Microscopic pK_a Values of *Escherichia coli* Thioredoxin†

Peter T. Chivers,‡,§ Kenneth E. Prehoda,‡,|| Brian F. Volkman,⊥,‡ Byung-Moon Kim,‡ John L. Markley,⊥,‡ and Ronald T. Raines*,‡

Department of Biochemistry and National Magnetic Resonance Facility at Madison, University of Wisconsin—Madison, Madison, Wisconsin 53706

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ABSTRACT: Thiol:disulfide oxidoreductases have a CXXC motif within their active sites. To initiate the reduction of a substrate disulfide bond, the thiolate form of the N-terminal cysteine residue (CXXC) of this motif performs a nucleophilic attack. *Escherichia coli* thioredoxin [Trx (CGPC)] is the best characterized thiol:disulfide oxidoreductase. Previous determinations of the active-site pK_a values of Trx have led to conflicting interpretations. Here, ^{13}C-NMR spectroscopy, site-specific isotopic labeling, and site-directed mutagenesis were used to demonstrate that analysis of the titration behavior of wild-type Trx requires the invocation of *microscopic* pK_a values for two interacting active-site residues: Asp26 (7.5 and 9.2) and Cys32 (CXXC; 7.5 and 9.2). By contrast, in two Trx variants, D26N Trx and D26L Trx, Cys32 exhibits a pK_a near 7.5 and has a well-defined, single-pK_a titration curve. Similarly, in oxidized wild-type Trx, Asp26 has a pK_a near 7.5. In CVWC and CWGC Trx, Cys32 exhibits a single pK_a near 6.2. In all five enzymes studied here, there is no evidence for a Cys35 (CXXC) pK_a of <11. This study demonstrates that a comprehensive approach must be used to unravel complex titration behavior of the functional groups in a protein.

Thiol:disulfide oxidoreductases that contain the Cys-Xaa-Xaa-Cys (CXXC)[1] motif are common enzymes found in diverse intracellular environments (Chivers et al., 1997, 1998). In reactions catalyzed by thiol:disulfide oxidoreductases, the thiols of the CXXC motif can act as nucleophiles, electrophiles, or leaving groups. The N-terminal cysteine residue (Cys_N) of known CXXC motifs is exposed to solvent, and it is this residue that initiates catalysis by performing an intermolecular nucleophilic attack. Accordingly, the pK_a of Cys_N has a strong influence on catalysis.[2]

In aqueous solution, thiols are most reactive as nucleophiles when their pK_a values are matched to the pH of the reaction mixture: pK_a = pH (Szajewski & Whitesides, 1980; Gilbert, 1990). This optimum arises from the balance between being unprotonated (and therefore reactive) and being nucleophilic. A normal thiol has a pK_a near 9 and is largely protonated [and therefore unreactive (Bednar, 1990)] at physiological pH.

One well-characterized thiol:disulfide oxidoreductase is thioredoxin from *Escherichia coli* [Trx (Holmgren, 1985)]. Previous workers have reported that Cys_N of Trx has a pK_a slightly >7 (Dyson et al., 1991, 1997; Li et al., 1993; Jeng et al., 1995; Wilson et al., 1995). In human Trx, Cys_N has a pK_a of 6.35 (Forman-Kay et al., 1992). Recently, however, Takahashi and Creighton (1996) proposed that Cys32 of *E. coli* Trx has pK_a ≥ 9. Disagreement also exists about the pK_a of Asp26 in reduced Trx (Wilson et al., 1995; Jeng & Dyson, 1996). This residue is proximal to the CXXC motif in the native enzyme (Figure 1).

Here, we have used ^{13}C-NMR spectroscopy, site-specific isotopic labeling, and site-directed mutagenesis to characterize the pH-titration behavior of Trx. Our data provide new insights into the controversial titration behavior of the enzyme. Specifically, we find that the interaction of functional groups within the active site of Trx are best modeled in terms of *microscopic* pK_a values for the side chains of Asp26 and Cys32.

MATERIALS AND METHODS

Strains and Reagents. *E. coli* strain BL21(DE3) was from Novagen (Madison, WI). *Taq* polymerase and restriction endonucleases were from Promega (Madison, WI). Deuterium oxide, deuterium chloride, and potassium deuteroxide were from Isotec (Miamisburg, OH). [3-^{13}C]Cysteine was from Cambridge Isotopes (Andover, MA). Potassium phosphate was from Fisher.

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* Author to whom correspondence should be addressed.

‡ Department of Biochemistry, University of Wisconsin—Madison.

§ WARF and Steenbock Predoctoral Fellow. Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

|| Present address: Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143.

⊥ National Magnetic Resonance Facility at Madison.

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[1] Abbreviations: CXXC, Cys-Xaa-Xaa-Cys; Cys_C, C-terminal cysteine of CXXC; Cys_N, N-terminal cysteine of CXXC; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LB, Luria broth; NMR, nuclear magnetic resonance; *pdi*, protein disulfide isomerase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Trx, *Escherichia coli* thioredoxin.

[2] In addition to their effect on kinetics, the pK_a values of the thiols contribute to the value of $E^{\circ\prime}$ for the corresponding disulfide bond (Chivers et al., 1997). For example, lower pK_a values give rise to higher $E^{\circ\prime}$ values for the CXXC motif of DsbA (Grauschopf et al., 1995).

