

## Accelerated Publications

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### Direct Measurement of the Aspartic Acid 26 $pK_a$ for Reduced *Escherichia coli* Thioredoxin by $^{13}\text{C}$ NMR<sup>†</sup>

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**ABSTRACT:** Because of interference from the pH-dependent behavior of nearby groups in the active site of *Escherichia coli* thioredoxin, the  $pK_a$  of the buried carboxyl group of the aspartic acid at position 26 has been difficult to quantitate. We report a direct measurement of this  $pK_a$  using an NMR method utilizing the correlation between the  $\text{C}^\beta\text{H}$  proton resonances and the  $^{13}\text{CO}$  of the titrating carboxyl group. The experiments show unequivocally that the  $pK_a$  is 7.3–7.5, rather than the value of 9 or greater recently proposed by Wilson, N. A., *et al.* [(1995) *Biochemistry* 34, 8931–8939]. The assignment of the titrating resonances to Asp 26 is unambiguous: the values of the  $\text{C}^\beta\text{H}$  chemical shifts correspond exactly to those of Asp 26, and their titration in the pH range 5.7–10.0 is the same as that observed previously for the proton resonances alone. In addition, the chemical shift of the carboxyl  $^{13}\text{C}$  resonance at pH 5.7 is upfield of those of the other carboxyl and carboxamide resonances, diagnostic for a protonated carboxyl group. The resonances assigned to Asp 26 are the only ones that titrate in the pH range 5.7–10.5. None of the other aspartate and glutamate residues in the molecule are titrated in this pH range, consistent with their positions on the surface of the molecule. The  $pK_a$  measured for Asp 26 in reduced thioredoxin is identical within experimental error to that measured in the oxidized form of the protein. This is significant for the reductive mechanism of thioredoxin: the buried salt bridged/hydrogen-bonded side chains of Asp 26 and Lys 57 are likely to contribute to the facility of the reaction by providing a convenient source and sink for protons in the hydrophobic environment of the complex between thioredoxin and its substrates.

A number of studies have recently addressed the issue of the  $pK_a$  of the buried aspartic acid in *Escherichia coli* thioredoxin. This residue is highly conserved among all thioredoxins (Eklund *et al.*, 1991) and appears to be completely buried in both prokaryotic (Katti *et al.*, 1990; Jeng *et al.*, 1994) and eukaryotic (Qin *et al.*, 1994) thioredoxins. The  $\epsilon$ -amino group of a lysine residue at position 57 is also buried in close proximity to Asp 26 and takes part in a loose (Jeng *et al.*, 1994) or water-mediated (Katti

*et al.*, 1991) hydrogen-bonding interaction. This residue is highly conserved only among prokaryotes, but its function is apparently duplicated in eukaryotic thioredoxins by the presence of a lysine residue at position 39 (Eklund *et al.*, 1991). The  $pK_a$  of the buried aspartate residue has been determined by a number of methods for the oxidized form of thioredoxin ( $\text{Trx-S}_2$ ) (Dyson *et al.*, 1991; Langsetmo *et al.*, 1991a) to be considerably shifted from “normal” values: to 7.5 instead of  $\sim 4.0$ . The  $pK_a$  determination for the reduced form of the protein [ $\text{Trx-(SH)}_2$ ] is complicated by the effect of the titration of the nearby thiol groups of the active site cysteine residues, Cys 32 and Cys 35, but was inferred from NMR studies to be also in the vicinity of 7 (Dyson *et al.*, 1991). The relationship of these three groups is shown in Figure 1.

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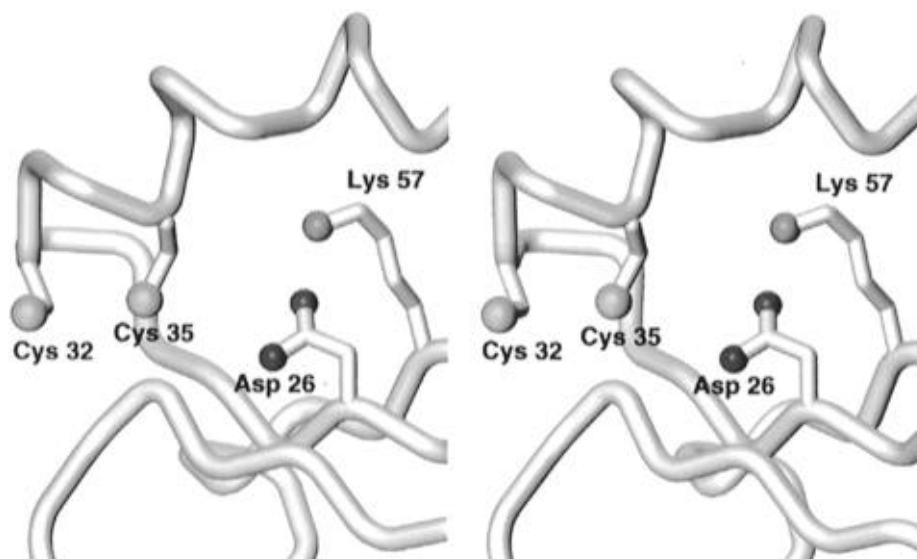


FIGURE 1: Portion of one of the solution structures of Trx-(SH)<sub>2</sub> (Jeng *et al.*, 1994) showing the spatial relationship between the two cysteines and the buried Asp 26 and Lys 57 side chains.

