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Conformation, stability, and active-site cysteine titrations of *Escherichia coli* D26A thioredoxin probed by Raman spectroscopy

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Abstract

The active-site cysteines (Cys 32 and Cys 35) of *Escherichia coli* thioredoxin are oxidized to a disulfide bridge when the protein mediates substrate reduction. In reduced thioredoxin, Cys 32 and Cys 35 are characterized by abnormally low pK_a values. A conserved side chain, Asp 26, which is sterically accessible to the active site, is also essential to oxidoreductase activity. pK_a values governing cysteine thiol–thiolate equilibria in the mutant thioredoxin, D26A, have been determined by direct Raman spectrophotometric measurement of sulfhydryl ionizations. The results indicate that, in D26A thioredoxin, both sulfhydryls titrate with apparent pK_a values of 7.5 ± 0.2, close to values measured previously for wild-type thioredoxin. Sulfhydryl Raman markers of D26A and wild-type thioredoxin also exhibit similar band shapes, consistent with minimal differences in respective cysteine side-chain conformations and sulfhydryl interactions. The results imply that neither the Cys 32 nor Cys 35 SH donor is hydrogen bonded directly to Asp 26 in the wild-type protein. Additionally, the thioredoxin main-chain conformation is largely conserved with D26A mutation. Conversely, the mutation perturbs Raman bands diagnostic of tryptophan (Trp 28 and Trp 31) orientations and leads to differences in their pH dependencies, implying local conformational differences near the active site. We conclude that, although the carboxyl side chain of Asp 26 neither interacts directly with active-site cysteines nor is responsible for their abnormally low pK_a values, the aspartate side chain may play a role in determining the conformation of the enzyme active site.

Keywords: cysteine; pK_a; Raman spectra; structure; thiol; thiolate; thioredoxin

The thioredoxin fold is an extensively studied structural motif found in a variety of proteins that interact with cysteine-containing substrates (Edman et al., 1985; Martin, 1995). Both the thioredoxin fold and the local sequence at the active site (-W-C-G-P-C-) are highly conserved among thioredoxins of different species (Holmgren, 1985; Martin, 1995). In *Escherichia coli*, thioredoxin functions not only as an oxidoreductase, but also as a host regulator of prokaryotic viral assembly (reviewed by Holmgren, 1985). The active-site cysteines 32 and 35 in *E. coli* thioredoxin are oxidized to form a cystine bridge when the protein mediates the reduction of a suitable substrate. The reduced form of thioredoxin is subsequently regenerated by the enzyme thioredoxin reductase.

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Abbreviations: D26A, *E. coli* thioredoxin with Asp 26 replaced by alanine; ν, Raman band frequency (cm^{-1} units); $\Delta\nu_{1/2}$, Raman band half-width measured as the full band width at half the maximum band intensity.

The X-ray crystal structure of oxidized, wild-type thioredoxin has been solved at 1.68 Å resolution (Katti et al., 1990) and a solution structure has been determined by multidimensional NMR methods (Dyson et al., 1990; Jeng et al., 1994). The protein fold comprises a five-stranded β-sheet core and four α-helical segments. Structural information on the reduced form of the protein is available from both NMR and Raman spectroscopy (Dyson et al., 1990; Li et al., 1993; Chandrasekhar et al., 1994; Jeng et al., 1994). The spectroscopic results show that the tertiary structure is largely conserved between oxidized and reduced forms of thioredoxin, although some conformational rearrangements occur within and near the active site.

The only two cysteine residues of *E. coli* thioredoxin form a disulfide bond in the oxidized protein and are present as free cysteines in reduced thioredoxin. In the NMR structure of reduced *E. coli* thioredoxin, Cys 32 is more exposed to solvent than is Cys 35 (Chandrasekhar et al., 1994). Raman spectroscopy shows that the SH group of each cysteine is a hydrogen bond donor, Cys 35 being assigned as a marginally stronger donor than Cys 32

(Li et al., 1993). This conclusion is based upon the observation that the Raman SH stretching frequency of Cys 35 (2,562 cm^{-1}) is slightly lower than that of Cys 32 (2,571 cm^{-1}) (Fig. 5 of Li et al., 1993) and the fact that the Raman SH stretching frequency is correlated with the strength of hydrogen bond donation (Li & Thomas, 1991).

Considerable interest has been focused recently on the titrating groups in the vicinity of the active site, including the side chain of Asp 26, which is conserved among most thioredoxins. It is widely accepted that oxidoreductase activity requires a thiolate ion in reduced thioredoxin at physiological pH (Kallis & Holmgren, 1980), which would be provided if at least one cysteine has a lowered pK_a value. The pK_a values of Cys 32, Cys 35, and Asp 26 are critical issues in the biological function of the enzyme, and are presently the subject of a literature controversy (Wilson et al., 1995; Jeng & Dyson, 1996; LeMaster, 1996; Takahashi & Creighton, 1996; Dyson et al., 1997). Chemical alkylation (Kallis & Holmgren, 1980) and NMR spectroscopy (Dyson et al., 1991; Wilson et al., 1995) suggest that the pK_a value of at least one active-site cysteine is perturbed well below the normal cysteine pK_a of 8.5. The titration of both cysteines of *E. coli* thioredoxin with $7 < pK_a < 8$ has been demonstrated by Raman spectroscopy, which monitors directly the thiol–thiolate equilibria of the native structure (Li et al., 1993). In the X-ray and NMR structures of *E. coli* thioredoxin, the Asp 26 side chain is buried and appears to be an unlikely neighbor for an active site that functions through a thiolate intermediate. The energetically unfavorable effect of an aspartate (Asp 26) carboxylate anion in the vicinity of the cysteines is apparently compensated by an elevation of the Asp 26 side chain pK_a to well above the typical value around 4 (Langsetmo et al., 1991a; Wilson et al., 1995). The ionization of Asp 26, with a pK_a of 7.5 in oxidized thioredoxin, is linked to the lower stability of the reduced protein at high pH (Langsetmo et al., 1991a, 1991b; Wilson et al., 1995). It has been proposed that reduction potential in thioredoxin-related enzymes is linked, in general, to the relative thermodynamic stabilities of reduced and oxidized species, and that reduction potential in *E. coli* thioredoxin, in particular, may be modulated by titration of Asp 26 (Langsetmo et al., 1991a, 1991b).