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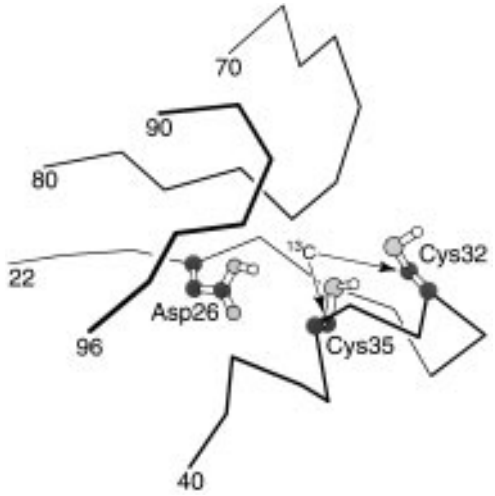

FIGURE 1: Functional groups in the active site of reduced wild-type *E. coli* thioredoxin (Jeng et al., 1994). This figure was created using MOLSCRIPT v1.2 (Kraulis, 1991). To enhance the resolution of ^{13}C NMR experiments, enzymes were enriched with ^{13}C at C^{β} of the two cysteine residues.

Construction of pTRX. YepWL.TRX is a plasmid that directs the expression of wild-type thioredoxin in the endoplasmic reticulum of *Saccharomyces cerevisiae* (Chivers et al., 1996). The gene for Trx was amplified from YepWL.TRX by the PCR with oligonucleotides PC26 (5′-AAGAAGGAGTTATACATACATATGAGCGATA-AAATTATT-3′) and PC27 (5′-GGGGCACCCAACGTC-GACATTCCCTTACGCCAGGTTAGCGTC-3′). The PCR products were cleaved at the underlined sites with *Nde*I and *Sal*I, and the resulting fragments were inserted into pET22b(+) that had been cleaved with the same enzymes. The resulting plasmid, which directs the production of wild-type Trx in the cytoplasm of *E. coli*, is called pTRX.

Previously, we had isolated two variants of *E. coli* Trx that complement *pdi1*Δ *S. cerevisiae* (Chivers et al., 1996). These variants have altered CXXC sequences (CWGC and CVWC) compared to that of wild-type Trx (CGPC). Genes coding for CWGC Trx and CVWC Trx (without the HDEL tails used in the complementation experiments) were constructed by a procedure analogous to that used for the construction of pTRX.

To probe the behavior of Asp26 during the titration of Trx, we created enzymes in which Asp26 was replaced with an isologous asparagine or leucine residue. The genes for D26L and D26N Trx were produced by oligonucleotide-mediated site-directed mutagenesis as described by Chivers (1996) and Chivers and Raines (1997).

Production and Purification of Trx in E. coli. pTRX or a mutant thereof was transformed into *E. coli* BL21(DE3). Plasmid copy number was amplified according to the protocol of Hoffman et al. (1995). Transformed bacteria were used to inoculate fresh LB media (20 mL) containing ampicillin (0.40 mg/mL). For expression of *trxA*, three sequential 1:10^3 dilutions were made into LB media (20 mL) containing ampicillin (0.40 mg/mL), and all three resulting cultures were grown overnight at 37 °C. An aliquot (10 mL) of the least dense culture ($A < 1$ at 600 nm) was used to inoculate 0.5 or 1.0 L cultures of TB media. Expression of *trxA* was induced, when $A = 1.9$ at 600 nm, by the addition of IPTG (to 1 mM). After a 3 h induction period, cells were harvested by centrifugation at 7000*g* for 10 min. The cell pellet was resuspended in lysis buffer (125 mL per 0.5 L of cell culture), which was 20 mM Tris-HCl buffer, pH 7.8, containing urea (6.0 M) and EDTA (1.0 mM). The suspension was shaken for 20 min at 37 °C to ensure complete lysis. The lysate was centrifuged at 30000*g* for 15 min, and the urea-soluble fraction (125 mL) was dialyzed against 4 L of 20 mM Tris-HCl buffer, pH 7.8, containing EDTA (1.0 mM). The dialysate was then centrifuged at 30000*g* for 30 min to remove any precipitate. The resulting supernatant was concentrated using a microconcentrator from Amicon (Beverly, MA) with a YM10 ultrafiltration membrane. The concentrate (<10 mL) was passed through (flow rate = 1.5 mL/min) a Pharmacia FPLC Hi-Load Sephadex G-75 column, which had been equilibrated with 0.10 M potassium phosphate buffer, pH 7.6, containing EDTA (1.0 mM). PAGE performed in the presence of SDS showed that the enzyme was >98% pure at this stage (data not shown). Although this purification method is not sufficient to remove endogenous wild-type Trx, any such contamination was not detectable in the NMR experiments on the variants.

Solutions of enzyme were concentrated for NMR experiments using a Centriprep 10 concentrator from Amicon. For experiments in D_2O, the concentrated enzyme solution was lyophilized, and the lyophilizate was resuspended in D_2O and incubated at 37 °C for ≥4 h to allow for proton exchange. Then, the enzyme solution was lyophilized again, and this lyophilizate was resuspended in D_2O (0.5 mL). When necessary (*e.g.*, to remove DTT or excess salt), samples were desalted using a Pharmacia FPLC Fast Desalt 10/10 column, which had been equilibrated with 0.10 M potassium phosphate buffer, pH 6.2.