The unusual pH dependence of the two active site thiols in reduced thioredoxin is crucial for the reductive mechanism. Reduction of a substrate disulfide by thioredoxin involves a two-electron, two-proton reduction. pH control at the active site is therefore important to the control of the rate of reduction by thioredoxin. We have recently site-specifically determined the  $pK_a$ s of the two active site thiols in Trx-(SH)<sub>2</sub> using direct NMR methods to be 7.5 and 9.5 (Jeng *et al.*, 1995). The <sup>13</sup>C resonance of the atom attached to the titrating group should primarily show the influence only of the directly bonded titrating group. Surprisingly, the C<sup>β</sup> resonances of the two cysteine residues show a double titration. We have interpreted this as evidence of a shared proton between the sulfur atoms of the two cysteines at neutral pH, consistent with the otherwise unexplained shifted  $pK_a$  of the solvent-exposed Cys 32 thiol.

At the same time, another group was working on the same system (Wilson *et al.*, 1995). Their <sup>13</sup>C-<sup>1</sup>H data for the cysteine thiols of Trx-(SH)<sub>2</sub> appear identical to our own, but a very different interpretation was placed on them. The higher of the two  $pK_a$ s observed in the <sup>13</sup>C<sup>β</sup>-<sup>1</sup>H<sup>β</sup> titration, at 9.5, was attributed to the buried Asp 26 carboxyl group on the basis of a comparison of the behavior of a mutant in which Asp 26 had been replaced by alanine (D26A). In this paper we present a direct measurement of the Asp 26  $pK_a$ , using a modified two-dimensional H(CA)CO NMR experiment to estimate directly the <sup>13</sup>C chemical shift of the carboxyl carbon of Asp 26. These measurements establish unequivocally that the  $pK_a$  of Asp 26 is between 7.3 and 7.5, rather than 9, as inferred by Wilson *et al.* (1995). This has profound implications for the mechanism of pH control in the reductive and oxidative reactions of thioredoxin.

## MATERIALS AND METHODS

Reduced thioredoxin uniformly labeled with <sup>15</sup>N and <sup>13</sup>C was prepared as previously described (Chandrasekhar *et al.*, 1991, 1994), utilizing an algal homogenate (Martek Co.) as a basis for an enriched medium. Purification of thioredoxin and preparation of D<sub>2</sub>O samples of the reduced protein using dithiothreitol were performed as previously described (Dyson *et al.*, 1989). The pH of the sample was varied between 5.7

and 10.6 by the addition of small aliquots of 0.1 M NaOD or DCl in D<sub>2</sub>O. pH values quoted are meter readings uncorrected for the deuterium isotope effect.

NMR experiments were carried out at 308 K on a Bruker spectrometer operating at 500 MHz for protons. The behavior of the <sup>13</sup>CO of the carboxyl and carboxamide groups in the protein as a function of pH was monitored using a two-dimensional H(CA)CO experiment (Kay *et al.*, 1990), modified to optimize the detection of the coupling between the <sup>13</sup>CO of a carboxyl or carboxamide and the adjacent <sup>13</sup>C<sup>β</sup>H or <sup>13</sup>C<sup>γ</sup>H (Yamazaki *et al.*, 1993; Oda *et al.*, 1994). Spectra were referenced in  $\omega_2$  to the pH-independent backbone <sup>13</sup>CO-<sup>1</sup>H cross peaks of Gly 84 at 4.38 ppm and Ala 108 at 4.09 ppm (Dyson *et al.*, 1989) and indirectly in  $\omega_1$  (Wishart *et al.*, 1995). Spectral widths were 6250 Hz with 2048 complex points in  $\omega_2$  and 6250 Hz with 128 complex points in  $\omega_1$ . Quadrature detection was achieved in  $\omega_1$  by a combined States-TPPI method. Spectra were Fourier transformed using Gaussian and exponential window functions on a Sun workstation using the FTNMR software of Dennis Hare.