In order to further assess the role of Asp 26 in the structure and stability of the enzyme active site, we have investigated the thioredoxin mutant, D26A, by Raman spectroscopy. The goals of this study are, first, to determine the cysteine pK_a values of the mutant protein by direct Raman spectrophotometric titration for comparison with the Raman-determined cysteine pK_a values of wild-type thioredoxin, and second, to monitor any structural differences between D26A and wild-type thioredoxin.

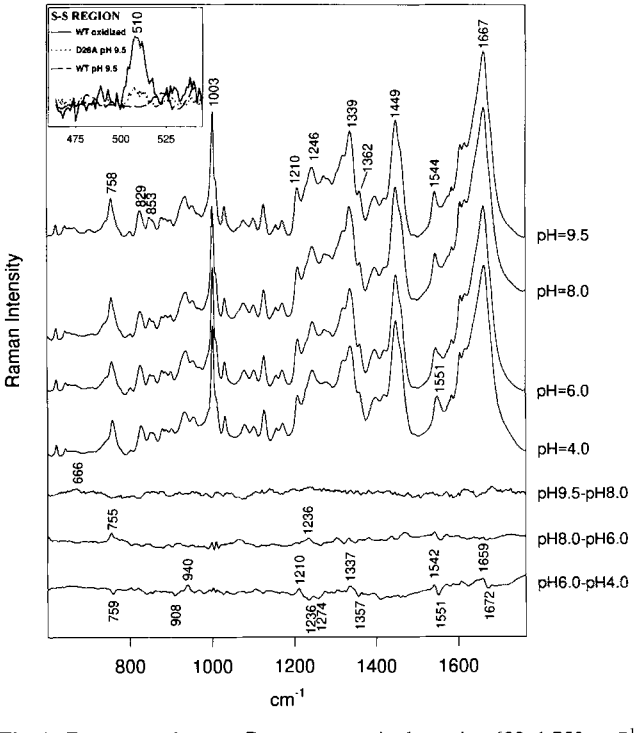

Fig. 1. From top to bottom: Raman spectra in the region 600–1,750 cm^{-1} of reduced D26A *E. coli* thioredoxin in H_2O solution at pH values of 9.5, 8.0, 6.0, and 4.0, and corresponding difference spectra. In each case, the protein concentration is ≈80 mg/mL, sample temperature is 10 °C, and the spectrum is corrected for contributions of the aqueous buffer (see text). Labels indicate frequencies of prominent and pH-sensitive Raman bands, which are assigned as indicated in Table 1. The inset compares the Raman profile of D26A at pH 9.5 in the region of S-S stretching markers with corresponding profiles of fully reduced and fully oxidized wild-type thioredoxin to illustrate the sensitivity of D26A to partial oxidation at high pH.

Results

Figure 1 shows Raman spectra of reduced D26A at pH values between 4.0 and 9.5, along with difference spectra corresponding to the indicated intervals of pH. Alterations in structure detected by the difference spectra occur mainly in the range $4 < pH < 8$. Raman assignments for reduced D26A (Table 1) are based upon previous results for the wild-type protein (Li et al., 1993). As in the case of the wild-type protein, many bands in the Raman spectrum of D26A (region 600–1,800 cm^{-1}) are pH dependent. In all D26A samples, the protein was reduced by equilibration with excess dithiothreitol, as described previously for wild-type thioredoxin (Li et al., 1993). The thiol reagent was removed by chromatography on a size-exclusion column in N_2-equilibrated buffers. During the course of the Raman experiments, any re-oxidation was detected by bands near 510 and 666 cm^{-1}, assigned respectively to S-S and C-S stretching vibrations (Sugeta et al., 1973; Li et al., 1993) that reflect the formation of disulfide bonds. In very similar Raman experiments with wild-type thioredoxin, no indication of disulfide bond formation was observed (Li et al., 1993). In D26A, however, partial (≤15%) oxidation of samples investigated at pH > 9 was detected by the emergence of Raman markers near 510 and 666 cm^{-1} (Fig. 1). D26A is susceptible to cysteine oxidation even in the presence of high concentrations of β-mercaptoethanol (up to ~5 mM), indicating greater sensitivity for disulfide bond formation in D26A than in wild-type thioredoxin above pH 9.

