Effects of Buried Charged Groups on Cysteine Thiol Ionization and Reactivity in *Escherichia coli* Thioredoxin: Structural and Functional Characterization of Mutants of Asp 26 and Lys 57[†]

H. Jane Dyson,*[,‡] Mei-Fen Jeng,[‡,§] Linda L. Tennant,[‡] Ivan Slaby,[||] Monica Lindell,[||] Dao-Shan Cui,[||] Sergei Kuprin,[||] and Arne Holmgren*[,||]

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-17177 Stockholm, Sweden

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ABSTRACT: To investigate the role of Asp 26 and Lys 57, two conserved, buried residues, in the redox mechanism of *Escherichia coli* thioredoxin (Trx), three mutant proteins, Asp 26 → Ala (D26A), Lys 57 → Met (K57M), and the double mutant D26A/K57M, were prepared, replacing the charged amino acids with hydrophobic residues with similar sizes. Both the oxidized (Trx-S_2) and reduced [Trx-$(SH)_2$] forms of the mutant thioredoxins are fully folded and similar in overall structure to the wild-type protein (wt). The structure of the active site hydrophobic surface is unchanged by the mutation of Asp 26 and Lys 57, since DNA polymerase activity in the 1:1 complex of the T7 gene 5 protein and mutant Trx-$(SH)_2$ shows similar K_d values (∼5 nM) for both mutants and wt. In contrast, redox reactions involving thioredoxin as a catalyst of the reduction of disulfides or oxidation of dithiols are strongly affected by the mutations. In the reaction of Trx-S_2 with thioredoxin reductase at pH 8.0, the k_{cat}/K_m value for the D26A mutant is decreased by a factor of 10 from that of wt, while the value for the D26A/K57M mutant is reduced 40-fold. The activity of Trx-$(SH)_2$ as a protein disulfide reductase was measured with insulin, using fluorescence to detect oxidation of thioredoxin. At 15 °C and pH 8.0, both the D26A and K57M mutants showed 5−10-fold decreases in rates of reaction compared to those of the wild type, and the pH−rate profiles for the mutants were shifted 1 (K57M) and 2 (D26A) units to higher pH compared with the wt curve. NMR measurements for the three mutant proteins indicate that the proteins have the same global fold as that of the wild type, although changes in the chemical shifts of a number of resonances indicate local structural changes in the active site region. The resonances of oxidized D26A and D26A/K57M are pH-independent between pH 6.0 and 10.0, confirming the identification of the active site group titrating with a pK_a of 7.5 in wt Trx-S_2 as Asp 26. A profound change in the pK_a of Asp 26, from 7.5 in the wild type to 9.4 in the mutant, is observed for K57M Trx-S_2. The pH-dependent behavior of the resonances is affected in all mutant Trx-$(SH)_2$ proteins. A single pK_a shifted to higher values is observed on both the Cys 32 and Cys 35 C^β resonances. Ultraviolet absorbance measurements (A_{240}) as a function of pH for wt Trx-$(SH)_2$ demonstrate that the cysteine thiols titrate with apparent pK_as of about 7.1 and 9.9. The mutant proteins each show a single transition in the A_{240} measurements, with a midpoint at pH 7.8−8.0, consistent with the NMR results. The change in absorbance at 240 nm with increasing pH indicates that the number of thiols titrating in each mutant is greater than one but less than two. It is clear that both thiol pK_as have been significantly shifted by the mutations. The Cys 32 pK_a is moved from 7.1 in wt to 7.8−8.0 in the mutants. The value of the Cys 35 pK_a either is indistinguishable from that of Cys 32, thus accounting for more than one thiol titrating in the UV absorbance measurements or else is shifted to much higher pHs (>10) where its transition is masked in both UV and NMR measurements by the effects of ionization of the tyrosine residues and unfolding of the protein. Our results strongly suggest that the buried Asp 26 carboxyl and Lys 57 ϵ-amino groups significantly affect the pK_as of the active site thiols, particularly that of the exposed low-pK_a thiol Cys 32, thereby enhancing the rates of thiol−disulfide reactions at physiological pH.

Thioredoxins, ubiquitous in all living cells, are small (M_r ∼ 12 000) proteins which catalyze a large number of oxidation−reduction reactions via thiol−disulfide exchange by two cysteine residues at the active site (Holmgren, 1985). The reduction potential of thioredoxin is relatively low ($E°$ = −270 mV). Oxidized thioredoxin (Trx-S_2)[1] has a disulfide bridge which is usually reduced by thioredoxin reductase (TR) and NADPH (reaction 1). Reduced thioredoxin [Trx-

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* Authors to whom correspondence should be addressed.

[‡] The Scripps Research Institute.

[§] Present address: Graduate Institute of Cell and Molecular Biology, Taipei Medical College, Taipei, Taiwan, ROC.

[||] Karolinska Institute.

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[1] Abbreviations: NMR, nuclear magnetic resonance; Trx-$(SH)_2$, reduced thioredoxin; Trx-S_2, oxidized thioredoxin; D26A, mutant with Asp 26 converted to Ala; K57M, mutant with Lys 57 converted to Met; D26A/K57M, double mutant with Asp 26 converted to Ala and Lys 57 converted to Met; wt, wild-type; TR, thioredoxin reductase; G5p, gene 5 protein of bacteriophage T7; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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$(SH)_2$] contains two thiol groups and is an efficient protein disulfide reductase (reaction 2).

$$\text{Trx-}S_2 + \text{NADPH} + H^+ \overset{\text{TR}}{\rightleftharpoons} \text{Trx-}(SH)_2 + \text{NADP}^+ \quad (1)$$

$$\text{Trx-}(SH)_2 + \text{protein-}S_2 \rightleftharpoons \text{Trx-}S_2 + \text{protein-}(SH)_2 \quad (2)$$

Thioredoxin from *Escherichia coli* contains 108 amino acids, with the active site sequence $Cys_{32}Gly_{33}Pro_{34}Cys_{35}$. The active site residues and Asp 26 are completely conserved among thioredoxins, while Lys 57 is conserved in prokaryotic thioredoxins and in homologous proteins with thioredoxin domains such as mammalian protein disulfide isomerase (Eklund et al., 1991; Kemmink et al., 1996).

The structures of both oxidized (Katti et al., 1990; Jeng et al., 1994) and reduced (Dyson et al., 1990; Jeng et al., 1994) *E. coli* thioredoxin have been determined at high resolution. The thioredoxin fold consists of a core five-stranded β-sheet flanked by four helices. The active site residues Ala 29−Lys 36 occupy the end of β2, two or three linking residues, and the beginning of helix α2. The structures show that the sulfur of Cys 32 is located on the surface of the protein, while the sulfur of Cys 35 is buried. The structures also show Asp 26 completely buried and in close proximity to the active site, with the closest approach being the carboxyl group of Asp 26 to the sulfur of Cys 35, 5.9 Å in Trx-$(SH)_2$ and 5.6 Å in Trx-S_2 (Jeng et al., 1994).

The reaction of thioredoxin with iodoacetate as a function of pH reveals that only the SH group of Cys 32 is reactive at neutral pH, with a pK_a of 6.7 (Kallis & Holmgren, 1980; Takahashi & Creighton, 1996). Detailed information about the structure and titration behavior of the active site groups in both Trx-S_2 and Trx-$(SH)_2$ has been provided by NMR analysis (Dyson et al., 1991; Jeng et al., 1995; Jeng & Dyson, 1996). An NMR determination of the pH-dependent behavior of both Trx-$(SH)_2$ and Trx-S_2 showed that an additional group besides the two sulfhydryls titrated in the active site region over the same pH range (Dyson et al., 1991). The group was inferred to be the carboxyl group of Asp 26, titrating with a pK_a of ~7. Since the aspartate carboxyl group is completely buried (Katti et al., 1990; Jeng et al., 1994), a greatly shifted pK_a is to be expected. Identification of the titrating groups in Trx-$(SH)_2$ is complicated by the linkage of the thiol titrations to that of Asp 26. Determination of the pK_a values of the thiol groups and of Asp 26 is extremely important since their ionization state influences the stability of the thiolate anion that is required for the formation of a transient mixed disulfide with a target protein disulfide (Kallis & Holmgren, 1980; Holmgren, 1985). These pK_a values also determine the intrinsic reactivities of the sulfur atoms even when they form a disulfide bond (Creighton, 1975; Szajewski & Whitesides, 1980). Recent NMR experiments using selectively labeled thioredoxin showed pK_a values of 7.4 and 9.5 for Cys 32 and Cys 35, respectively, and the presence of a shared proton between the Cys 35 thiol and the Cys 32 thiolate was proposed (Jeng et al., 1995). A different interpretation was made on the basis of the same measurements made by Wilson et al. (1995); both thiol pK_as were interpreted to be below 7.5, and that of Asp was 26 >9. While we believe that the latter interpretation is incorrect in detail, since the pK_a of Asp 26 is demonstrably 7.5 (Jeng & Dyson, 1996), the fact remains that there is strong evidence of thiol titrations at lowered pHs from two independent NMR groups as well as from chemical modification studies (Kallis & Holmgren, 1980) and Raman spectroscopy (Li et al., 1993). A contrasting view arises from a recent equilibrium study using UV absorbance and the equilibrium of thioredoxin with glutathione (Takahashi & Creighton, 1996); it is proposed that the cysteine thiol groups in Trx-$(SH)_2$ both have pK_a values in the region of 9−10 and that their ionization is not strongly linked to that of any other groups. Clearly, there is a need for a clarification of these apparently conflicting interpretations in order to form a correct idea of the mechanism of protein disulfide reduction by Trx-$(SH)_2$.

The stability of wild-type thioredoxin is thermodynamically linked to the titration of the buried Asp 26 (Langsetmo et al., 1991b), and its replacement by Ala markedly enhances the temperature stability of the mutant protein D26A (Langsetmo et al., 1990, 1991a,b). These results indicate that the buried Asp 26 side chain is required for the function of thioredoxin, rather than for structure or stability. Gleason (1992) characterized the D26A thioredoxin and showed a 10-fold higher K_m value for thioredoxin reductase and an unchanged redox potential compared with those of the wild-type protein. In the present paper, we present an extension of Gleason's results, together with a comparison of enzymatic and structural characteristics for two other mutant proteins.

As a possible hydrogen-bonding or salt bridge partner for the buried Asp 26 carboxyl group (which is ~50% charged at neutral pH since its pK_a is 7.5), Lys 57, which is completely buried in close proximity to Asp 26, is also a potentially important residue in influencing the redox mechanism of *E. coli* thioredoxin. By contrast, Asp 26 has been thought to be unimportant in the mechanism of mammalian thioredoxins, in part because of the absence of Lys 57 and in part because the Asp 26 does not appear to titrate abnormally in a M75T mutant human thioredoxin (Forman-Kay et al., 1992). Results with a quadruple mutant (M75T/C62A/C69A/C73A) human thioredoxin indicate that the pK_a of Asp 26 in this system is greatly perturbed, to ~9.9 (Qin et al., 1996), but the structure of the human mutant protein is clearly different from that of the wild-type human protein (Weichsel et al., 1996). Mammalian thioredoxins have a highly conserved lysine residue in place of the Ala at position 39 of bacterial proteins (Eklund et al., 1991) which could provide the required stabilization of the charged or polar Asp 26 carboxyl group. In the present study, the role of Lys 57 has been explored by replacing it with methionine (K57M), which has a similar chain length without the charged ϵ-amino group. We have also generated the double mutant D26A/K57M to test whether the removal of both of these hydrophilic, potentially charged groups would affect the structure and function of thioredoxin. In many ways, the double mutant is the most stringent test of the hypothesis that these charged groups are important to the redox mechanisms of thioredoxin.