Preparation of Site-Specifically ^{13}C-Labeled Trx. To enhance the resolution of NMR experiments, Trx was prepared with an enrichment of ^{13}C at C^{β} of the two cysteine residues (Figure 1). This ^{13}C-labeled enzyme was prepared as described above from growth medium containing [3-^{13}C]-cysteine (40 μg/mL). The resulting enzyme had levels of ^{13}C incorporation that were low, but adequate for the analysis of wild-type Trx. To produce ^{13}C-labeled Trx variants, pTRX was transformed into *E. coli* strain JM15, a cysteine auxotroph. Transformed cells were grown in LB medium containing [3-^{13}C]cysteine (40 μg/mL), and enzyme was produced as described above. The use of an auxotrophic host increased the incorporation of label into the Trx variants above that of the wild-type enzyme.

Enzyme Concentrations. Concentrations of enzyme were determined by measuring *A* at 280 nm. The extinction coefficients of the CWGC and CVWC Trx were estimated to be $\epsilon_{280} = 18\,470\ M^{-1}\ cm^{-1}$ using the method of Gill and von Hippel (1989). The extinction coefficients of D26N and D26L Trx were assumed to be the same as that of wild-type Trx [$\epsilon_{280} = 13\,700\ M^{-1}\ cm^{-1}$ (Krause et al., 1991)].

NMR Spectroscopy. NMR experiments were performed on solutions (0.5 mL) of D_2O containing enzyme (1−3 mM), potassium phosphate (0.1 M), and DSS (1 mM), which served as a chemical shift reference. In experiments on reduced enzymes, DTT was added to a final concentration of 5 mM. The pH of solutions was adjusted by adding aliquots (≤4 μL) of either 1.0 M KOD or 1.0 M DCl. The pH of the sample was measured before and after each experiment, and the latter reading was used for data analysis. pH measurements of D_2O solutions were corrected for the deuterium isotope effect on the glass electrode (Glasoe &

Long, 1960). The pK_a of a thiol usually exhibits a large inverse solvent isotope effect (Schowen, 1977). Unfortunately, solvent isotope effects on thiol pK_a values in Trx are likely to be difficult to interpret because many protonic sites are near the two cysteine residues [Figure 1 (Kresge, 1973; Chivers, 1996)]. ^{13}C-NMR spectra were collected at 308 K on a Bruker DMX500 instrument (^{13}C 125.68 MHz) using WALTZ-16 proton-decoupling (Shaka et al., 1983). NMR data were analyzed with the program FELIX 95 from Biosym (San Diego, CA).

Data Fitting. Titration data were fitted to either one- or two-pK_a titration curves with the program MACCURVEFIT. Data from simple proton titrations were fitted to eq 1:

$$\delta = \delta_{HA} - \frac{(\delta_{HA} - \delta_{A^-})}{(1 + 10^{(pK_a - pH)})} \quad (1)$$

Complex titration data were fitted to equations that take into account multiple protonation steps (Shrager et al., 1972). Sequential, two-proton titrations of noninteracting functional groups were fitted to eq 2:

$$\delta = \frac{((\delta_{HAH})[H^+]^2 + (\delta_{HA^-})K_1[H^+] + (\delta_{^-AH})K_2[H^+] + (\delta_{A^{2-}})K_1K_2)}{([H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2)} \quad (2)$$

In the proteins studied herein, $K_1 \gg K_2$. Such titrations produce predominantly one monoprotic species, which has a chemical shift of δ_{HA^-}.[3] Branched, two-proton titrations of interacting functional groups were fitted to eq 3:

$$\delta = \frac{((\delta_{HAH})[H^+]^2 + (\delta_{HA^-})K_1[H^+] + (\delta_{^-AH})K_2[H^+] + (\delta_{A^{2-}})cK_1K_2)}{([H^+]^2 + [H^+]K_1 + [H^+]K_2 + cK_1K_2)} \quad (3)$$

Such titrations produce two monoprotic species. These species have chemical shifts of $\delta_{^-AH}$ and δ_{HA^-}, depending on which site (1 or 2, respectively) is protonated. Equations 2 and 3 differ in the last term of both the numerator and the denominator. These terms account for a change in the proton affinity of the second protonation site (site 2) due to the presence (K_2) or absence [cK_2 (Wyman & Gill, 1990)] of a proton at the first protonation site (site 1).

RESULTS

Production and Purification of Thioredoxin Variants. Our protocol for the production of Trx in *E. coli* is similar to that used for the production of ribonuclease A (delCardayré et al., 1995), except that Trx was produced in a soluble form. Chromatography on a gel filtration column yielded enzyme that was of sufficient purity (>98%) for NMR experiments. Wild-type Trx was isolated at 150 mg/L of culture. Pure D26L Trx and D26N Trx were isolated at slightly lower levels. Pure CVWC Trx and CWGC Trx were isolated at 50 mg/L of culture.

[3] If $K_1 \gg K_2$, then fits are not sensitive to the third term in the numerator of eq 2. Indeed, some workers omit this term entirely (Dyson et al., 1991; Jeng et al., 1995). Although this term has a negligible effect, it was included in our fits. Values of $\delta_{^-AH}$ were estimated by assuming that $\delta_{HAH} + \delta_{A^{2-}} = \delta_{HA^-} + \delta_{^-AH}$.