Amide bands

One of the most distinctive spectral changes induced in reduced D26A by elevating the pH from 4 to 6 is a shift of amide I intensity from 1,672 cm^{-1} (β-strand) to 1,659 cm^{-1}. It is accompanied by a shift of amide III intensity from 1,236 to both 1,274 and 1,337 cm^{-1}. These small amide I and amide III perturbations indicate a conversion of some (≈2%) β-strand structure into different, probably less ordered, conformations (Bandekar, 1992). Such concerted amide I and amide III band changes are not observed when the pH is further elevated from 6 to 9.5, suggesting no further secondary structure change above pH 6. The conformational

Table 1. *Raman frequencies, intensities, and assignments of the mutant thioredoxin D26A*[a]

Frequency	Intensity	Assignments[b]
622	1.1	F
644	0.9	Y
668	0.7	C-S stretch
758	3.9	W
829	2.7	Y
853	2.2	Y
880	2.2	W
900	2.1	A
930–950	3.2	C-C stretch
955	2.8	N-C stretch
1,003	9.5	F
1,010	1.0 s	W
1,031	2.6	F
1,060–1,080	1.9	D,E,K, C-C-C stretch
1,102	1.5	CH_3 def
1,127	3.0	W, CH_3 def
1,158	1.3	CH_3 def
1,173	1.8	Y, CH_3 def
1,210	3.6	Y,W
1,246	6.5	Amide III
1,275	5.1	Amide III, Y
1,283	5.0	Amide III
1,322	6.5	CH_2 def
1,339	7.6	W, amide III
1,401	3.4	CO_2^- stretch
1,449	9.7	CH_2 def
1,551	3.0	W
1,585	1.6	F, W
1,607	3.4	F
1,620	3.5	W, Y
1,667	10	Amide I
1,740	—	C=O stretch of COOH
2,331	<1	N=N stretch (frequency and intensity standard)
2,567	<1 b	S-H stretch

[a]Frequencies, in cm^{-1} units, are from the spectrum of D26A thioredoxin collected at pH 4; intensities are arbitrary on a 0–10 scale; s, shoulder; b, broad band.

[b]Only major contributors are indicated; one-letter codes are used for amino acids.

change in D26A between pH 4 and 6 is not observed in wild type (Li et al., 1993). These results show that, unlike wild-type thioredoxin, the overall secondary structure of reduced D26A is invariant to titrations above pH 6.

Tryptophan markers

Difference bands in Figure 1 near 755, 1,340, and 1,360 cm^{-1} coincide with prominent marker bands of the tryptophan side chains (residues Trp 28 and Trp 31) that are diagnostic of indole ring amphipathic environment (Miura et al., 1989). Sensitivity of the tryptophan marker bands to changes of pH in the range $4 < pH < 8$ (Fig. 1) indicates that, upon titration of reduced D26A in this pH range, structural changes take place affecting the average environments and interactions of Trp 28 and Trp 31. Similar, although less dramatic, spectral changes have been noted for tryptophan markers of wild-type thioredoxin (Li et al., 1993). Both Trp 28 and Trp 31 also contribute to a Raman marker in the 1,540–1,555 cm^{-1} interval that is diagnostic of the indole side-chain torsion $|\chi^{2,1}|$ (Miura et al., 1989). The data of Figure 1 indicate that the average value of $|\chi^{2,1}|$ in D26A thioredoxin changes from ~100° at pH 4 (1,551 cm^{-1} marker) to ~80° at pH 8 (1,544 cm^{-1} marker). However, in the range $8 < pH < 9.5$, none of the tryptophan markers of D26A is altered significantly, whereas those of the wild-type enzyme exhibit large changes (Li et al., 1993).

The above results show that, unlike wild-type thioredoxin, the local structure in the vicinity of Trp 28 and Trp 31 in D26A is sensitive to titration occurring between pH 4 and pH 8, but is invariant to titrations above pH 8.

Thiol markers

We have applied to reduced D26A the methodology developed previously for determination of cysteine pK_a values by direct Raman spectrophotometric measurement of thiol–thiolate equilibria (Li et al., 1993; Tuma et al., 1993). The left panel of Figure 2 shows the pH dependence of the complex Raman band at 2,567 ± 2 cm^{-1}, assigned to the SH bond stretching frequencies of thiol groups of Cys 32 and Cys 35. The frequency, width, and pH dependence of the composite sulfhydryl band of D26A are very similar to the corresponding spectral features of the wild-type protein, indicating that the strength of SH hydrogen bonding and the respective pK_a values of C32 and C35 are very similar in mutant and wild type. Additionally, the integrated intensity of the composite SH band at pH 3.5, when both cysteine thiols are fully protonated, is equal to that of two thiol groups per D26A molecule. On the other hand, the bands of D26A and wild type are not completely superimposable. For example, even at pH 3.5, it is not possible to distinguish in D26A two discrete band components, as was the case for wild-type thioredoxin (see following section). Although some band asymmetry is suggested in Figure 2, it is insufficient to serve as a reliable basis for decomposition of the band envelope into two components, as was employed for wild type (Li et al., 1993). Also, unlike wild-type thioredoxin, which showed a progressive shift of the SH band center as pH was increased, the sulfhydryl band of D26A does not exhibit a significant shift of the band center with increasing pH. In the right panel of Figure 2, the normalized and integrated intensity of the Raman SH band is plotted as a function of pH. The data indicate an apparent pK_a value of 7.5 ± 0.2 for the average thiol of D26A. Based upon the statistical significance of the Figure 2 data, we conclude that pK_a values for Cys 32 and Cys 35 SH groups in D26A are separated by less than 0.5 pH unit. For comparison, in wild-type thioredoxin, similarly determined pK_a values are 7.1 ± 0.2 and 7.9 ± 0.2, with the former ascribed to Cys 32 and the latter to Cys 35 (Li et al., 1993; see also Fig. 4).