Each of the mutants, D26A, K57M, and D26A/K57M, has been characterized in several ways. Both the oxidative and reductive reactions were monitored using standard procedures (Holmgren, 1985); changes in the environment of the two tryptophan residues, which are close to the active site, were detected by changes in fluorescence behavior, and structural changes at the binding site of thioredoxin with the T7 gene 5 protein were monitored by assaying generation of T7 DNA polymerase activity (Slaby & Holmgren, 1991). It is

important to establish whether the mutations at Asp 26 and Lys 57 have caused significant structural changes in the active site region. Structural changes were evaluated by examination of the NMR spectrum, and the pK_as on the titrating groups in the active site were evaluated by changes in NMR chemical shifts with pH. The results obtained for the three mutants of thioredoxin show that the two buried charged groups, Asp 26 and Lys 57, strongly influence the optimum function of thioredoxin as a redox catalyst.

MATERIALS AND METHODS

The D26A mutant thioredoxin was obtained using the *E. coli* JF521 strain with plasmid pUC118-trxA-D26A (Langsetmo et al., 1991a), a generous gift of J. Fuchs (Department of Biochemistry, Gortner Laboratory, St. Paul, MN). The K57M mutant and the D26A/K57M double mutant were prepared by site-directed mutagenesis using the method of Slaby et al. (1996) and expressed in JF521 which is a trxA- mutant strain. The mutations were confirmed by sequencing of the plasmids using a Pharmacia ALF DNA sequencer. Thioredoxin reductase (Russel & Model, 1985) and wt Trx were expressed in overproducing *E. coli* strains and purified as described elsewhere (Dyson et al., 1989; Holmgren & Björnstedt, 1995). Phage T7 gene 5 protein (G5p) was purified from *E. coli* BH 215/pRS 101 (Reutimann et al., 1985) by the Affigel-Trx affinity chromatography method (Slaby & Holmgren, 1991).

Protein concentrations were determined from the absorbance at 280 nm using molar extinction coefficients of 13 700 M^{-1} cm^{-1} for Trx (Holmgren, 1985) and 134 000 M^{-1} cm^{-1} for G5p (Slaby & Holmgren, 1991). Polyacrylamide gel electrophoresis was run on 8 to 12% gradient gels and isoelectric focusing on preformed pH 4.6 to 6.5 gradient gels using the Phast System of Pharmacia Biotechnology (Uppsala, Sweden).

Preparation of Protein for NMR Experiments. Overproduction and labeling of the mutant proteins for NMR spectroscopy were performed in a manner similar to that previously described for the wild-type protein (Chandrasekhar et al., 1991, 1994). For the production of D26A mutant thioredoxin uniformly labeled at levels of ~90−95% with both ^{13}C and ^{15}N, an algal homogenate (Martek Co.) was used as a basis for an enriched medium, since the yield of cells and of thioredoxin from minimal medium containing labeled glucose did not give amounts of material sufficient for NMR experiments. Samples of the mutant proteins semispecifically labeled with [β-^{13}C]cysteine were prepared as previously described (Jeng et al., 1995). Thioredoxin was purified from cell extracts, and samples of the reduced protein were prepared using dithiothreitol as previously described (Dyson et al., 1989). Yields of the protein were comparable to those for the wild-type protein in the same expression system, about 20 mg/L.

Samples for NMR spectroscopy were prepared as previously described (Dyson et al., 1988), in 90% ^1H_2O−10% ^2H_2O or 99% ^2H_2O at pH 6.0 and a protein concentration of 2−4 mM. The pH is slightly higher than that for NMR experiments with the wild-type protein (5.7), as the D26A mutant protein appears to be slightly more susceptible to aggregation at this pH. 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 calibrated at 298 or 308 K for all experiments.

Enzyme Activity Measurements. The activity of Trx-S_2 with *E. coli* thioredoxin reductase was measured at 25 °C with DTNB as a disulfide substrate, using 0.1 M Tris-HCl (pH 8.0)−1 mM EDTA, 0.1 mg/mL bovine serum albumin, 0.4 mM NADPH, 1 mM DTNB, and 5% (v:v) ethanol (Krause & Holmgren, 1991; Holmgren & Björnstedt, 1995). Alternatively, the measurements were conducted using 0.10 M potassium phosphate−EDTA buffer at pH 7.0 with the same substrates. Thioredoxin reductase either in excess (0.10 μM) or at a limiting concentration (0.01 μM) was used for determination of K_m. The background reaction of the enzyme was automatically subtracted from the measurements by adding the enzyme to the blank. Activity was followed at 412 nm in 1 cm cuvettes using a Zeiss PMQIII system with automatic zero control as previously described (Krause & Holmgren, 1991; Holmgren & Björnstedt, 1995).

Insulin was used to measure the protein disulfide reduction activity of thioredoxin, and the reactions were monitored at 340 nm as oxidation of NADPH in the presence of excess thioredoxin reductase (0.1 μM). The mixture contained 0.1 M potassium phosphate buffer−1 mM EDTA (pH 6.0−9.0), 160 μM insulin, and 0.4 mM NADPH. Thioredoxin or mutant proteins were added at 1 μM, and activity was monitored at 340 nm. By using a molar extinction coefficient of 6200 M^{-1} cm^{-1} for NADPH, the rate of the reactions was calculated. From the increase in A_{412}, the rate of reduction of DTNB was calculated using a molar extinction coefficient of 27 200 M^{-1} cm^{-1}, since reduction of DTNB by 1 mol of Trx-$(SH)_2$ yields 2 mol of 3-carboxy-4-nitrobenzenethiol each with a molar extinction coefficient of 13 600 M^{-1} cm^{-1} (Holmgren & Björnstedt, 1995).

The rate of insulin disulfide reduction by Trx-$(SH)_2$ was directly followed under second-order conditions using tryptophan fluorescence measurements as previously described (Krause et al., 1991). The fluorescence at 350 nm of 0.6 μM Trx-$(SH)_2$, prepared by reduction with excess DTT and desalting (Holmgren & Björnstedt, 1995), was recorded vs time after addition of 0.6 μM bovine insulin. All reactions were carried out in 0.1 M potassium phosphate−1 mM EDTA buffer of the desired pH. The rate was calculated from the decrease in fluorescence since insulin lacks tryptophan fluorescence. Second-order rate constants were calculated as described (Krause et al., 1991). T7 DNA polymerase activity generated from mixing T7 gene 5 protein and reduced thioredoxin was examined by the method of Krause and Holmgren (1991).

Fluorescence Measurements. Protein fluorescence was determined with a thermostated Shimadzu RF-510LC spectrofluorimeter at 18 °C. The excitation wavelength was 283 nm, and emission spectra from 300 to 400 nm were recorded. Reduction of Trx-S_2 was achieved by addition of DTT (final concentration of 1 mM). The background of buffers and DTT was subtracted from the spectra. The relative fluorescence at 350 nm following excitation at 283 nm was followed as a function of pH using potassium phosphate buffers with 1 mM EDTA. At low pH, reduction by DTT takes time, and prereduced Trx-$(SH)_2$ was also used.

NMR Experiments. NMR experiments were carried out on Bruker spectrometers operating at 600 or 500 MHz for protons. In order to obtain sequential ^1H assignments of the

mutants by comparison with the spectra of wild-type thioredoxin, three spectra were acquired for a sample in 90% ^1H_2O−10% ^2H_2O: a double-quantum filtered COSY spectrum (2QF COSY; Rance et al., 1983), a two-dimensional nuclear Overhauser effect spectrum (NOESY; Jeener et al., 1979), and a double-quantum spectrum (2Q; Braunschweiler et al., 1983). A series of 2QF COSY spectra in ^2H_2O were acquired at a number of pH values as previously described (Dyson et al., 1991, 1994). Spectral widths were in the range of 5500−7042 Hz for the experiments in 90% ^1H_2O−10% ^2H_2O and 6250 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.

Several two- and three-dimensional spectra were used to obtain ^{15}N and ^{13}C assignments for the D26A mutant protein, including HSQC (Norwood et al., 1990), three-dimensional NOESY-HMQC (Bax et al., 1990), three-dimensional HCCH-COSY (Ikura et al., 1991), HCCH-TOCSY (Bax et al., 1990), and three-dimensional HN(CO)CA (Bax & Ikura, 1991; Grzesiek & Bax, 1992). Standard pulse sequences were used throughout. The spectra generally consisted of 64 (ω_1−ω_3) planes acquired with 128 × 2 free induction decays and 1024 points in the acquisition dimension, using a combined TPPI−States method of phase incrementation. Spectral widths were commonly 7500 Hz in ω_3 and 3600 Hz in ω_1. A spectral width of 5810 Hz was chosen in ω_2 to give maximum digital resolution of the folded data. For the ^{15}N NOESY-HSQC, a total of 64 (ω_1−ω_3) planes were acquired, over a ^{15}N spectral width of 2041 Hz. Spectral widths were 4032 Hz (2K points) and 8064 Hz (256 real points) in ω_3 and ω_1, respectively. For the HN(CO)CA, 64 planes were acquired, over a ^{13}C spectral width of 3771 Hz, with a ^{15}N spectral width (ω_1) of 2410.8 Hz and a ^1H spectral width (ω_3) of 5000 Hz. The data were acquired with a combined TPPI−States phase incrementation protocol (Marion et al., 1989).

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.

Measurement of Thiol Ionization by Ultraviolet Absorbance. Fresh samples of thioredoxin (wt or mutant proteins) (5 mM) were reduced by excess (50 mM) DTT in 0.1 M potassium phosphate−0.1 mM EDTA at pH 7.0 under argon and passed through a PD10 Sephadex column in the same buffer, carefully avoiding any contamination with DTT. Recycling of the same thioredoxin sample resulted in lower values of $\Delta\epsilon$ for the pH titration due to photo-oxidation. A dilution was made under argon to 1.0 mL in a semi-micro cuvette with a 1 cm light path giving a 2 cm column of liquid with a concentration of 30−40 μM ($A_{280} \sim 0.6$) in 0.1 M potassium phosphate−0.1 mM EDTA, and the pH was adjusted up and down by small additions of 1 M NaOH and HCl, respectively. The blank consisted of the same buffer and was treated with exactly the same amounts of HCl and NaOH. Full spectra were recorded from 240 to 340 nm at 25 °C against the blank, and the pH was recorded before and after the measurement using the same glass electrode as was used for the NMR pH titrations. The absorbance at 280 nm and 240 was recorded, together with the mean value of the pH before and after the absorbance measurement. After the final measurement (at pH ∼10), the pH was adjusted to 7.5 and 50 μL of 20 mM DTNB was added to both the sample and the blank in order to evaluate the state of reduction of the solution. The absorbance at 412 nm was determined, and the concentration of free thiol groups was calculated using an ϵ of 27 200 M^{-1} cm^{-1} for the two thiols in thioredoxin (Holmgren & Björnstedt, 1995).

pK_a Measurements. Chemical shift and UV absorbance data as a function of pH were fitted by the method of least squares to a titration curve of the form

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

or, where the data show the influence of two titrations, to a curve of the form

$$\delta = (1/[H^+]^2 + [H^+]K_1 + K_1K_2)(\delta_{H2A}[H^+]^2 + \delta_{HA}K_1[H^+] + \delta_A K_1K_2) \quad (2)$$

as previously described (Dyson et al., 1991, 1994; Jeng et al., 1995). Here, δ is the observed chemical shift or absorbance value at a given pH, n is the number of protons transferred in a given titration step, kept fixed at 1, δ_{H2A}, δ_{HA}, and δ_A are the chemical shifts or absorbances for the various protonated forms of the protein, and K_a, K_1, and K_2 are acid ionization constants for the single-proton titration and for the low-pH and high-pH proton transfers, respectively, in the two-proton titration.