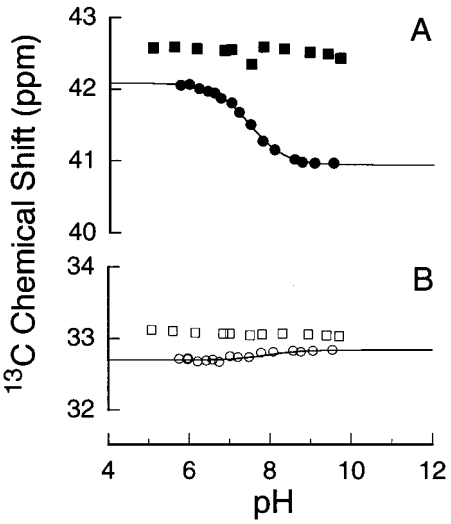

FIGURE 2: The pH dependence of the $^{13}C^{\beta}$ signals from oxidized [$^{13}C^{\beta}$-Cys]thioredoxins in D_2O. (A) Cys32 in wild-type thioredoxin (Trx; ●) and D26L Trx (■). (B) Cys35 in wild-type Trx (○) and D26L Trx (□). Titrations were carried out at 308 K in 0.1 M potassium phosphate buffer.

Table 1: Results from the Analysis of the pH Dependence of the Chemical Shifts of the $^{13}C^{\beta}$ Nuclei of Cys32 and Cys35 in Oxidized [$^{13}C^{\beta}$-Cys]Thioredoxins [a]

thioredoxin		Cys32	Cys35
wild-type	pK_1	7.45 (0.02)	7.51 (0.18)
	δ_{HAH}	42.08 (0.01)	32.70 (0.01)
	δ_{HA^-}	40.94 (0.01)	32.84 (0.01)
D26L	pK_1	nt[b]	nt

[a] Titrations were carried out at 308 K in 0.1 M potassium phosphate buffer. Values were determined by fitting the experimental data to eq 1. Chemical shift values are in parts per million. Errors were estimated from the deviation of the best fit to eq 1. [b] No titration.

^{13}C-NMR Spectroscopy. Wild-type Trx has only two cysteine residues, both of which are located in the enzyme's active site. To determine the pK_a values of the active-site thiols, we used enzymes that were labeled selectively with ^{13}C only at the β position of its cysteine residues. This technique enabled direct monitoring of the titration of the thiol (and other nearby) protons by following the change in ^{13}C-chemical shift (Jeng et al., 1995; Wilson et al., 1995).

The pH dependence of the $^{13}C^{\beta}$-Cys chemical shifts of Cys32 and Cys35 in oxidized [$^{13}C^{\beta}$-Cys]Trx is shown in Figure 2. The pH-dependent chemical shifts of oxidized [$^{13}C^{\beta}$-Cys]Trx could be fitted to a single pK_a of approximately 7.5 (Figure 2 and Table 1). We conclude that this value reflects the titration of Asp26 (Dyson et al., 1991; Langsetmo et al., 1991). This conclusion is supported by the titration curves for oxidized D26L Trx, which lack a pK_a in the entire pH range examined (Figure 2). Most importantly, these data demonstrate that the chemical shift of C^{β} of Cys32 (Figure 2A) is sensitive to the ionization state of Asp26. As had been observed in carbamoylmethylated Trx (LeMaster, 1996), this chemical shift is affected more by the ionization of Asp26 than is that of Cys35 (Figure 2B).

The pH dependence of the $^{13}C^{\beta}$-Cys chemical shift of Cys32 and Cys35 in reduced [$^{13}C^{\beta}$-Cys]Trx is shown in Figure 3. The pH dependence of the $^{13}C^{\beta}$-Cys signals from D26L Trx could be fitted to a single pK_a of 7.5, and that from D26N Trx could be fitted to a single pK_a of 7.4 (Figure 3B and Table 2). The similarity of these values as monitored

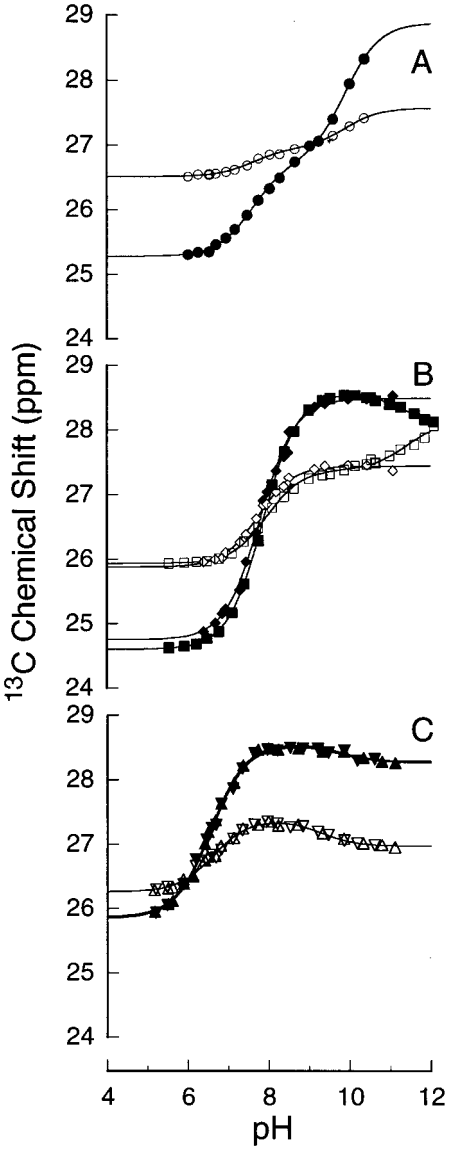

FIGURE 3: The pH dependence of the $^{13}C^{\beta}$ signals from reduced [$^{13}C^{\beta}$-Cys]thioredoxins in D_2O. Closed symbols, Cys32; open symbols, Cys35. (A) Wild-type thioredoxin (Trx; ●, ○). (B) D26L Trx (■, □) and D26N Trx (◆, ◇) Trx. (C) CWGC Trx (▲, △) and CVWC Trx (▼, ▽). The pK_a values determined from fitting the experimental data to eq 2 are listed in Table 2. Titrations were carried out at 308 K in 0.1 M potassium phosphate buffer containing DTT (5.0 mM).

by the chemical shifts from two different cysteine residues, Cys32 and Cys35, suggests that they report on the titration of the same functional group, which has a pK_a near 7.5 (Table 2). A similar pK_a value has been reported previously for D26A Trx (Wilson et al., 1995; Jeng & Dyson, 1996). Like the previous workers, we conclude that this value reflects the titration of Cys32.