Comparison of D26A and wild-type thioredoxin

Raman spectra in the region 600–1,750 cm^{-1} of wild-type thioredoxin and D26A at pH 4.0 are compared in the left panel of Figure 3. Both proteins are fully in the dithiol form. The observed low-intensity difference bands (bottom trace) are due to small structural changes involving both the main chain and side chains, and indicate that the mutation slightly perturbs the native secondary and tertiary structures. Corresponding data at pH 8 are compared

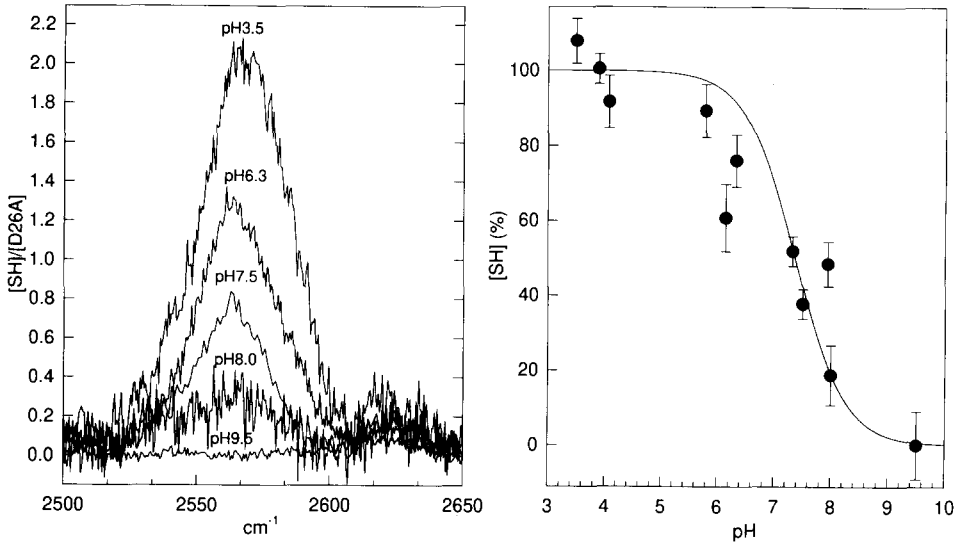

Fig. 2. Titration of SH groups of D26A thioredoxin. Left: Raman spectra in the region 2,500–2,650 cm^{-1} of reduced D26A thioredoxin at the indicated pH values. Below pH 9, no oxidation of the protein could be detected spectrophotometrically. At pH 9.5, up to 15% of the protein may exist in the oxidized form, as judged from the intensity of the Raman band near 510 cm^{-1} (Fig. 1 inset), which is assigned to the cystine disulfide bond. Right: Raman–pH titration curve obtained from spectra shown in the left panel and additional data not shown. The ordinate is the normalized, integrated intensity of the Raman SH band. The data points (●) are averages of multiple experiments performed at each pH, for which the corresponding standard deviations are indicated by error bars. The solid curve is the least-squares fit to the data points and yields an average pK_a of 7.5 for the two thiol groups. (Assuming up to 15% oxidation at pH 9.5, the average pK_a measurement would remain below 7.7.)