RESULTS

Properties of the Mutant Proteins. The protein product of the mutated *trxA*-D26A gene inserted into the pUC118 vector was expressed in the thioredoxin-negative *E. coli* strain JF 521 and the D26A Trx purified to homogeneity. A yield of 50 mg of the mutant protein per 10 g of wet weight bacteria was obtained. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and isoelectric focusing gels showed one band of D26A Trx with a p*I* of 4.7, identical to that of the wt protein (Krause & Holmgren, 1991) as also reported by Langsetmo et al. (1991a). Thus, replacement of Asp 26 by the neutral alanine residue does not affect the apparent p*I* of the protein, as expected from its low value (p*I* = 4.5) (Holmgren, 1985) and the unusual pK_a of Asp 26; in the wt protein, the Asp 26 carboxyl is uncharged at pH 4.5. Site-directed mutagenesis was used to create the K57M mutant. In a second step, the D26A mutation was also introduced, creating the D26A/K57M double mutant. Expression and purification of the K57M and D26A/K57M mutant proteins in the JF521 strain of *E. coli* produced yields similar to that for D26A, and the proteins were readily purified to produce single bands on SDS−PAGE and isoelectric focusing gels. All of the mutant proteins were free of wild-type thioredoxin since they were produced in Δ*trxA E. coli* cells lacking thioredoxin.

Tryptophan Fluorescence of the Mutant Thioredoxins. The tryptophan fluorescence emission of wt Trx-S_2 increases 3.5-fold upon reduction to Trx-$(SH)_2$ with DTT at pH 7.3 and 18 °C using excitation at 283 nm (Holmgren, 1985) and is shown for comparison in Figure 1. The corresponding tryptophan fluorescence emission intensity of D26A Trx-S_2 is about 50% of that of the oxidized wt protein and increases only 2.7-fold upon reduction. The fluorescence emission maximum of both the oxidized and reduced form of D26A Trx is shifted toward shorter wavelengths. This was also

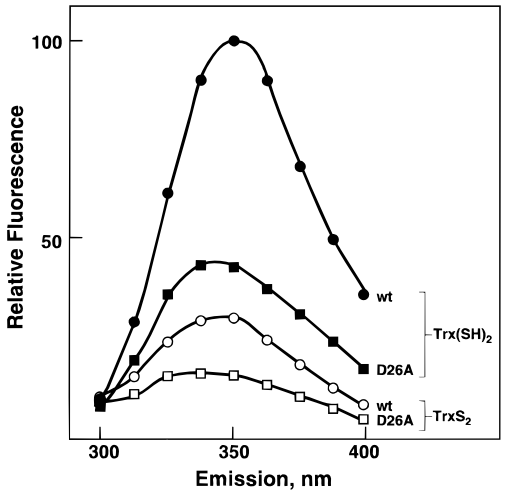

FIGURE 1: Tryptophan fluorescence spectra of D26A thioredoxin at pH 7.3 and 18 °C. Excitation was at 283 nm, and emission spectra of D26A Trx-S_2 (□) and D26A Trx-$(SH)_2$ (■) are presented in relation for equimolar concentrations (1 μM) of wild-type Trx-S_2 (○) and Trx-$(SH)_2$ (●).

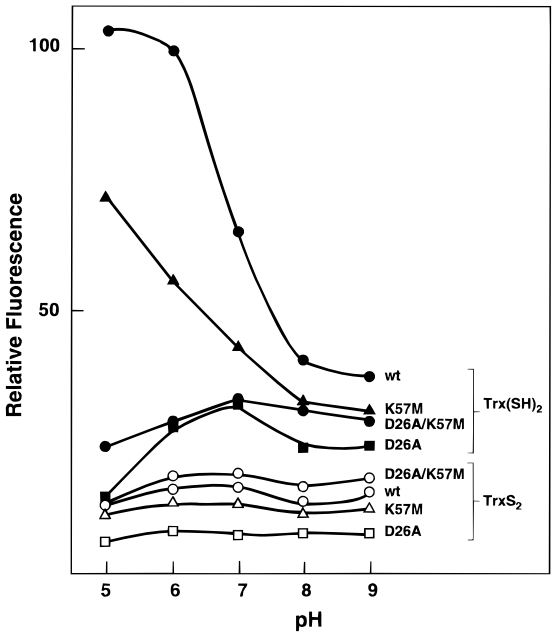

FIGURE 2: Tryptophan fluorescence at 350 nm of 1 μM solutions of wild-type and mutant thioredoxins as a function of pH: wild-type Trx-S_2 (○), wild-type Trx-$(SH)_2$ (●), D26A Trx-S_2 (□), D26A Trx-$(SH)_2$ (■), K57M Trx-S_2 (△), K57M Trx-$(SH)_2$ (▲), D26A/K57M Trx-S_2 (○), and D26A/K57M Trx-$(SH)_2$ (●).

observed by Gleason (1992), who measured the fluorescence properties of D26A Trx at 30 °C.

One striking property of the fluorescence intensity of wild-type Trx-$(SH)_2$ is a dependence on pH (Holmgren, 1985) (Figure 2). The slope of decreasing intensity between pH 5.0 and 8.0 has been attributed to titration of a group with a pK_a of 6.7, which has been attributed to the thiol of Cys 32 (Kallis & Holmgren, 1980). However, the pK_a of Asp 26 is also close to this value, and the crystallographic (Katti et al., 1990) and NMR (Dyson et al., 1990; Jeng et al., 1994) structural data indicate a close proximity between the side chain of Asp 26 and that of Trp 28, which is responsible for the major increase in fluorescence upon reduction of Trx-S_2 (Slaby et al., 1996). The titration of the fluorescence

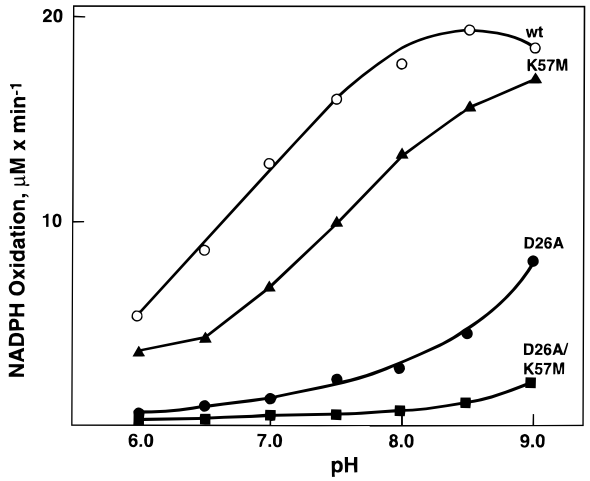

FIGURE 3: Rate of insulin disulfide reduction as a function of pH. The reaction mixture contained 1 μM Trx-$(SH)_2$, 160 μM insulin, 0.1 μM thioredoxin reductase, and 0.4 mM NADPH: wild type (○), D26A (●), K57M (▲), and D26A/K57M (■).

intensity in wild-type Trx-$(SH)_2$ may therefore be due to the effects of titration of Asp 26 rather than to that of Cys 32, which the structures show is far from the Trp 28 side chain. The latter hypothesis is borne out by the behavior of the fluorescence intensity as a function of pH in D26A Trx-$(SH)_2$, which does not exhibit the marked pH-dependent increase between pH 7 and 5 seen in wild-type Trx (Figure 2). The fluorescence emission intensity of K57M Trx-$(SH)_2$ is also reduced compared to that of wt Trx but shows a similar pH-dependent behavior and an increase between pH 7 and 5. Significantly, the behavior of the double mutant D26A/K57M is the same as that of D26A, with no increase in fluorescence yield between pH 7 and 5. We conclude that the large pH-dependent change in fluorescence intensity of wild-type Trx is probably caused mainly by the titration of the carboxyl group of Asp 26, with a possible contribution from Cys 32; the involvement of His 6 as suggested by Jeng et al. (1994) is not confirmed by these results.

Reactions of Oxidized Mutant Thioredoxins with Thioredoxin Reductase. The activity of the oxidized mutant proteins as substrates in a standard thioredoxin assay was compared with that of wt Trx-S_2. Each Trx-S_2 was at a concentration of 1.0 μM, and relatively high (0.1 μM) thioredoxin reductase concentrations were used, together with excess (160 μM) insulin as a protein disulfide acceptor. As seen from Figure 3, D26A Trx-S_2 showed about 10% of the activity of wt Trx-S_2 at pH 7.0, in agreement with the published data of Gleason (1992), who reported that the K_m value for D26A Trx was increased by a factor of 10 and the V_{max} was unchanged at pH 8.0. The K57M Trx-S_2 had about 50% of the activity of wt, but the activity of the double mutant was only about 2% at pH 7.0 (Figure 3). A series of measurements were made at pH values between 6.0 and 9.0 (Figure 3). Clearly, both K57M and D26A, and particularly the D26A/K57M double mutant, show activity profiles that are shifted toward higher pH values, with much reduced activity at all pH values.

Michaelis−Menten kinetics for thioredoxin reductase require assays at lower thioredoxin reductase concentrations (∼10 nM) for observation of substrate saturation behavior and use of a fast oxidant of Trx-$(SH)_2$ such as DTNB for keeping the concentration of Trx-S_2 constant, since the K_m

Table 1: K_m and V_{max} Values for the Reduction of Wild-Type and Mutant Thioredoxins by Thioredoxin Reductase[a]

Trx protein	K_m (μM)	V_{max}[b] (μM min^{-1})	k_{cat}[c] (s^{-1})	k_{cat}/K_m[d] (M^{-1} s^{-1})
wild type	2.5	20	33	8×10^6
D26A	16	12	20	7.5×10^5
K57M	5.0	20	33	4×10^6
D26A/K57M	25	3.7	6	2×10^5

[a] Data were obtained by using the standard DTNB assay at pH 8.0 and 25 °C, using 10 nM thioredoxin reductase and Trx-S_2 concentrations of 1.0, 2.5, 5.0, and 10.0 μM. K_m and V_{max} values were derived from Lineweaver−Burke plots of 1/[S] vs 1/V. [b] Micromolar NADPH oxidized per minute calculated from the ΔA_{412} (inverse minutes) using a molar extinction coefficient of 27 200 M^{-1} cm^{-1}. [c] Calculated per 10 nM enzyme. [d] Apparent second-order rate constant assuming one active site dithiol in thioredoxin reductase (half-site reactivity).

value of thioredoxin for the enzyme is low (~2 μM) (Holmgren, 1985). A set of data for both the wt and the three mutant proteins was generated at pH 8.0 in Tris-HCl−EDTA buffers as shown in Table 1. The values were also used to calculate the k_{cat} of the enzyme and from that the k_{cat}/K_m or apparent second-order rate constant for the reaction between the dithiol of thioredoxin reductase and the disulfide of Trx-S_2 (Holmgren, 1985). The D26A protein clearly has its K_m and V_{max} values changed, with a 10-fold reduction in k_{cat}/K_m. The K_m value for K57M is about twice that of the wt protein, and k_{cat}/K_m is approximately halved. Both K_m and V_{max} are changed for D26A/K57M, and the k_{cat}/K_m value is about 2.5% of the value for wt thioredoxin.