The titration curves in Figure 3, panels A and C, reveal the presence of two pK_as. The titration of the wild-type enzyme has an obvious inflection (Figure 3A), which is absent in the titrations of CWGC Trx and CVWC Trx (Figure 3C). The physical basis for this inflection is discussed below. In the titration of the variants (Figure 3C), a distinct second transition was observed above pH 8.5. ^{1}H-NMR spectra in this pH range gave no indication that the enzymes are unfolding (data not shown). This second transition therefore results from the deprotonation of a functional group in the variants. This functional group could be either Asp26 or Cys35. The similarity of this upfield chemical shift change to that observed for oxidized Trx (Figure 2A) leads us to conclude that the second transition arises from the titration of Asp26. Thus, a pK_a corresonding to Asp26 is apparent in the titrations of reduced CWGC Trx and reduced CVWC Trx (Figure 3C). This pK_a, which is also evident in the titration of oxidized Trx (Figure 2A), is concealed in the titration of the reduced wild-type enzyme (Figure 3A).

Finally, a second titration was observed at pH >10 for D26L Trx (Figure 3B and Table 2). This pK_a could arise from the titration of Cys35. Yet, without a well-defined titration endpoint at high pH, it is only possible to determine that this pK_a has a lower limit of 11. No such titration at high pH was observed for D26N Trx.

Table 2: Results from the Analysis of the pH Dependence of the Chemical Shifts of the $^{13}C^{\beta}$ Nuclei of Cys32 and Cys35 in Reduced [$^{13}C^{\beta}$-Cys]Thioredoxins[a]

thioredoxin		Cys32	Cys35
wild-type	pK_1	7.59 (0.04)	7.62 (0.07)
	pK_2	9.88 (0.06)	9.89 (0.10)
	δ_{HAH}	25.28 (0.03)	26.51 (0.01)
	δ_{HA^-}	26.72 (0.04)	26.94 (0.02)
	$\delta_{A^{2-}}$	28.88 (0.09)	27.57 (0.04)
CVWC	pK_1	6.12 (0.36)	6.22 (0.06)
	pK_2	nd[b]	8.88 (0.02)
	δ_{HAH}	25.84 (0.06)	26.26 (0.03)
	δ_{HA^-}	28.48 (0.03)	27.38 (0.03)
	$\delta_{A^{2-}}$	nd	26.97 (0.06)
CWGC	pK_1	6.14 (0.024)	6.16 (0.04)
	pK_2	9.45 (0.30)	9.44 (0.14)
	δ_{HAH}	25.84 (0.03)	26.22 (0.02)
	δ_{HA^-}	28.56 (0.03)	27.38 (0.02)
	δ_{A2^-}	28.26 (0.04)	26.96 (0.02)
D26L	pK_1	7.46 (0.01)	7.56 (0.03)
	pK_2	10.92 (0.09)	11.0 (0.1)
	δ_{HAH}	24.60 (0.01)	25.94 (0.02)
	δ_{HA^-}	28.59 (0.01)	27.39 (0.02)
	δ_{A2^-}	28.06 (0.03)	28.13 (0.06)
D26N	pK_1	7.39 (0.03)	7.32 (0.03)
	pK_2	nt[c]	nt[c]
	δ_{HAH}	24.75 (0.06)	25.87 (0.02)
	δ_{HA^-}	28.49 (0.04)	27.45 (0.02)
	$\delta_{A^{2-}}$	nt[c]	nt[c]

[a] Titrations were carried out at 308 K in 0.1 M potassium phosphate buffer containing DTT (5 mM). Values were determined by fitting the experimental data to eq 2, which does not account for interaction between titrating groups. Values for D26N Trx were determined by fitting to eq 1. Chemical shift values are in parts per million. Errors were estimated from the deviation of the best fit to eq 2 or eq 1 (for D26N Trx). [b] Not determined. [c] No titration.

DISCUSSION

The titration behavior of reduced thioredoxin has defied straightforward interpretation (Holmgren, 1972; Reutimann et al., 1981; Dyson et al., 1991, 1997; Li et al., 1993; Jeng et al., 1995; Wilson et al., 1995; Jeng & Dyson, 1996; Takahashi & Creighton, 1996). The difficulty in determining pK_a values in reduced Trx results largely from the proximity of three titrating groups (Asp26, Cys32, and Cys35) in the active site (Figure 1). Here, we have used ^{13}C-NMR spectroscopy, site-specific isotopic labeling, and site-directed mutagenesis to address this complexity.

Nearby titrating groups with similar pK_a values can exhibit *microscopic* pK_as (Edsall & Wyman, 1958). [For a recent example of a thorough analysis of microscopic pK_as, see McIntosh et al. (1996).] Microscopic pK_as arise from

Coulombic interactions that create either positive or negative cooperativity. Wyman and Gill (1990) have presented a comprehensive analysis of such cooperativity in the titration of protein functional groups. In addition, equations for fitting independent (eq 2) and dependent (eq 3) titration curves are known (Shrager et al., 1972). Previous interpretations of the pH-titration of Trx have not considered the possibility of microscopic pK_as (Dyson et al., 1991, 1997; Jeng et al., 1995; Wilson et al., 1995; Jeng & Dyson, 1996), despite the proximity of Cys32, Cys35, and Asp26 (Figure 1).