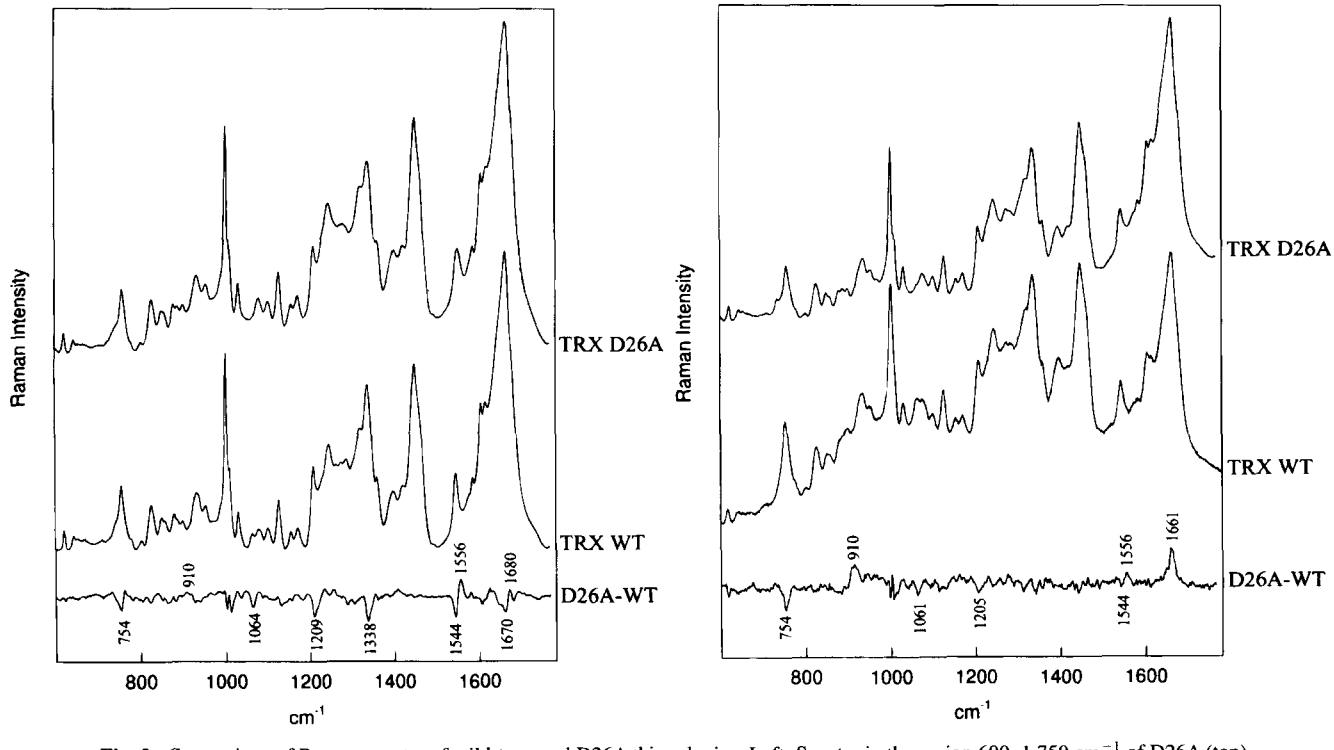

Fig. 3. Comparison of Raman spectra of wild-type and D26A thioredoxins. Left: Spectra in the region 600–1,750 cm^{-1} of D26A (top) and wild type (middle), each at pH 4.0 and 10 °C, and their difference spectrum (bottom). Right: Same as at left, except that data were collected from pH 8.0 solutions.

in the right panel of Figure 3. Here, both proteins are substantially in the dithiolate form. In this case, the observed difference bands (bottom trace) reflect only the side-chain substitution, Asp 26 with alanine, and give no compelling evidence of a change in either secondary or tertiary structure.

Comparison of the difference bands of the dithiol proteins (Fig. 3, left) with assignments of Table 1 shows that the structural effects of the mutation can be summarized as follows. A few peptides (~2% of the total amide I intensity) are converted from β-strand (1,670 cm^{-1} trough) to other conformations; tryptophan environments and indole orientations (754, 1,209, 1,338, and 1,540–1,560 cm^{-1}) are altered; and the change in primary structure is also evident (910 cm^{-1} Ala, 1,064 cm^{-1} Asp). A weak negative band at 1,740 cm^{-1} is detected in the pH 4 difference spectrum. This band could originate from the COOH moiety of Asp 26. Conversely, for the dithiolate proteins (pH 8), only the difference in primary structure is apparent, typified by the positive difference band at 910 cm^{-1} due to Ala 26 in the mutant (Aubrey & Thomas, 1991). At pH 8, no significant differences are observed in either the 1,700–1,800 cm^{-1} interval (COOH) or in the 1,390–1,420 cm^{-1} (COO^-) interval. However, the higher noise level in the pH 8 difference spectrum precludes reaching a firm conclusion about the ionization state of the Asp 26 side chain from the present data.

Because the differences at pH 4 are dominated by tryptophan markers, it seems clear that Trp 28 and Trp 31 are located within or close to a region of the protein that is perturbed structurally by the mutation, viz. the active site. A similar conclusion has been reached from fluorescence studies of D26A (Hanson, 1995; Dyson et al., 1997).

Figure 4 compares the Raman SH band profiles (2,500–2,650 cm^{-1} interval) of wild-type thioredoxin and D26A, each normalized appropriately to the same intensity standard (Tuma et al., 1993). In D26A, the band is broader, but more symmetrically shaped than its wild-type counterpart, leading to small positive difference lobes toward the high and low frequency sides of the band center. For D26A, the bandwidth $\Delta\nu_{1/2}$ is 37 cm^{-1}, which is 5 cm^{-1} greater than $\Delta\nu_{1/2}$ of wild-type thioredoxin. One explanation for the slightly broader SH band in D26A is that it represents a somewhat greater distribution of SH chemical environments than exists in the wild-type protein. This is consistent with results obtained on cysteine model compounds (Li et al., 1992) and could result from heterogeneity in the conformations of active-site cysteines. This interpretation is also consistent with the pH dependence of tryptophan marker bands of D26A, noted above.

Discussion

In reduced D26A, and in reduced wild-type thioredoxin, both active-site cysteines have pK_a values < 8

The integrated intensity of the Raman SH band of thioredoxin, appropriately normalized (Tuma et al., 1993), is a direct measure of the total molar concentration of the thiol groups of Cys 32 plus Cys 35. Figure 2 (right panel) represents the best fit of the reduced D26A data points to a single thiol–thiolate equilibrium with pK_a = 7.5 ± 0.2. Because the Raman method monitors directly the ionization of both SH bonds, the diminution of the integrated SH band intensity to less than 25% of its maximal value at pH 8 (Fig. 2, left panel) provides firm evidence that both cysteines in reduced D26A have pK_a values near 7.5, well below the typical cysteine pK_a of about 8.6. Similar experiments with wild-type thioredoxin give cysteine pK_a values of 7.1 and 7.9 (Li et al., 1993). Thus, in D26A, as in wild-type thioredoxin, Raman data indicate that both active-site cysteines titrate anomalously, and both have pK_a values between 7 and 8. This is in agreement with the wild-type and D26A cysteine pK_a assignments of Wilson et al. (1995). It is also consistent with D26A data of Dyson et al. (1997), although these authors propose that either both thiols titrate with pK_a between 7 and 8, or one thiol titrates with pK_a near 7.8 and the other with higher than their experimental pH (>10).

