibility for the active-site sequence and its contacting loops, consistent with the reported decreased thermal stability of Trx-$(SH)_2$ compared to that of Trx-S_2 [30]. This flexibility was suggested as a possible explanation for the differential reactivity of the oxidized and reduced forms of thioredoxin particularly in complex formation with the gene 5 protein of T7 DNA polymerase: conformations other than the ground-state structure would be accessible for Trx-$(SH)_2$ in the active site region with small energy cost. The similarity between the NMR spectra of Trx-$(SH)_2$ and the C32S,C35S mutant suggests that a similar flexibility is also present in the mutant protein. Despite the slightly altered side-chain packing in the vicinity of the active site in the mutant, the polypeptide chain is still sufficiently flexible to be capable of taking up the (possibly higher-energy) conformations required in T7 DNA polymerase and in the assembly of filamentous phage to give the observed activity of the mutant protein in these systems.

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