Eliminating the Asp26 titration by mutation or the Cys32 titration by oxidation simplifies the titration behavior. Replacing the carboxyl group in residue 26 produces Trx variants with well-defined titration curves (Figure 3B). These curves can be fitted to a single pK_a value for Cys32 [Table 2 (Wilson et al., 1995; Dyson et al., 1997)]. The pK_a of 7.5 determined from these curves is likely to be similar to that of Cys32 in wild-type Trx when Asp26 is protonated because the net charge on both enzymes is identical. In an analogous manner, the pK_a of Asp26 when Cys32 and Cys35 are protonated can be estimated from its titration in oxidized Trx. This pK_a (as reported by signals from the nearby cysteine residues) is also about 7.5 [Table 1 (Dyson et al., 1991; Langsetmo et al., 1991)]. Previous titrations of Trx in which Cys32 had been modified with a carbamoylmethyl group revealed a pK_a of 7.58 for Asp26 (LeMaster, 1996). The structure of this modified Trx was virtually identical to that of the reduced enzyme (LeMaster, 1996). The similar pK_a values for Asp26 and Cys32 together with the proximity of Asp26 and Cys32 (Figure 1) and the sensitivity of the NMR chemical shift of $^{13}C^{\beta}$ of Cys32 to the ionization state of Asp26 (Figure 2A) necessitate the consideration of microscopic pK_a values for Asp26 and Cys32. Here, Coulombic interactions between Asp26 and Cys32 are likely to exhibit *negative* cooperativity because both residues become anionic upon deprotonation.

The titration behavior of CVWC Trx and CWGC Trx support an analysis based on microscopic pK_a values. The titration observed in the variants at pH >9 (Figure 3C) must be reporting on the ionization of an enzymic group. This ionization is unlikely to be that of Cys35 because the change in chemical shift of the $^{13}C^{\beta}$ nuclei of Cys35 is not downfield (as expected for a thiol titration) but upfield. Indeed, the change in chemical shift is similar to that observed in oxidized Trx (Figure 2A) as well as the carbamoylmethylated enzyme (LeMaster, 1996). So, we conclude that at pH >9 the $^{13}C^{\beta}$ nuclei are reporting on the titration of Asp26. The ionization of Asp26 is apparent in the titration of CVWC Trx and CWGC Trx because it is not obscured by the ionization of Cys32, which has a low pK_a in these variants [Table 2 (Chivers et al., 1996)]. In contrast, Asp26 and Cys32 have similar pK_a values in wild-type Trx. Accordingly, the titration of Asp26 is indistinct in the change in chemical shift of the $^{13}C^{\beta}$ nuclei of Cys32 and Cys35 in the wild-type enzyme (Figure 3A).

Microscopic pK_a values are related by the thermodynamic box depicted in Figure 4. Because pK_a is a state function, complete titration by the path **I** → **II** → **IV** is identical to that by the path **I** → **III** → **IV**. In other words, the four distinct titrations are related by eq 4:

$$pK_{32SH} + p(cK_{CO_2H}) = pK_{CO_2H} + p(cK_{32SH}) \qquad (4)$$

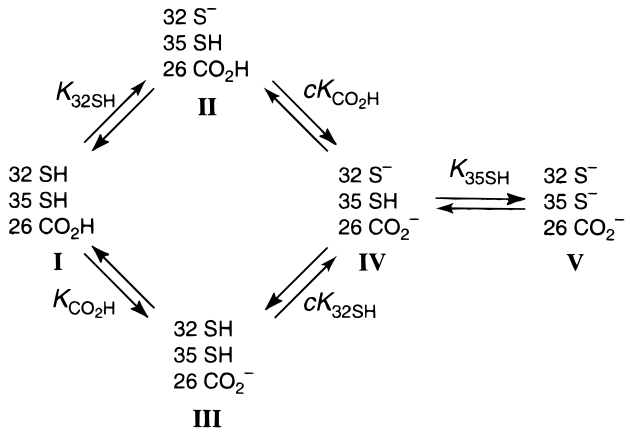

FIGURE 4: Proposed microscopic acid dissociation equilibria for the active-site residues of reduced thioredoxin. K_{32SH} and K_{CO_2H} are equivalent to K_1 and K_2 in eq 3, respectively.

Equation 4 and the values of pK_{32SH} and pK_{CO_2H} can be used to determine the value of c, which provides a measure of the extent of the interaction between Asp26 and Cys32 (vide infra).

Microscopic pK_a values and chemical shift values for species **I**−**IV** can be determined by fitting experimental data to eq 3 (Shrager et al., 1972). To determine these values, we fitted the pH dependence of the chemical shifts of the $^{13}C^{\beta}$ nuclei of Cys32 in reduced wild-type Trx to eq 3 by assigning pK_2 = pK_{CO_2H} = 7.5 and Δ^-AH = 1.1 ppm.[4] These constraints were derived from the values for oxidized wild-type Trx (Figure 2A and Table 1). The resulting fits are shown in Figure 5A, and the corresponding pK_a and chemical shift values are listed in Table 3. A fit of the same data with pK_2 = pK_{32SH} = 7.5 and ΔHA$^-$ = 4.0 ppm, which are the values determined for D26L Trx (Figure 3B and Table 2), yields values for pK_1 = pK_{CO_2H} and c that differ insignificantly from those in Table 3. Thus, the system is robust, yielding the same values if either pK_1 and ΔHA$^-$ or pK_2 and Δ^-AH are constrained. The two other curves in Figure 5A were also constructed with eq 3, but by assuming that c = 1 and that the pK_a of Asp26 is always 7.5 (lower curve) or always 9.5 (upper curve). The poor quality of these fits confirms that Cys32 interacts with another residue during its titration. Finally, Figure 5B depicts a similar analysis of the microscopic pK_a values and chemical shift values of CVWC Trx, which behaves in a manner similar to that of CWGC Trx (Figure 3C).