Reactions of Reduced Mutant Proteins with Insulin Disulfides. The catalytic activity of Trx-$(SH)_2$ as a disulfide reductase (reaction 2) can be recorded directly by using the change of tryptophan fluorescence upon oxidation of Trx-$(SH)_2$ at 350 nm under second-order conditions (Krause et al., 1991). Using this method, 0.6 μM Trx-$(SH)_2$ was mixed with 0.6 μM insulin and the time-dependent change in fluorescence was recorded and converted to rate constants at different pH values for the wt, D26A, and K57M Trx. The rate at 15 °C and pH 8.0 for wt Trx is 2.08×10^4 M^{-1} s^{-1}, equivalent to a $t_{1/2}$ of 80 s. The reaction shows a strong dependence on pH with an apparent pK_a value of 6.7 (Figure 4). The rate for wt thioredoxin at pH 5.0 is 1.13×10^3 M^{-1} s^{-1}, still significant. The mutant proteins also show a strong pH dependence, different from that of the wt protein (Figure 4). D26A Trx-$(SH)_2$ shows a low activity at pH 5 and 6 and an increase between pH 7.0 and 8.0, where wt Trx has reached a maximum; this represents an approximate shift of the midpoint by about 2 pH units toward the alkaline side. The increase in the rate for K57M is also shifted relative to that of wt, by about 1 pH unit. Measurements were attempted with the D26A/K57M double mutant, but activity was very low (below 1% of the wt value), and data were difficult to reproduce due to the long observation times required to obtain $t_{1/2}$ measurements. We conclude that both Asp 26 and Lys 57 play a major role in the redox activity of Trx-$(SH)_2$ as a disulfide reductase, particularly in the acceleration of the reaction under physiological conditions (pH 6.0−7.5).

Interactions of the Mutant Proteins with the T7 Gene 5 Protein. Reconstitution of the gene 5 protein−Trx-$(SH)_2$ complex *in vitro* was measured as the stimulation of T7 DNA polymerase activity. Figure 5 shows the T7 DNA polymerase activity generated by addition of the increasing amounts of thioredoxin to a constant (3 nM) concentration of G5p. A numerical expression derived from Scatchard plots for the reconstituting efficiency of Trx gives an observed equilibrium dissociation constant K_{obs} corresponding to the Trx concentration yielding a half-maximum

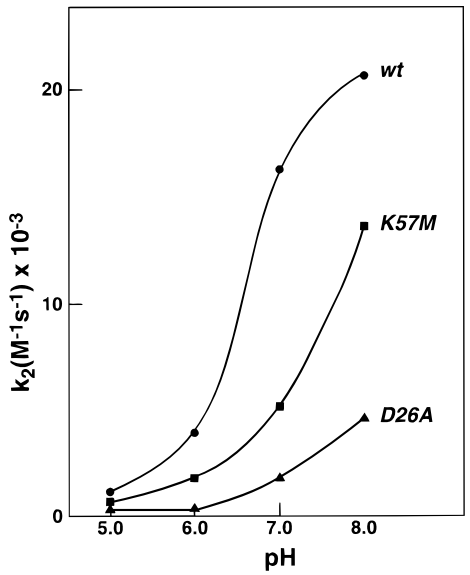

FIGURE 4: Rate of insulin disulfide reduction by wild-type and mutant Trx-$(SH)_2$ as a function of pH. Each reaction contained 0.6 μM prereduced Trx-$(SH)_2$ which was mixed with 0.6 μM insulin at time zero. The decrease in tryptophan fluorescence of thioredoxin was recorded for 5 s each 30 s at 350 nm for up to 10 min. The temperature was 15 °C, and N_2-equilibrated buffers with the designated pH values were used. From the progress curves, half-times in seconds were calculated and second-order rate constants (k_2) were derived: (●) wild-type Trx-$(SH)_2$, (■) D26A mutant Trx-$(SH)_2$, and (▲) K57M mutant Trx-$(SH)_2$. Reaction rates for D26A/K57M were too slow at all pH values for reliable results to be obtained.

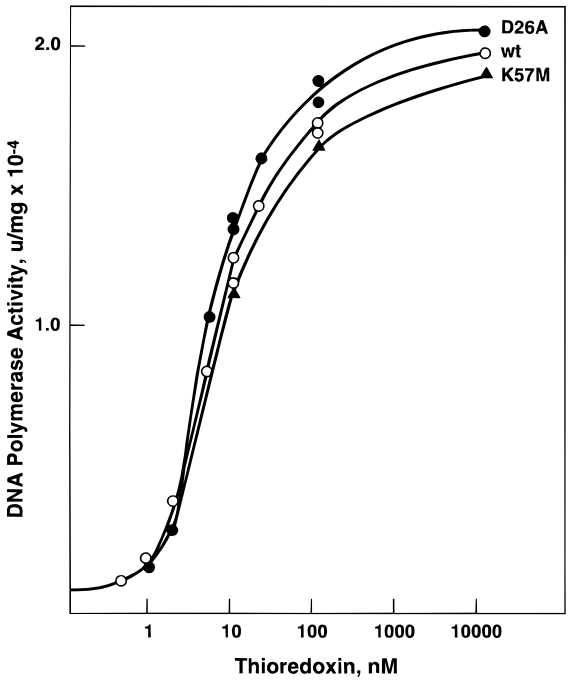

FIGURE 5: Stimulation of DNA polymerase activity of T7 gene 5 protein by thioredoxin. Reaction mixtures contained 3 nM G5p and increasing amounts of wild-type Trx-$(SH)_2$ (○), D26A Trx-$(SH)_2$ (●), or K57M Trx-$(SH)_2$ (▲). Similar results (not shown) were obtained for D26A/K57M.

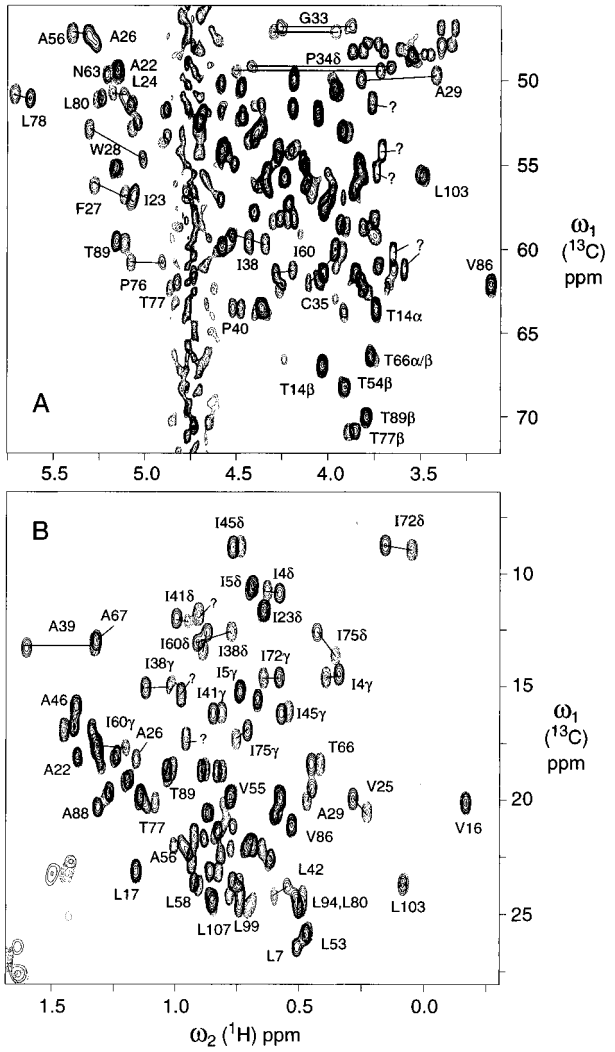

FIGURE 6: Portions of a 500 MHz ^{13}C HSQC spectrum of D26A Trx-$(SH)_2$ (dark lines) superimposed on the spectrum of wild-type Trx-$(SH)_2$ (light lines). Cross-peaks that are significantly changed in the mutant are labeled, with a connecting line to the corresponding cross-peak in the wild-type spectrum. Peaks labeled with ? indicate an impurity: (A) C^α region and (B) methyl region.

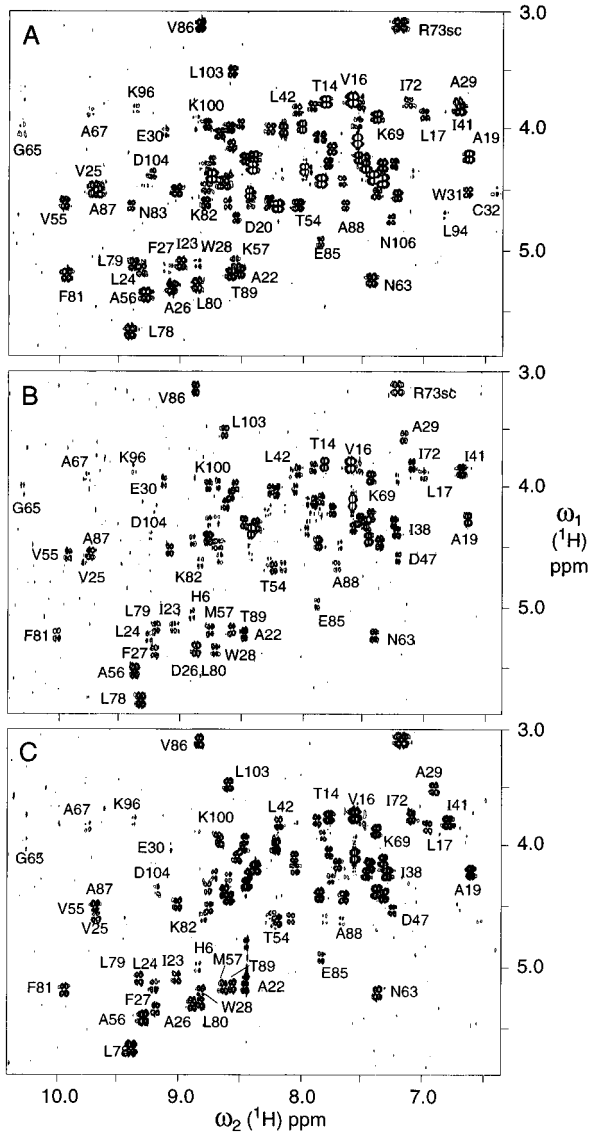

FIGURE 7: Fingerprint region of the 2QF COSY spectrum of (A) D26A Trx-$(SH)_2$, (B) K57M Trx-$(SH)_2$, and (C) D26A/K57M Trx-$(SH)_2$. Selected cross-peaks are labeled. sc is side chain.

polymerase activity (Huber et al., 1986; Krause & Holmgren, 1991). K_{obs} for wild-type Trx-$(SH)_2$ is 5 nM, and values of 4, 5, and 6 nM were measured for the D26A, K57M, and D26A/K57M mutants, respectively. These results indicate that the Asp 26 and Lys 57 residues are not involved in the binding interaction with the T7 gene 5 protein. We conclude that the mutation of charged residues in the hydrophobic core of the protein close to the active site thiols does not affect binding of the gene 5 protein and activation of T7 DNA polymerase which involves the active site surface (Krause & Holmgren, 1991).