The pK_a values of Cys 32 and Cys 35 in wild-type thioredoxin are presently the subject of controversy (Wilson et al., 1995; Jeng & Dyson, 1996; LeMaster, 1996; Takahashi & Creighton, 1996). The issue includes the pK_a of the Asp 26 side chain in reduced thioredoxin. In oxidized thioredoxin, the active-site cysteines are in a disulfide bridge, and the only ionizable active-site residue is Asp 26, which has a pK_a of ~7.5 (Langsetmo et al., 1991a). In reduced thioredoxin, there are three ionizable active-site residues, Asp 26, Cys 32, and Cys 35. Although it is generally agreed that Cys 32 has a pK_a value in the range 6.7–7.5, the pK_a values of Cys 35 and Asp 26 obtained from NMR data are in dispute. Cys 35 and Asp 26 have been assigned values of 7.9 and >9, respectively, by Wilson et al. (1995), and values of ~9 and 7.4, respectively, by Jeng and Dyson (1996). The discrepancy arises from different interpretations of similar NMR data. Complex ^1H and ^{13}C chemical shift versus pH curves of the cysteine CβH group clearly indicate the effects of titration of a group with $pK_a \sim 9$, as well as effects of titrations of other groups with pK_a values in the range 7–8. Comparison of cysteine CβH chemical shift versus pH data in

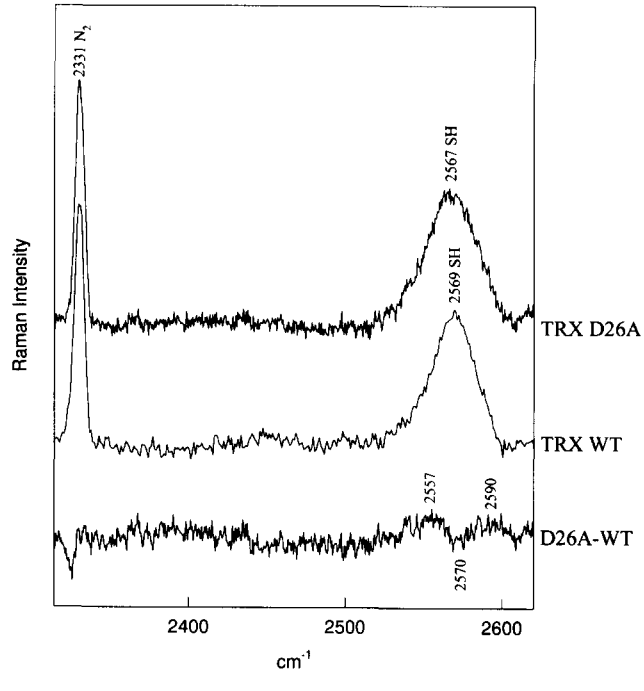

Fig. 4. Raman spectra in the region 2,300–2,650 cm^{-1} of D26A thioredoxin (top) and wild-type thioredoxin (middle) at pH 4.0 and 10 °C. Bottom trace represents the computed difference between top and middle traces. The band at 2,331 cm^{-1} is due to ambient N_2 and serves as a frequency and intensity standard (Tuma et al., 1993). Its residual intensity in the difference spectrum reflects the slightly different protein concentrations in the two samples.

D26A and wild-type thioredoxin led Wilson et al. (1995) to assign the $pK_a > 9$ to Asp 26, because the mutant lacks the titration with $pK_a \sim 9$, and because the Raman-determined pK_a values of wild-type and D26A thioredoxin are consistent with this. A similar pK_a value was measured for Asp 26 in human thioredoxin (Qin et al., 1996). On the other hand, NMR measurement of aspartate chemical shift versus pH for wild-type thioredoxin led Jeng and Dyson (1996) to assign the titration with $pK_a \sim 9$ to Cys 35 and the pK_a of 7.4 to Asp 26. However, we propose that the second inflection in the plot of chemical shift versus pH for Asp 26 (Jeng & Dyson, 1996) is consistent with a higher pK_a for that residue. Further, the pK_a assignments of Dyson and associates for Cys 32, Cys 35, and Asp 26 would mean that the Cys 35 pK_a is normal (~9) in wild type, but anomalous (~7.5) in D26A. A third pK_a value of 11.1 for Cys 35 has been suggested recently on the basis of ^{13}C NMR chemical shift measurements (LeMaster, 1996). In this case, we believe it is likely that the extraordinarily large Cβ chemical shift (2 ppm) assigned to the Cys 35 titration reflects denaturation of thioredoxin above pH 10.

In order for the wild-type pK_a assignments of Jeng and Dyson (1996) to be correct, the Raman data on wild-type thioredoxin (Li et al., 1993) would have to be incorrect. In questioning the Raman-determined thiol pK_a values of reduced wild-type thioredoxin, it has been claimed by Jeng and Dyson (1996) that other Raman data in the same paper indicate that titrations of all carboxyl groups (presumably including Asp 26) are complete below pH 8.0. However, this is a misrepresentation of the results of Li et al. (1993), who make no such assertion. Li et al. (1993), observing the pH behavior of the Raman band (1,710 cm^{-1}) of titrated carboxyls, noted that their data do not permit accurate determination of carboxyl pK_a values, but do show clearly that the titration (of carboxyls) is not complete at pH 7.0. There is no reason to doubt the Raman-determined pK_a values of cysteines in reduced wild-type thioredoxin.