We thus conclude that the titrations of Asp26 and Cys32 in wild-type Trx are described better in terms of microscopic pK_as than by two noninteracting pK_as. When Asp26 is protonated, the microscopic pK_a of Cys32 is 7.5. When Cys32 is protonated, the microscopic pK_a of Asp26 is 7.5, similar to that of the oxidized enzyme. In contrast, when Cys32 is unprotonated, the microscopic pK_a of Asp26 is 9.2. Likewise, when Asp26 is unprotonated, the microscopic pK_a of Cys32 is 9.2. The extent of the interaction between Asp26 and Cys32 can be described by the parameter c, as defined in Figure 4 (Wyman & Gill, 1990). In the wild-type enzyme c = 0.020 (Table 3), which means that strong negative

[4] Δ refers to a chemical shift difference between two protonation states. For example, Δ^-AH refers to the difference between 26CO$_2$H and 26CO$_2^-$, as observed at C$^{\beta}$ of Cys32. For each protein examined, values of Δ vary much less than do values of δ.

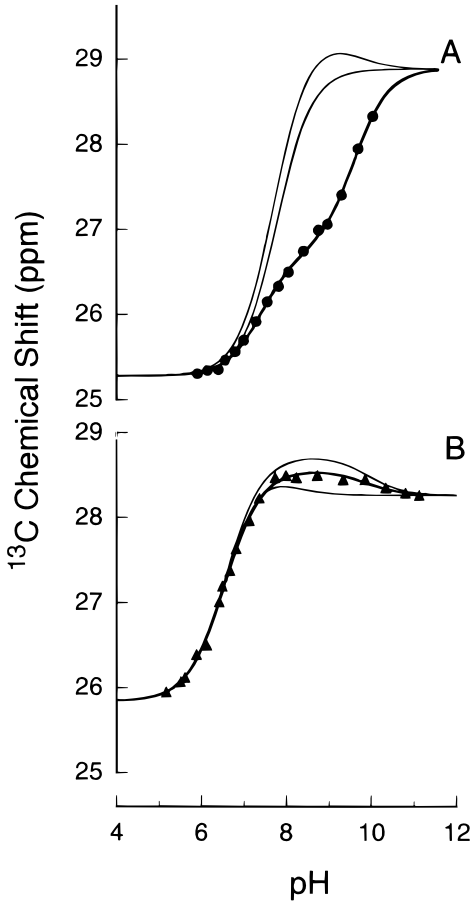

FIGURE 5: Fit of experimental data to titration curves with dependent pK_as (eq 3). (A) Wild-type thioredoxin (Trx). (B) CVWC Trx. The pK_a and ^{13}C chemical shift values are listed in Table 3. The other curves are fits of the experimental data to eq 3, assuming that $c = 1$ and that the pK_a of Asp26 is always 7.5 (lower curve) or 9.2 (upper curve).

Table 3: Microscopic pK_a Values and Chemical Shift Values from the Analysis of the pH Dependence of the Chemical Shifts of the $^{13}C^{\beta}$ Nuclei of Cys32 and Cys35 in Reduced [$^{13}C^{\beta}$-Cys]Thioredoxins[a]

	thioredoxin	
parameter	wild-type	CVWC
$pK_{32SH}^{(I \rightarrow II)}$	7.50 (0.04)	6.16 (0.02)
$pK_{CO_2H}^{(I \rightarrow III)}$	7.50 (0.04)	7.50 (0.04)
$pK_{32SH}^{(III \rightarrow IV)}$	9.20 (0.06)	8.08 (0.32)
$pK_{CO_2H}^{(II \rightarrow IV)}$	9.20 (0.07)	9.42 (0.33)
c[b]	0.020 (0.003)	0.012 (0.009)
δ_{HAH}	25.28 (0.03)	25.84 (0.05)
δ_{HA^-}	29.28 (0.20)	28.74 (0.10)
δ^-_{AH}	24.18 (0.20)	24.80 (0.10)
$\delta_{A^{2-}}$	28.88 (0.16)	28.26 (0.05)

[a] Titrations were carried out at 308 K in 0.1 M potassium phosphate buffer containing DTT (5 mM). Values were determined by fitting the experimental data to eq 3, which accounts for interaction between titrating groups. Here, K_1 is K_{32SH}, and K_2 is K_{CO_2H}. Species **I**−**IV** refer to Figure 4. Chemical shift values are in ppm. Errors were estimated from the deviation of the best fit to eq 3. [b] $c = K_{32SH}^{(III \rightarrow IV)}/K_{32SH}^{(I \rightarrow II)}$.

cooperativity exists for the deprotonation of Asp26 and Cys32.[5] Indeed, deprotonation of Cys32 is impeded by a deprotonated Asp26 (and vice versa) by an interaction energy, $\Delta G = -RT \ln c = 2.4$ kcal/mol at 308 K.