NMR Resonance Assignments. An extensive NMR study was made of the reduced D26A mutant thioredoxin, including complete assignment of the backbone ^{1}H, ^{13}C, and ^{15}N resonances and of many of the side chains. Figure 6 shows a comparison of parts of the ^{13}C HSQC spectrum of D26A Trx-$(SH)_2$ with that of the wild-type protein (Chandrasekhar et al., 1994). As for the ^{15}N HSQC spectrum (Jeng & Dyson, 1996), many of the cross-peaks occur in similar or identical positions in the two spectra, indicating that the overall structure of the two molecules is the same. However, there is sufficient difference in the area of interest, close to the active site, that a complete assignment of the spectrum was necessary. This was done using a number of NMR spectroscopic techniques, including triple-resonance spectra. Due to the expense of producing uniformly labeled material using algal homogenates, only unlabeled material was used for ^{1}H backbone assignments of K57M and D26A/K57M. A comparison of the "fingerprint" region of the COSY spectra of the three mutants is shown in Figure 7. Tables of resonance assignments are not included here but have been deposited in the BioMagResBank. In addition, samples of K57M and D26A/K57M semispecifically labeled with [β-^{13}C]cysteine were prepared in order to evaluate the pK_as of the thiols in the Trx-$(SH)_2$ forms of these two mutants.

The magnitude of the chemical shift differences between wild-type and D26A Trx-$(SH)_2$ is illustrated in Figure 8. As observed for all three mutant proteins, the majority of the resonances remain close to those of the wild-type protein, indicating that the overall structure of the protein has not been significantly changed by the mutations. There is a change of 1−2 ppm in the $^{13}C^\alpha$ and 2−4 ppm in the ^{15}N shift for residues 26−28 and smaller changes in the contacting β-strand residues 57−61. Apart from the electronic

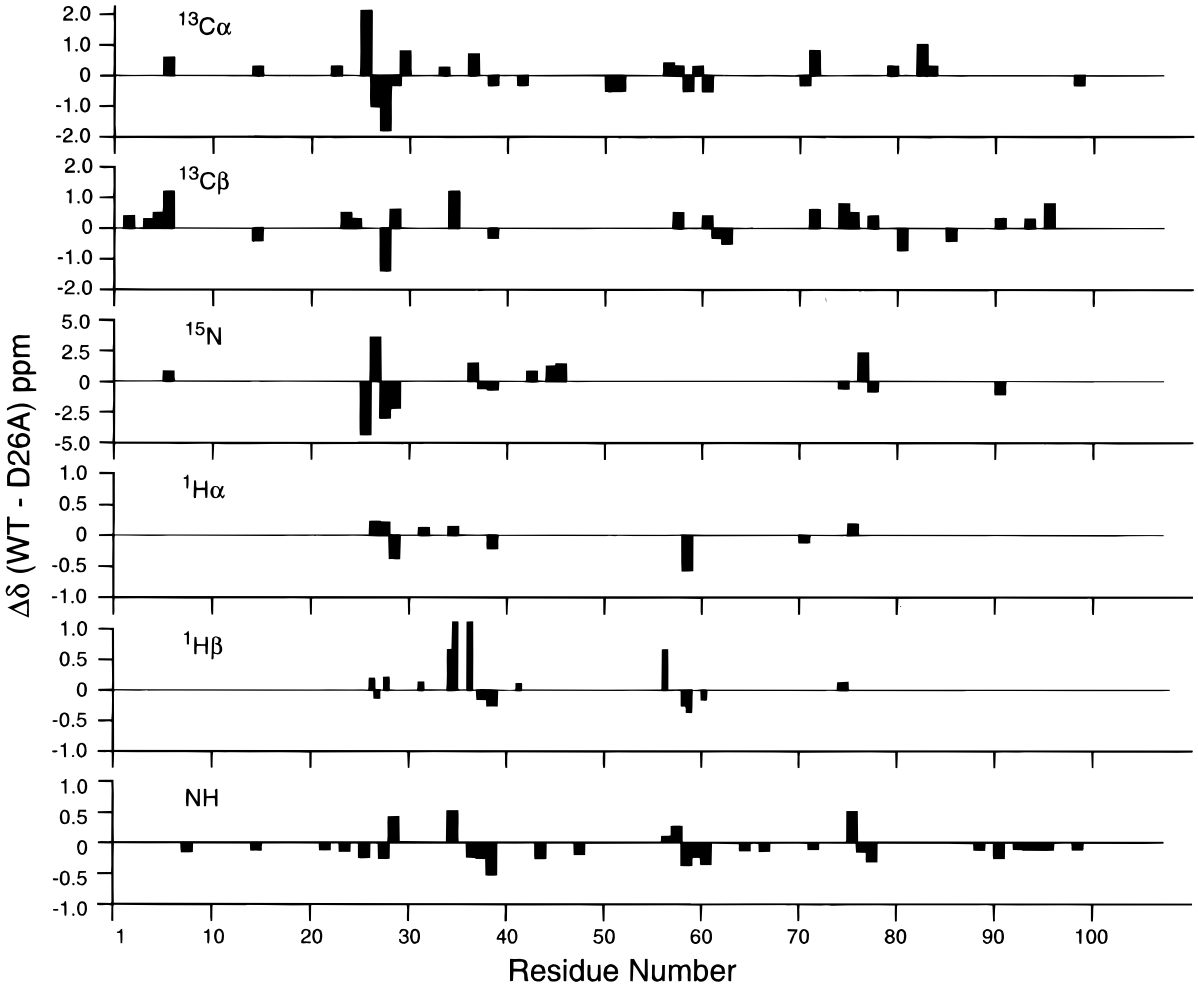

FIGURE 8: Comparison of the chemical shifts of the wild type and D26A mutant Trx-$(SH)_2$ for backbone and β resonances. Each box represents the difference between the value found for D26A subtracted from that of the wild-type protein for the same resonance.

effects directly related to the loss of the Asp 26 carboxyl group, these chemical shift changes may indicate a small rearrangement in the β-sheet in the immediate vicinity of the mutation. Changes have occurred in the structure of the hydrophobic core containing the Asp 26 carboxyl upon mutation of the residue to alanine. The most noticeable changes are in the residues in the helix opposite the residue at position 26 and in the adjacent strand; for example, the largest changes in NH chemical shift apart from the mutated residue and its immediate neighbor are for Ala 29, Ala 39, and Thr 77. The largest changes in proton chemical shifts for D26A Trx-$(SH)_2$ (Figure 8) occur for the $C^{\beta}H$ of Cys 35 and Met 37. This is significant, as it shows that, while the secondary structure of the molecule is preserved, a rearrangement has taken place in the packing of the hydrophobic pocket behind the active site when Asp has been replaced by Ala. This emphasizes the connection between the dynamics of the active site and of this helix, as seen in the hydrogen exchange behavior of thioredoxin (Jeng & Dyson, 1995). Rearrangement of the region behind the active site is consistent with the observed increase in thermodynamic stability of the D26A mutant compared to that of the wild type, which implies that a local structural change has been caused by the mutation (Langsetmo et al., 1990).

Effect of the Mutations on the Solubility of the Proteins at Extremes of pH. One of the more immediately noticeable properties of the mutant proteins is a lower solubility at low pH values compared to that of wt. The wild-type protein aggregates below pH 5.5 (Dyson et al., 1988); for the mutants, the pH below which aggregation is observed, for example, in increased line widths in the NMR spectrum, is shifted upward to approximately 6.0. Most of the resonance assignments for these mutants were therefore made above pH 6. The D26A/K57M mutant protein also has low solubility at high pH, and no NMR measurements were possible above pH 9.

Titrating Groups in the Mutant Thioredoxins. A summary of the pK_as observed for the titrating groups in the three mutant thioredoxins, as calculated from the pH-dependent behavior of a number of ^{1}H and ^{13}C resonances, is shown in Table 1, together with published results for the wild-type protein (Dyson et al., 1991; Jeng et al., 1995) and for a two-cysteine mutant (Dyson et al., 1994). The only groups that titrate in thioredoxin in the pH range of 6−10, apart from the cysteine thiols and Asp 26, are the N-terminal amine group and the side chain of His 6. The N-terminal amine group in all of the mutant proteins titrates with a pK_a indistinguishable from that of wild-type thioredoxin (Dyson et al., 1991). The pK_a for the titration of His 6 was not generally well determined by the mutant data, since it is below the lowest pH that could be used for the mutants, but the behavior of the resonances affected by the His 6 titration appears to be little changed by the mutations.

pH-Dependent Behavior of the Oxidized Mutant Thioredoxins. None of the resonances in the active site region of the oxidized D26A and D26A/K57M mutant proteins was

Table 2: pK_as of Mutant and Wild-Type Thioredoxins from NMR and UV Absorbance Data

	wild type			D26A		K57M		D26A/K57M	
residue	ox	red	C32S/C35S[b]	ox	red	ox	red	ox	red
S1	7.4[a]	7.4[a]	7.6	7.6	7.3	7.7	7.8	7.8	7.8
H6	6.2[a]	5.9[a]	6.3	6.9	6.9	6.3	6.1	6.3	6.2
D26	7.5[a]	7.4[a]	8.4	–	–	[10][d]	9.4	–	–
C32 (NMR)	–	7.4[a]	–	–	8.0[c]	–	8.0[c]	–	8.1[c]
C32 (UV)	–	7.1	–	–	7.8	–	8.1	–	8.0
C35 (NMR)	–	9.5[a]	–	–	[7.7 or >10][c]	–	[8.1 or >10][c]	–	[7.7 or >10][c]
C35 (UV)	–	9.9	–	–	[7.8 or >10]	–	[8.1 or >10]	–	[8.0 or >10]

[a] Data from Dyson et al. (1991) and Jeng et al. (1995). [b] Data from Dyson et al. (1994). [c] Values measured from the C$^\beta$ titrations for C32 and C35 (Figure 9). Compare published values of 7.8 and 7.9 for the two cysteine thiols in D26A (Wilson et al., 1995). The UV absorbance results are equivocal with regard to the number of thiols titrating with a pK_a of ~8.0 in the mutants (see the text). [d] This value is not well-determined by the data; the value given represents an average of several determinations derived from proton data using methods similar to published ones (Dyson et al., 1991).

affected by pH (data not shown). This provides an unequivocal demonstration that the group that titrates with a pK_a of 7.5 in wild-type Trx-S_2 is indeed Asp 26, as has been indicated by a number of previous studies (Langsetmo et al., 1991a,b; Dyson et al., 1991).

A further corroboration of this conclusion is provided by the behavior of the oxidized form of the K57M mutant. This protein retains the Asp 26 carboxyl group, and in the oxidized form, a titration is observed in a number of resonances of protons in the vicinity of the active site. The apparent pK_a of the titrating group, presumably Asp 26, is greatly increased, from 7.5 in the wild-type protein to 9.4. This is a strong indication that the buried salt bridge or hydrogen bond acts in the wild-type protein to lower the pK_a of the buried Asp 26 carboxyl group from the extremely high values to be expected from the extent of burial of the side chain. The solvent exposure of the carboxyl group of Asp 26 in wild-type thioredoxin is ~0.3% in both Trx-S_2 and Trx-$(SH)_2$ (Jeng et al., 1994), which leads to the expectation of a pK_a of >10 from simple electrostatic considerations (D. Bashford, unpublished observations; Langsetmo et al., 1991b). However, our data suggest that, in wild-type thioredoxin, the salt-bridging/hydrogen-bonding interaction of the Asp 26 carboxyl with the Lys 57 ϵ-amino group causes the pK_a to be lowered to the observed value of 7.5. These results are consistent with recent studies on human thioredoxin, which lacks the lysine at position 57; titration of the reduced form of a quadruple mutant form of human thioredoxin showed that Asp 26 had a pK_a of 9.9 in this system (Qin et al., 1996).