NMR and Raman experiments are complementary; the NMR data reflect all titrating groups influencing the chemical shift of the ^1H or ^{13}C nucleus being monitored, whereas the Raman sulfhydryl marker arises exclusively from the SH species and the loss of Raman band intensity with increasing pH measures directly the dissociation of SH into S^- and H^+. The pK_a values derived from NMR chemical shift titration experiments vary with the nucleus (^1H or ^{13}C) being monitored and, appropriately, have been considered estimates rather than direct determinations of sulfhydryl pK_a (Wilson et al., 1995). A further caution regarding the interpretation of NMR titrations is the possibility that the data may reflect a conformational change distal to the titrating group.

In addition to the controversy over thioredoxin cysteine pK_a values arising from NMR experiments, Takahashi and Creighton (1996) have reached a third conclusion, namely, that both thiol pK_a values are in the region 9–10. They employed UV absorption spectroscopy to measure the pH dependence of the equilibrium constant for reaction of thioredoxin cysteine thiols with glutathione and thiolate. To explain their disagreement with Raman-detected pK_a values, Takahashi and Creighton (1996) suggest that the protein concentrations in the Raman experiment may introduce artifacts. However, this seems unlikely in view of the fact that the Raman experiments were repeated on several thioredoxin samples over a wide range of protein concentrations (50–120 mg/mL) with no detectable change in the thiol–thiolate concentration ratio. Further, we note that agreement between the Raman-detected and NMR-detected pK_a values for D26A supports the notion that the concentration of protein (<10 mg/mL in the latter) is not a determining factor in the Raman experiments. Further, recent UV absorption measurements employing experimental protocols that minimize thiol group photo-oxidation artifacts (Dyson et al., 1997) give data indicating cysteine pK_a values between 7 and 8 for both wild-type and D26A thioredoxins.

Comparison of the cysteine pK_a values in D26A and wild-type thioredoxin provides insight into the role of Asp 26 in determining the pK_a of active-site cysteines. Although the average pK_a values for both cysteines are similar in wild type and D26A, the spread of pK_a values is slightly larger in the wild-type protein (Li et al., 1993; Wilson et al., 1995). From this we conclude that, although Asp 26 influences the spread of cysteine pK_a values in thioredoxin, it is not the sole or major determinant of the depression of cysteine pK_a values. Kortemme and Creighton (1995) suggest that favorable electrostatic interaction of a thiolate ion with a helix macrodipole may facilitate SH ionization and account for a lower cysteine pK_a in thioredoxin. The present results are consistent with such an explanation.

Structural effects of the D26A mutation

Crystal structure data are not available at present for D26A thioredoxin, although NMR spectra indicate that the wild-type and D26A solution structures are quite similar. Nevertheless, small but important differences in side-chain environments may exist between the two. The prominent Raman secondary structure markers (amide I at 1,664 cm^{-1} and amide III at 1,245 cm^{-1}) of wild type and D26A confirm no significant conformational change in thioredoxin with replacement of Asp 26 by alanine. However, although the overall α/β fold is preserved, significant differences are observed in Raman bands sensitive to side-chain environments. As noted above, the SH band of D26A is slightly broader and more symmetrically shaped than that of the wild-type protein (Fig. 4). This difference, although small, is consistent with a broader distribution of Cβ-S-H conformers (Li et al., 1992) in the mutant.

In addition to SH groups, the conformations of tryptophan side chains are detectably different in wild-type and D26A thioredoxin. In the wild-type protein, only a small change in tryptophan conformation is observed between pH 4 and 8 (Li et al., 1993), whereas D26A exhibits a significant change in tryptophan environment in this pH range. Evidently, in wild-type thioredoxin over the range $4 < pH < 8$, Asp 26 participates in an interaction that prevents facile rotation of a tryptophan indole ring. This could result from interaction of the indole with Asp 26 directly, or from an interaction involving Asp 26 and another neighboring protein group that titrates with pK_a in the 4–8 range. Specifically, His 6 is close to van der Waals contact with the indole of Trp 28 (Katti et al., 1990; Chandrasekhar et al., 1994), as can be seen in Figure 5. Involvement of His 6 with an indole is also supported by tryptophan fluorescence measurements conducted on the thioredoxin mutants H6A and D26A (Hanson, 1995). Mutation of Asp 26 presumably eliminates the stabilizing interaction and results in greater sensitivity of indole environment to pH change.

Conclusions

The D26A mutation does not appreciably change either the secondary structure of *E. coli* thioredoxin or the chemistry of the active-site cysteines. In D26A, residues Cys 32 and Cys 35 titrate with anomalously low pK_a values near 7.5, as determined directly by Raman spectrophotometry. The cysteine pK_a values of D26A