The effect of this cooperativity is apparent in comparisons of wild-type Trx and the CVWC enzyme. When Asp26 is

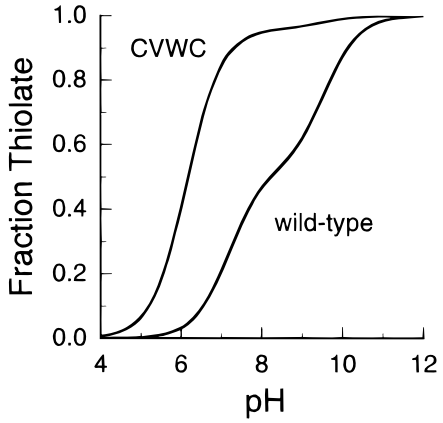

FIGURE 6: Calculated fraction of molecules with deprotonated Cys32 as a function of pH for wild-type thioredoxin and CVWC thioredoxin. Curves were calculated from the sum of species **II** and **IV** divided by the sum of species **I**−**IV** (Figure 4) using data in Figure 5 (Table 3).

protonated in this variant, the pK_a of Cys32 is 6.16. This value is within 0.2 pH units of that determined by fluorescence titration (Chivers, 1996; Chivers et al., 1996). As a consequence of this low pK_a, only a small fraction (≤4%) of reduced CVWC Trx is ever found as species **III** (Figure 4). Although strong negative cooperativity still exists during the titration of the CVWC enzyme, which has $c = 0.012$, the greater separation of the Asp26 and Cys32 pK_a values diminishes the influence of this cooperativity and yields two almost independent titrations (Figure 6). The low pK_a of Cys32 in reduced human Trx could have a similar effect (Forman-Kay et al., 1992).

None of the published NMR titration data is in discord with our analysis. Two titration steps were observed when the pH dependence of the chemical shift of Asp26 was monitored directly in reduced Trx (Jeng & Dyson, 1996), but these data were fitted with an equation for one pK_a. The second pK_a is likely to be from the dissociation of a proton from Asp26 in those molecules in which Cys32 had titrated first. The ^{13}C chemical shift of Cys32 in the [$^{13}C^{\beta}$-Cys]Trx variants increases by 3−4 ppm upon increasing the pH from 4 to 12 (Table 2). A similar increase was found for wild-type [$^{13}C^{\beta}$-Cys]Trx when the experimental data were fitted to eq 3.

The pH dependence of the $^{13}C^{\beta}$ resonance from Cys35 in Trx in which Cys32 is carbamoylmethylated and thus neutral, rather than anionic, at high pH, reveals a pK_a of 11.1 (LeMaster, 1996). Our titration data show no evidence for the deprotonation of Cys35 in the pH range studied for any of the Trx variants with the exception of D26L Trx. Even in that enzyme, Cys35 has p$K_a \geq 11$. Such a high pK_a value for this residue is not surprising. Cys35 is a buried residue in an environment of low dielectric constant (LeMaster, 1996). In addition, as the pH of the solution is increased, both Asp26 and Cys32 will become anionic, making the deprotonation of Cys35 difficult. A high pK_a value for Cys35 does not conflict with the mechanistic role of this residue.

[5] For comparison, $c = 0.006$ for the complete ionization of thioglycolic acid, HO_2CCH_2SH [Kellogg et al. (1996) and R. S. Brown, personal communication]. The magnitude of the value of c for the Asp26···Cys32 interaction is striking because, unlike in thioglycolic acid, the inductive effect in thioredoxin has a negligible through-bond component.

Detailed kinetic studies indicate that Asp26 acts as a base, removing a proton from Cys35 during catalysis by Trx (Chivers, 1996; Chivers & Raines, 1997).

Our analysis of the titration behavior of reduced Trx conflicts with the interpretation, but not the experimental data, of Takahashi and Creighton (1996). The interpretation of equilibrium measurements, such as the pH dependence of the equilibrium constant for the reaction of reduced Trx and oxidized glutathione (Takahashi & Creighton, 1996), without full knowledge of the titration behavior of *all* relevant species is prone to (sometimes large) error. A comprehensive approach is necessary to unravel the complex titration behavior in the active site of Trx. For example, Dyson et al. (1997) recently reported UV titrations of wild-type and D26A Trx. Their data resemble closely those obtained for ^{13}C NMR titrations of the same proteins.

The values of the microscopic pK_as of Trx are a consequence of electrostatic interactions within the enzymic active site (Warshel & Åqvist, 1991). An unperturbed cysteine residue has a pK_a of 8.7 (Calvin, 1954). One of the microscopic pK_a values of Cys32 in Trx is 7.5. This value is remarkably similar to that determined for a cysteine residue at the N-terminus of an α-helical peptide (Kortemme & Creighton, 1995; Kortemme et al., 1996). Thus, the sharing of a proton between Cys32 and Cys35 (Jeng & Dyson, 1996) need not be invoked to explain the low pK_a value of Cys32 relative to that of an unperturbed cysteine residue. Rather, the net stabilization of the thiolate form of Cys32, which initiates catalysis by the enzyme, could derive solely from its proximity to the dipole at the N-terminus of an α-helix (Blagdon & Goodman, 1975; Wada, 1976). The other microscopic pK_a value of Cys32 in Trx is 9.2. This value is likely to be a consequence of electrostatic interactions with both the α-helix dipole and an anionic side chain at position 26. These interactions have opposing effects on the titration of Cys32, with the result being a microscopic pK_a value of 9.2, which is close to that for an unperturbed cysteine residue.

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