The double C32S/C35S mutant (Dyson et al., 1994) shows a pK_a for the Asp 26 carboxyl that is consistent with this; the value is raised to 8.4 (Table 1), which indicates that the carboxyl group continues to be buried in this mutant and that the close proximity of the Cys 35 thiol also influences the pK_a of the Asp 26 carboxyl group in the wild-type protein. A similar effect is seen in a C35A mutant thioredoxin; the Asp 26 pK_a is 8.6, while that of the Cys 32 thiol has been raised to 9.1 (M.-F. Jeng and H. J. Dyson, manuscript in preparation).

pH-Dependent Behavior of the Reduced Mutant Thioredoxins. The pH-dependent behavior of the reduced mutant proteins is more complex than that of the oxidized forms, due to the titration of the two cysteine thiol groups. The pK_as of the cysteine thiols in wild-type Trx-$(SH)_2$ have recently been determined by ^{13}C NMR spectroscopy to be 7.4 and 9.7 (Jeng et al., 1995), and these values were thought to indicate that the two thiols interact closely at neutral pH, probably sharing one of the thiol protons. Similar determinations were made for the three mutants, using ^{13}C-labeled samples of D26A (uniformly labeled) and K57M and D26A/K57M (semispecifically labeled). The pK_a values obtained for each of the mutants are shown in Table 2. The titration behavior of the C$^\beta$ resonances of the two cysteines is shown for each mutant in Figure 9, together with data for the wild-type protein (Jeng et al., 1995), included for comparison. Significant differences from the behavior of the wild-type protein are observed for all three mutant proteins.

For D26A Trx-$(SH)_2$, either the two thiols may have very similar pK_as, close to 8, or the second thiol may titrate out of the accessible pH range following conformational changes of the protein at high pH. Both pK_as at 8 is consistent with our ideas of the assignment of the three pK_as in wild-type Trx-$(SH)_2$; if the Cys 35 thiol titrates at ~9.5−9.9 in the wild-type protein, then the removal of the nearby Asp 26 group will act to lower the Cys 35 thiol pK_a by electrostatic effects. There is evidence for a small pH-dependent change in the cysteine C$^\beta$ chemical shift at high pH in D26A (Figure 9). While it is possible that this may belong to one of the thiols, the change is rather small and in the opposite direction with respect to the other thiol titrations monitored in Figure 9. In addition, the resonances of the Lys 57 side chain begin to shift greatly at pH >9, indicating that the Lys 57 $N^\epsilon H_3^+$ group is probably beginning its titration (see the following section). The observation that changes in chemical shift of the cysteine C$^\beta$ resonances occur at pH 8 for both Cys 32 and Cys 35 is consistent with the two groups titrating with similar pK_as as suggested by Wilson et al. (1995). However, particularly in view of the results of the UV absorbance measurements (see a later section), the possibility that one of the thiols is titrating out of the pH range accessible in these experiments cannot be excluded. If this is so, then the two titration curves may represent the behavior of only one of the thiols, which influences the other because of the close spatial proximity of the two groups.

The pH-dependent behavior of the reduced K57M mutant is more difficult to interpret, due to the three titrations that occur in the active site region. However, the pH-dependent behavior of the two cysteine thiols can be unequivocally determined using a "semispecifically" labeled sample (Jeng et al., 1995), and values of 7.9 and 8.0 are obtained for the cysteine thiol pK_as of K57M (Table 2). As for D26A Trx-$(SH)_2$, the similarity of these values raises the possibility that they represent the titration of only one of the thiols, most likely Cys 32, while Cys 35 titrates out of the pH range

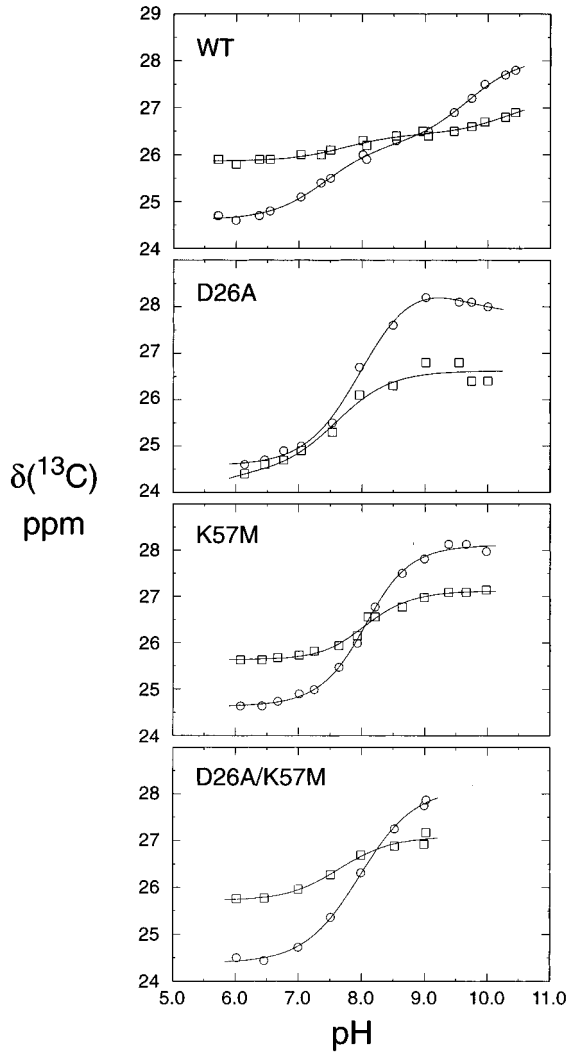

FIGURE 9: Titration data for the $^{13}C^{\beta}$ resonances of Cys 32 (○) and Cys 35 (□) in the reduced state of wild-type (Jeng et al., 1995), D26A, K57M, and D26A/K57M thioredoxin. Data were obtained from semispecifically ^{13}C-labeled material as previously described (Jeng et al., 1995) (wild type, K57M, and D26A/K57M) and from fully ^{13}C-labeled material (D26A). Solid curves were obtained from a nonlinear least-squares fit to the data.

on the alkaline side as a result of protein unfolding or else that the pK_as are coincident.

No direct ^{13}CO measurement of the Asp 26 pK_a was made for K57M. Instead, the value was inferred from the pH-dependent behavior of a number of ^1H resonances in the vicinity of the active site, a method that has been used previously (Dyson et al., 1991). This is illustrated in Figure 10, which clearly shows the effect of two titrating groups on the ^1H resonances of Asp 26 and Pro 76. The lower pK_a in each case is close to 8.0, while the higher pK_a varies between 9.7 and 10.8. This variation is not unexpected since the higher pK_a is not well-determined by the data. It can be seen from Figure 10 that the higher-pK_a titration potentially has a greater amplitude ($\Delta\delta$) for the two Asp 26 resonances than the lower-pK_a titration, especially for the $C^{\beta}H$. This is in contrast to the results for the wild-type protein (Dyson et al., 1991; Jeng & Dyson, 1996), where the lower pK_a observed on the $C^{\beta}H$ has the greater amplitude, consistent with the assignment of the pK_a of the Asp 26 carboxyl to this value, 7.5, in the wild-type protein. For K57M, it appears that the Asp 26 carboxyl pK_a is much higher, at a

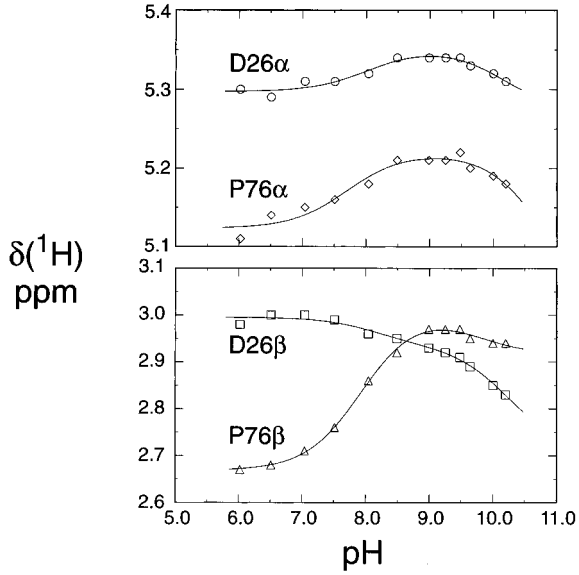

FIGURE 10: Titration curves for selected proton resonances of K57M, showing the effects of the cysteine pK_as at pH ~8 and of an additional pK_a at pH ~10, most probably the Asp 26 pK_a. Solid curves were obtained from a nonlinear least-squares fit to the data.

value of about 10.0. As observed for the wild-type protein (Jeng & Dyson, 1996), the Asp 26 pK_a is quite similar in the oxidized and reduced forms of the K57M mutant. An alternative hypothesis cannot be discounted: that the high-pK_a titration observed for the Asp 26 and Pro 76 ^1H resonances is due to the Cys 35 pK_a. However, there is little apparent change at the high-pH end of the $^{13}C^{\beta}$ titration for Cys 35 itself (Figure 9), leading to the inference that, if not coincident with the first thiol pK_a at 8.0, the second pK_a must be higher than 10.

For the D26A/K57M double mutant, the low stability of the protein at high pH prevented the observation of NMR data above pH 9. A single pK_a was observed on the ^{13}C resonances of both Cys 32 and Cys 35 (Figure 9), and it therefore appears likely either that the two cysteines are titrating with the same pK_a, or that the second pK_a is too high to be observed in this system. It is noticeable that the observed thiol pK_as for the three mutant proteins are all rather similar, suggesting that the mutations of Asp 26 and Lys 57 have similar effects on the pH dependence of the active site cysteines.

Behavior of the Lys 57 ϵ-Amino Group at High pH. The resonances of Lys 57 are distinctive in both the D26A mutant and wild-type proteins (Dyson et al., 1989; Chandrasekhar et al., 1994). In particular, the ^1H and ^{13}C resonances at the γ, δ, and ϵ positions are well-resolved in a ^{13}C HSQC spectrum and can be followed as a function of pH. Representative spectra including some of these resonances are shown for D26A Trx-$(SH)_2$ in Figure 11. Negligible changes are seen in the chemical shifts of the Lys 57 side chain protons as the pH is raised to 9.0. Above this, there are significant changes, especially in the δ and ϵ resonances. We interpret this as evidence of the beginning of the deprotonation of the Lys 57 ϵ-amino group. This interpretation is also consistent with the observed shape of the D26A titration curves in Figure 9, which show a small decrease above pH 9 that might be caused by the titration of another nearby group. Since Lys 57 is not immediately adjacent to Cys 32, it is likely that the observed chemical shift changes

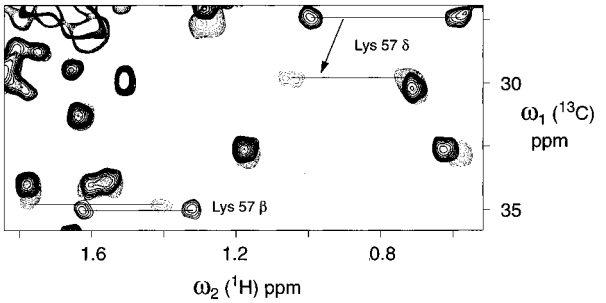

FIGURE 11: Superposition of $^1H-^{13}C$ HSQC spectra of D26A Trx-$(SH)_2$ at pH 7.04 (dark lines) and 10.0 (light lines), showing the change in position of the cross-peaks of Lys 57 with pH. The spectrum remains the same as that of the low-pH form until approximately pH 8.5.

at high pH presage the unfolding of the protein. An alternative explanation is that these changes reflect the titration of the Cys 35 SH at a pK_a of >10. However, we believe that the specificity and magnitude of the changes in the Lys 57 chemical shifts probably indicate that the Lys 57 transition is being monitored here.