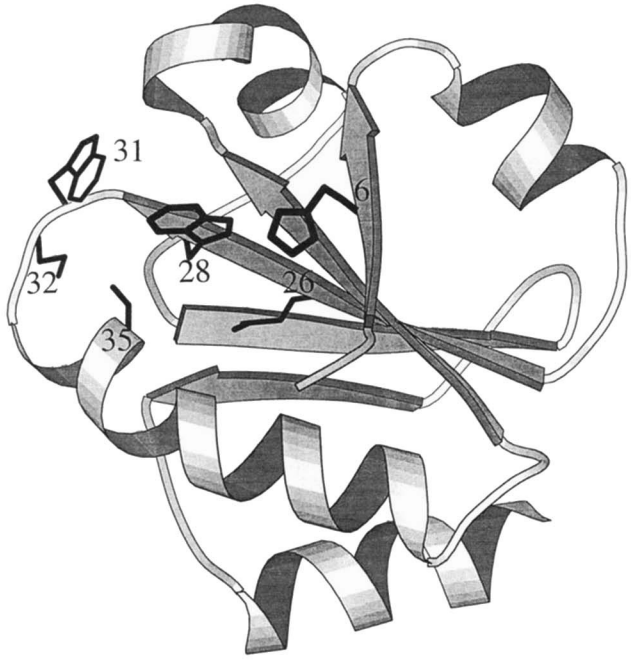

Fig. 5. MOLSCRIPT (Kraulis, 1991) representation of the NMR solution structure of reduced *E. coli* thioredoxin, illustrating the approximate locations of side chains of residues His 6, Asp 26, Trp 28, Trp 31, Cys 32, and Cys 35. From coordinates of the wild-type protein (Jeng et al., 1994).

are close to the pK_a values (7.1 and 7.9) of the wild-type protein, also determined by Raman spectrophotometric titration (Li et al., 1993). Depression of the cysteine pK_a values in *E. coli* thioredoxin is, therefore, not determined primarily by the ionization state of Asp 26.

The Raman SH band profiles of D26A and wild-type thioredoxin are very similar to one another, indicating similar hydrogen bonding environments for their respective thiol groups. On the basis of this finding, residue Asp 26 of wild-type thioredoxin is unlikely to be a direct hydrogen bonding partner for either Cys 32 or Cys 35. The very small difference between Raman SH band profiles of D26A and wild-type thioredoxin can be attributed to a slightly more heterogeneous population of active-site conformations for the mutant.

Environments and orientations of tryptophan side chains (Trp 28 and Trp 31) are sensitive to active-site cysteine titrations in both D26A and wild-type thioredoxin. However, the effects are more pronounced in D26A, particularly over the range $4 < pH < 8$. This finding supports the observation (Langsetmo et al., 1991a) that the replacement of Asp 26 with alanine leads to conformational disorder near the active site. Thus, although the side-chain carboxyl of Asp 26 does not interact directly with an active-site cysteine and apparently is not responsible for the abnormally low cysteine pK_a values, the Asp 26 carboxyl may play a role in determining the enzyme redox potential by modulating local structure and global stability (Langsetmo et al., 1990).

Materials and methods

The mutant (D26A) thioredoxin gene was expressed using a taq-kan vector in *E. coli* host cells grown on rich media. The expression was induced with isopropylthio-β-D-galactoside for 3 h. Cells were harvested and the protein purified according to protocols used previously for wild-type thioredoxin (Langsetmo et al., 1990; Li et al., 1993). The resulting protein was dialyzed against one of the following buffers: 10 mM sodium citrate ($pH \approx 4$), 10 mM sodium acetate ($4 < pH < 6$), or 10 mM Tris-HCl ($pH > 6$), each including a low concentration (<3 mM) of β-mercaptoethanol to prevent oxidation. The protein solution was concentrated under N_2 to ≈80–100 mg/mL using an Amicon concentrator and the final protein concentration was determined by measuring A_{280} using a molar extinction coefficient of 13,700 M^{-1} cm^{-1} (Reutimann et al., 1981). The final pH was measured for the concentrated solution, which was quickly loaded into a Kimax #34504 glass capillary and sealed under N_2. All samples were thermostated at 10 °C during collection of Raman spectra (Thomas & Barylski, 1970).

Raman spectra were excited with the 514.5 nm line of an argon laser (Innova 70, Coherent, Inc.) employing 200 mW of radiant power at the sample. Spectra were collected on a model 1877 triple spectrograph (Spex Industries, Inc.) equipped with a liquid nitrogen-cooled charge-coupled device detector (Model ST130, Princeton Instruments). Signal-to-noise ratios were improved by repetitive spectral collections using 1 min accumulation time. The spectral resolution of signal-averaged data is 6 cm^{-1}. Reported Raman frequencies are accurate to ±1 cm^{-1} for sharp bands and ±2 cm^{-1} for broad bands or poorly resolved shoulders. Additional spectra in the regions 400–700 and 2,300–2,700 cm^{-1} were recorded on a Spex 1401 double spectrophotometer as described previously (Li et al., 1993). The Raman band of N_2 gas (2,330.5 cm^{-1}) was exploited as an external frequency and intensity standard, as described elsewhere (Tuma et al., 1993). The N_2 intensity standard and the known protein concentration of the solutions served as the basis for calculating the thiol–thiolate equilibrium dissociation constant from the Raman SH band at 2,567 cm^{-1}. The apparent pK_a for the thiol–thiolate equilibrium was calculated using the equation: $I_{2567}(pH)/I_{2567}(pH\ 3.5) = 1/(1 + 10^{pH\text{-}pK_a})$, where $I_{2567}(pH)$ indicates the normalized integrated intensity of the Raman SH stretching band (i.e., experimental band area divided by thioredoxin concentration and area of the N_2 band) at a particular pH value, and $I_{2567}(pH\ 3.5)$ is the corresponding intensity at pH 3.5. The data were fit to the above equation by means of a nonlinear least-squares procedure (Pelikán et al., 1994) using commercially available software (NCSS Inc., Kaysville, Utah).

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