Measurements of Thiol Ionization in Wild-Type and Mutant Thioredoxins by UV Spectroscopy. The difference in the ultraviolet extinction coefficient of the thiol and the thiolate anion is about 4000 M^{-1} cm^{-1} at 240 nm (Benesch & Benesch, 1955; Polgar, 1974) and has recently been used to assign pK_a values to the thiols of DsbA (Nelson & Creighton, 1994) and thioredoxin (Takahashi & Creighton, 1996). Since the results of Takahashi and Creighton (1996) do not support a lowered pK_a value for the Cys 32 thiol and since this method should potentially yield quantitative results, we have repeated these measurements for wt Trx-$(SH)_2$, as well as making the measurements for the three mutant proteins. The results are shown in Figure 12. A titration is clearly visible for wt Trx-$(SH)_2$, with a midpoint of 7.1 and a $\Delta\epsilon_{240}$ of 3800 M^{-1} cm^{-1} between pH 6.5 and 8.5. In addition, a second titration is visible with a pK_a of 9.9 and a $\Delta\epsilon_{240}$ of 5500 M^{-1} cm^{-1}. The measurements were performed at a concentration of Trx-$(SH)_2$ of about 40 μM under strict anaerobic conditions and with 0.1 mM EDTA present to prevent oxidation of thioredoxin which would remove the thiolate anion by either disulfide formation or oxidation of the thiol to form a sulfoxide or sulfone. After the final measurements at high pH, the state of reduction of the protein was checked by the addition of DTNB. The wt and D26A proteins were >90% reduced according to this measurement, but a significant amount of oxidation (~20−30%) was observed for the K57M and D26A/K57M mutants after the completion of the pH titration. Lower values of $\Delta\epsilon_{240}$ obtained in successive measurements on the same thioredoxin sample regenerated between runs with DTT indicated that some of these changes are irreversible, probably due to the formation of a sulfoxide or sulfone, which is not capable of reduction by DTT. The results shown in Figure 12 were all obtained with fresh thioredoxin samples. Extrapolation of these results to the low concentrations of protein and EDTA in the samples of Takahashi and Creighton (1996) indicates that the thioredoxin was most probably oxidized at their low protein and EDTA concentrations. Our data unequivocally support the titration of thiols with pK_as of 7.1 and 9.9 in wt Trx-$(SH)_2$. Determination of the pK_a of the second thiol is complicated by the large absorbance changes that occur at high pH as a result of the titration of the tyrosine OH groups

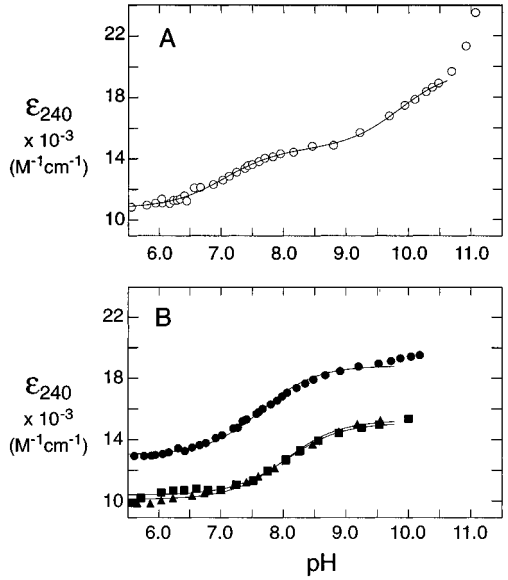

FIGURE 12: Measurements of thiol ionization in thioredoxin by ultraviolet absorbance for (A) wild-type (○) Trx-$(SH)_2$ and (B) D26A (●), K57M (▲), and D26A/K57M (■) Trx-$(SH)_2$. The spectrum from 240 to 320 nm of a solution of Trx-$(SH)_2$ containing 40 μM protein was recorded in 0.1 M potassium phosphate−0.1 mM EDTA at different pH values. ϵ_{240} (M^{-1} cm^{-1}) was calculated at each point from the absorbance and the protein concentration calculated from ϵ_{280} (=13700 M^{-1} cm^{-1}) and the A_{280} at each point, which remained the same throughout the measurements at 0.60 ± 0.03. Solid curves were obtained from a nonlinear least-squares fits to a two-pK_a titration (A) and one-pK_a titrations (B).

and unfolding of the protein, which may account for the relatively high value for the $\Delta\epsilon_{240}$ for the higher of the two pK_as. These results are consistent with published values obtained from NMR (Jeng et al., 1995), which are shown in Table 2 for comparison.

The mutant proteins show titrations with shifted pK_as (Figure 12B) that are entirely consistent with the NMR results of Figure 9; pK_as are shown in Table 2. The UV absorbance measurement has the advantage in that a quantitative estimate of the number of thiols titrating with a given pK_a can be made from the amplitude of the absorbance change at 240 nm ($\Delta\epsilon_{240}$) between the low- and high-pH sides of the curve. The $\Delta\epsilon_{240}$ values obtained for the mutants are greater than those expected for a single thiol (5900 M^{-1} cm^{-1} for D26A, 4700 M^{-1} cm^{-1} for K57M, and 5100 M^{-1} cm^{-1} for D26A/K57M) but are less than would be expected for two thiols (~8000 M^{-1} cm^{-1}). If the extent of photo-oxidation observed (~25% from the DTNB measurements) is taken into account for the K57M and D26A/K57M proteins, the $\Delta\epsilon_{240}$ values become significantly greater than 4000 M^{-1} cm^{-1} (6300−6800 M^{-1} cm^{-1}), equivalent to ~1.5 thiols titrating with a pK_a of ~8 in each mutant.

Two hypotheses could account for these results. In this first hypothesis, the $\Delta\epsilon_{240}$ values are taken to indicate that both of the thiols are titrating with the same or very similar pK_as, consistent with the NMR results (this work; Wilson et al., 1995). The Cys 32 thiol pK_a would be moved up from 7.1 to 8.0, and the Cys 35 pK_a would be moved down from 9.9 to 8.0. One of the major pieces of evidence for a shared proton is the separation of pK_as for two similar groups (Oda et al., 1994); the clear separation of over 2 pH units between the two thiol pK_as in wt Trx-$(SH)_2$ was used to suggest that a proton is shared between the two cysteine sulfur atoms at neutral pH (Jeng et al., 1995). If this is the case, then the

results for the D26A, K57M, and D26A/K57M mutants indicate that the shared proton has been lost in these systems.

An alternative hypothesis is that the high $\Delta\epsilon_{240}$ values obtained for the mutants reflect only uncertainty in the measurements and that the observed differences from the single-thiol value of ~4000 M^{-1} cm^{-1} are insignificant. If this is the case, then the results are consistent with the formation of a single thiolate with a pK_a of ~8.0; the pK_a of ~8.0 observed in the NMR experiments on the C^{β} resonances of both Cys 32 and Cys 35 (Figure 9) would then be a result of the titration of a single thiol group. The most likely group to be titrating is Cys 32, since the amplitude of the chemical shift change (Figure 9) is in all cases greater for Cys 32 C^{β} than for Cys 35. Measurements above pH ~10 were not attempted for the mutants, since at least one of them, D26A/K57M, aggregates at high pH (see the previous section); neither the wild type nor mutant proteins were examined below pH 5.5 for the same reason. If only a single thiol titrates at a pK_a of pH ~8.0 in the mutants, then the second thiol, probably Cys 35, must titrate with a pK_a of >10, out of the observable pH range for either the UV or NMR measurements. Although no information is available on the actual value of the second thiol pK_a for the mutants, a comparison of panels A and B of Figure 12 shows that it must be even higher than the second pK_a in wt Trx-$(SH)_2$, since the absorbance at pH 10 has not begun to rise to the same extent for the mutants (Figure 12B) as for wt (Figure 12A). The possibility that the pK_a of the second thiol is lower than 5 can be discounted in view of the pH dependence of the redox activity of the mutants. These results show that the major effect of the mutation of Asp 26 and Lys 57 is either causing the two thiol pK_as to be shifted, one up and one down, to give identical pK_as of ~8.0 or else causing both pK_as to be shifted to higher pH, one from 7.1 to 8.0 and the other from 9.9 to >10.

DISCUSSION

Effects of the Mutations on the Structure of Thioredoxin. The similarity of the NMR resonance assignments for the wild type and the three mutant proteins (Figures 6−8) is a strong indication that the mutants are folded correctly. The fact that redox activity is observed for all three mutants, albeit at lowered rates, is further corroboration of correct structure. The most compelling piece of evidence showing that the surface structure of these mutant proteins is unaffected by the mutation of the two buried charged residues comes from the T7 DNA polymerase activity measurements.

For T7 DNA polymerase, which is a strong 1:1 complex of Trx-$(SH)_2$ and the 80 kDa gene 5 protein (G5p) and where thioredoxin plays a structural role enhancing polymerase processivity (Tabor et al., 1987), enzyme activity is unaffected by mutation of either Asp 26 or Lys 57. This is significant and shows that the structure of the molecule outside the local hydrophobic pocket that contains these two charged side chains is unaffected by the mutations. The specificity of the binding for the reduced form of thioredoxin initially suggested that the active site thiols were involved in the binding, but later work (Russel & Model, 1986; Huber et al., 1986) showed that the cysteine thiols themselves were not absolutely required, and that the loops containing Gly 92 and Ile 75 were most important for this reaction (Nordström et al., 1981). We have recently suggested (Stone et al., 1993; Jeng & Dyson, 1995) that the difference between the two forms of thioredoxin in binding to gene 5 protein is due to the greater relative mobility of the polypeptide chain in this region of Trx-$(SH)_2$, which allows the molecule to bind to the G5p in a conformation other than the ground state structure found in solution, which is practically identical to that of Trx-S_2 (Jeng et al., 1994). If this is the case, then it is not unexpected that mutations that affect a region of the protein that is not immediately required in binding and is in any case buried would not affect the complex with G5p. Recent data from Biacore surface plasmon resonance experiments show similar K_d values for wt and mutant proteins, but some significant, partly compensating, changed k_{on} and k_{off} rates (N. Singha, A. Vlamis, and A. Holmgren, manuscript in preparation), consistent with changes in the conformational flexibility of wt and mutant proteins.

Effects of the Mutations on the Redox Activity of Thioredoxin. A major function of thioredoxin in cells is catalysis of fast thiol−disulfide exchange reactions in the cytosol, carrying electrons from NADPH via a dithiol in thioredoxin reductase to biosynthetic reactions such as *de novo* synthesis of deoxyribonucleotides by ribonucleotide reductase, another enzyme utilizing redox active cysteine residues (Holmgren, 1985; Mulrooney & Williams, 1994; Sjöberg, 1994). This behavior is in contrast to the specialized function of the protein disulfide isomerases such as mammalian protein disulfide isomerase (Krause et al., 1991; Kemmink et al., 1996) and DsbA from *E. coli* (Martin et al., 1993; Martin, 1995), which have thioredoxin domains that are structurally and functionally similar to thioredoxin but catalyze a process of net disulfide formation in extracytoplasmic proteins concomitant with folding. These proteins reside in a more oxidizing environment such as the lumen of the endoplasmic reticulum or the periplasmic space.

Since the irreversible formation of deoxyribonucleotides required for DNA synthesis in *E. coli* is rapid (>1000 dNTPs used per second in logarithmic growth), and both ribonucleotide reductase and thioredoxin reductase are present in concentrations between 0.1 and 1 μM, thioredoxin has evolved to operate fast in both oxidation of its dithiol and reduction of its disulfide. Thioredoxin is known to react faster by orders of magnitude in thiol−disulfide exchange reactions than low-molecular weight dithiols such as dithiothreitol or glutathione (Holmgren, 1985). Elucidation of the important factors contributing to the mechanism of thioredoxin is challenging, and has been the subject of recent study in several laboratories. The mechanism of disulfide reduction by thioredoxin is thought to involve the sharing of a proton between the sulfurs of the two active site cysteines, which facilitates both the initial nucleophilic attack of the Cys 32 thiolate on the substrate disulfide to form a transient mixed disulfide and the dissociation of the products at the end of the reaction (Kallis & Holmgren, 1980; Jeng et al., 1995). However, two buried, potentially charged groups, the side chains of Asp 26 and Lys 57, are present in the immediate vicinity of the active site. We have shown that removal of these groups strongly impairs the rate enhancement in the redox reactions of thioredoxin at physiological pH.

In its oxidized form, thioredoxin is reduced by the dithiol of thioredoxin reductase (reaction 1) and the apparent second-order rate constant calculated from the k_{cat}/K_m value for the wt protein is 8×10^6 M^{-1} s^{-1} (Table 1). Removing Asp 26 reduces this value to 7.5×10^5 M^{-1} s^{-1}, affecting both K_m

and V_{max}. The removal of Lys 57 has a smaller effect, but the combination of removal of both groups in the D26A/K57M double mutant protein lowers the k_{cat}/K_m value to 2×10^5 M^{-1} s^{-1}or about 2.5% of that of the wt protein. These data were obtained at pH 8.0. At lower pH, closer to physiological values, the reactions were even slower (Figure 3).

In its reduced form, thioredoxin operates as a powerful protein disulfide reductase. The fastest reactions occur with the acceptor disulfide (Cys 754−Cys 759) in ribonucleotide reductase, which is re-formed after each catalytic cycle of the enzyme (Sjöberg, 1994). The K_m value of ribonucleotide reductase for substrate Trx-$(SH)_2$ at the optimum pH of 7.5 is 1.3 μM (Holmgren, 1985). We did not measure the activity of the mutant proteins with ribonucleotide reductase, but Gleason (1992) characterized the D26A mutant protein, which gives a K_m of 33.3 μM and a k_{cat} of 170 min^{-1}, compared to a K_m of 2 μM and a k_{cat} of 178 min^{-1} for wt from the same set of measurements. The k_{cat}/K_m value for D26A was found to be 6% of that of the wt protein.

We have used the reaction of Trx-$(SH)_2$ with insulin to probe the effects of the mutations on another important general function of thioredoxin, protein disulfide reduction. Previous data (Holmgren, 1979) showed a K_m value for insulin of about 11 μM in thioredoxin reductase-coupled reactions and demonstrated that thioredoxin catalyzes the reduction of protein disulfides faster than low-molecular weight disulfides such as cystine or oxidized glutathione (GSSG), which has a K_m value of approximately 0.8 mM and a lower V_{max} (Holmgren, 1979). Figure 4 shows that the direct reaction (reaction 2) under second-order conditions has a rate constant of 2.08×10^4 M^{-1} s^{-1} at pH 8 and 15 °C for wt thioredoxin. Although this is about 2 orders of magnitude slower than for the estimated reaction rate with the disulfide in ribonucleotide reductase, it is still a fast reaction. As seen from Figure 4, there is still significant reaction of wt thioredoxin at pH 5 (1.13×10^3 M^{-1} s^{-1}); this rate is about 10-fold faster than the reaction between GSSG and thioredoxin ($<1 \times 10^2$ M^{-1} s^{-1}) at the high pH of 8.7 (Nikitovic & Holmgren, 1996; Takahashi & Creighton, 1996). The D26A and K57M mutants were both up to 10-fold slower at any pH value (Figure 4). More importantly, with a move up into the physiological pH range from 6.0 to 7.5, wt thioredoxin shows a highly increased reaction rate which is not seen for the mutants. The double mutant D26A/K57M was affected more than would be expected from simple additivity of the effects of the individual mutations; at a 0.6 μM concentration, the reaction was too slow to obtain reliable data. In order for efficient operation in the cell at pH 7.0, reaction rates of the cysteine thiols of wt thioredoxin with enzyme disulfides of 10^5-10^6 M^{-1} s^{-1} and of its disulfide with enzyme dithiols of 10^5-10^6 M^{-1} s^{-1} are required. Removing both Asp 26 and Lys 57 drops both values by factors between 10 and 100, and therefore, the D26A/K57M mutant should not be able to function as a thioredoxin in *E. coli*. One reaction of thioredoxin where these ideas could be tested *in vivo* is in the sulfate reduction system (Russel et al., 1990).

The redox activity of the K57M mutant is not as severely perturbed as in the D26A mutant. This may be rationalized in two ways. First is the fact that the Lys 57 side chain, while important in the overall operation of the redox reaction as shown by the behavior of the double mutant, is of secondary importance relative to that of Asp 26. This is consistent with its lower frequency of evolutionary conservation. Second, the relative extent of the change upon going from lysine to methionine is smaller than in changing aspartic acid for alanine; the side chain of lysine is already largely hydrophobic so that the percentage of the residue actually changed is quite small. Clearly, the positively charged group plays a role in the optimization of the rates of catalysis by thioredoxin, since its removal affects these rates, but as long as Asp 26 and the two cysteines are present, the effect can be compensated for, perhaps by recruitment of another positively charged group such as Lys 36 that does not normally participate in the reaction.

pK_as of the Active Site Thiols in Reduced Thioredoxin. The behavior of Trx-$(SH)_2$ as a function of pH is complicated by the presence of three titrating groups in close proximity in the active site region, the Asp 26 carboxyl and the two cysteine thiols. Site-specific NMR results (Jeng et al., 1995; Jeng & Dyson, 1996) indicate the active site pK_as in wild-type Trx-$(SH)_2$ are 7.4 and 9.4 for the two cysteine thiols and 7.5 for the Asp 26 carboxyl. A recent study by Takahashi and Creighton (1996) puts forward a different hypothesis, that both of the thiol pK_as in Trx-$(SH)_2$ are above 9, on the basis of measurements of the pH dependence of the equilibrium constant for the reaction with glutathione and by the detection of the thiolate anion by ultraviolet absorbance, as well as a repeat of the experiments of Kallis and Holmgren (1980) on chemical modification of the thiols with iodoacetamide. The pH dependence of the chemical modification and the glutathione equilibrium constants both show transitions with pK_as in the region of 6.5−7.5 (Takahashi & Creighton, 1996). However, the ultraviolet absorbance at 240 nm shows only a small transition in this pH range, about 20% of the expected absorbance change. A transition with much greater $\Delta\epsilon_{240}$ is observed at pH >9; this was interpreted as being representative of the ionization of the two cysteine thiols, the major evidence for both pK_as being greater than 9. We have measured the ultraviolet absorbance as a function of pH for wt Trx-$(SH)_2$ as well as for the three mutants. We observe a significant increase in absorbance at 240 nm above pH 10 which we interpret as being due to unfolding of the protein as well as ionization of the two tyrosine residues. In addition, two well-defined transitions with pK_as of 7.1 and 9.9 are observed, each with a $\Delta\epsilon_{240}$ of ∼4000 M^{-1} cm^{-1}, well within the expected range for the formation of a single thiolate (Benesch & Benesch, 1955; Polgar, 1974; Nelson & Creighton, 1994). The pH dependence of the ϵ_{240} (Figure 12A) in fact shows a striking resemblance to those observed by NMR (Figure 9; Jeng et al., 1995). Our results are clearly different from those of Takahashi and Creighton (1996), who observed only 20% of the theoretical $\Delta\epsilon_{240}$ in measurements using much lower final concentrations of both Trx-$(SH)_2$ (3−10 μM) and EDTA (10 μM). Because thioredoxin is sensitive to metal-catalyzed photo-oxidation in UV light, as observed in fluorescence measurements (S. Kuprin and A. Holmgren, unpublished observations), the failure of Takahashi and Creighton (1996) to observe the formation of thiolates with pK_as of 7.1−7.5 and 9.5−9.9 may have been due to photo-oxidation and consequent loss of the thiol(ate) from the solution at higher pH.

Function of the Buried Charged Groups in Thioredoxin. The Asp 26 carboxyl group is in close proximity to the

disulfide bond and to the ϵ-amino group of Lys 57 in the structure of Trx-S_2 (Katti et al., 1990; Jeng et al., 1994) and to the Cys 35 thiol and the Lys 57 amino group in Trx-$(SH)_2$ (Jeng et al., 1994). It is completely buried in both oxidation states. Removal of the Asp 26 carboxyl group increases the thermodynamic stability (Langsetmo et al., 1991b), so the burial of this group is not related to the structural integrity of thioredoxin. Both the pH dependence of the redox reaction rates and the pK_as of the thiol groups are changed in the D26A mutant. It is clear that the Cys 32 thiol pK_a is increased from 7.1 to 7.8−8.0 as a result of the Asp 26 → Ala mutation and that the Cys 35 pK_a is either decreased to 7.8−8.0, consistent with the loss of the shared thiol proton present in wt Trx-$(SH)_2$ at neutral pH, or increased to >10. This implies that the buried aspartate functions to stabilize the thiol pK_as of thioredoxin at appropriate values so that the redox reactions can occur rapidly.

The pK_a of the Asp 26 group in K57M Trx-S_2 is 9.4, considerably increased from the wild-type value of 7.5. This is most probably directly due to the loss of the positively charged ϵ-amino group, whose presence would tend to lower the pK_a of the buried carboxyl group. The pK_a of the Cys 32 thiol in K57M and D26A/K57M Trx-$(SH)_2$ is apparently increased in each case to ∼8, a value similar to that seen for D26A. These results hint that the functional significance of Lys 57 is the regulation of the ionization state of Asp 26, and hence indirectly the active site thiols, at neutral pHs. The absence of the Lys 57 amino group in mammalian thioredoxins may reflect a difference in the function and mechanism of thioredoxin reductase between *E. coli* and mammalian systems. Indeed, there are major differences in the reactivity and structures of *E. coli* and mammalian thioredoxin reductases (Holmgren & Björnstedt, 1995). *E. coli* thioredoxin reductase shows no activity with human thioredoxin and has 35 kDa subunits. In contrast, mammalian thioredoxin reductase has 58 kDa subunits, is active with both mammalian and *E. coli* thioredoxins (Luthman & Holmgren, 1982), and is a selenocysteine-containing enzyme (Tamura & Stadtman, 1996) with a completely different structure (Z. Liangwei, E. Arnér, J. Ljung, F. Åslund, and A. Holmgren, manuscript in preparation).

The mechanism for the reductive reaction of thioredoxin is based on an initial nucleophilic attack of a thiolate (Cys 32) on the substrate (Kallis & Holmgren, 1980; Jeng et al., 1995). In order for this step to be accomplished at neutral pH, the pK_a of the Cys 32 thiol must be sufficiently low so that an appreciable concentration of thiolate will be present. Raising the pK_a of the Cys 32 thiol would effectively lower the efficiency of the initial (probably rate-determining) step of the reaction. Such an increase in pK_a is observed for Cys 32 in the mutants; this is consistent with the observed lower rates of the thioredoxin redox reactions for these mutants at physiological pH.

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