Chemical shift values as a function of pH for Asp 26 were analyzed using the program Templegraph (Mihalisin Associates) in terms of a single titration curve of the form (Dyson *et al.*, 1991):

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

where  $\delta$  is the observed chemical shift at a given pH,  $\delta_{HA}$  and  $\delta_A$  are the chemical shifts for the various protonated forms of the protein,  $n$  is the number of protons transferred, and  $K_a$  is the acid ionization constant.

## RESULTS AND DISCUSSION

A modified H(CA)CO spectrum of Trx-(SH)<sub>2</sub> at pH 8.5 is shown in Figure 2. All of the cross peaks can be identified either with the backbone <sup>13</sup>CO-H<sup>α</sup> correlations of glycine and other residues whose <sup>13</sup>C<sup>α</sup> frequency is sufficiently low to be excited in the experiment or with the side-chain <sup>13</sup>CO-H<sup>β</sup> correlations of the aspartate and asparagine

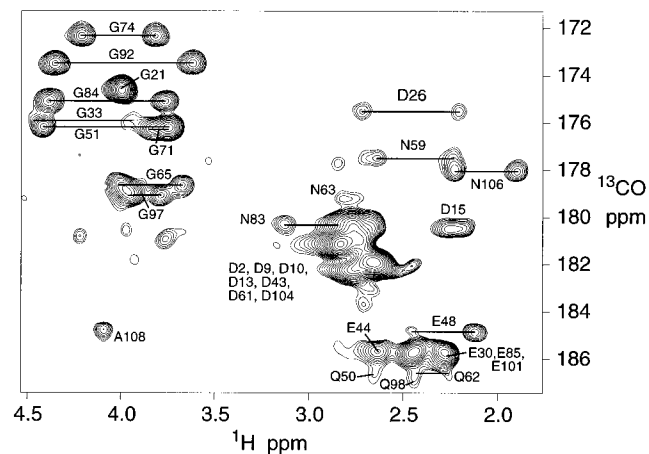


FIGURE 2: A 500 MHz modified two-dimensional H(CA)CO spectrum of reduced thioredoxin at pH 8.52, showing the cross peaks for the glycine C $\alpha$ H–CO (backbone) and the side-chain C $\beta$ H–CO and C $\gamma$ H–CO connectivities for the Asp, Glu, Asn, and Gln residues. Cross peaks are also visible for other residues for which (like glycine) the C $\alpha$  resonance is at a sufficiently low frequency to be excited during the pulse sequence, which has otherwise been optimized for the C $\beta$  and C $\gamma$  of the side chains.

Table 1:  $^{13}\text{CO}$  Assignments for Trx-(SH) $_2$  at pH 5.92, 308 K $^a$

Gly	C $\alpha$ H	$^{13}\text{CO}$
Gly 21	3.99, 3.99	174.7
Gly 33	3.98, 4.31	176.1
Gly 51	4.40, 3.74	176.2
Gly 65	3.98, 3.67	178.7
Gly 71	3.82, 3.67	176.3
Gly 74	4.20, 3.74	172.4
Gly 84	4.38, 3.75	175.1
Gly 92	3.59, 4.38	173.6
Gly 97	3.76, 3.92	179.1
Asp/Asn	C $\beta$ H	$^{13}\text{CO}$
Asp 15	2.27, 2.18	180.3
Asp 26	2.89, 2.23	173.7
Asn 59	2.65, 2.21	177.5
Asn 63	2.72, 2.78	179.1
Asn 83	2.85, 3.11	180.3
Asn 106	2.23, 1.88	178.1
Glu/Gln	C $\gamma$ H	$^{13}\text{CO}$
Glu 44	2.62, 2.68	185.2
Glu 48	2.44, 2.14	184.6
Gln 50	2.53, 2.50	186.6
Gln 62	2.28, 2.47	186.6
Gln 98	2.45, 2.45	186.6

$^a$  Assignments are presented only for those resonances that could be unambiguously identified from the 2D H(CA)CO spectrum.

residues and the  $^{13}\text{CO}$ –H $\gamma$  correlations of the glutamic acid and glutamine residues.

The  $^{13}\text{CO}$  chemical shifts for resonances that could be unambiguously assigned from the H(CA)CO spectrum are shown in Table 1. The cross peaks for the majority of the 11 aspartate residues are heavily overlapped in the region ( $^1\text{H}$  = 2.6–2.9 ppm and  $^{13}\text{C}$  = 180–182 ppm). The cross peaks due to Asp 26 are readily identifiable, since the C $\beta$ H resonances are distinctive (Dyson *et al.*, 1989), and the  $^{13}\text{CO}$  resonance is upfield-shifted, as expected for a protonated carboxyl carbon (Oda *et al.*, 1994). However, the cross peaks are of lower intensity than those for the other carboxyl groups of the molecule, presumably reflecting the unique position of the Asp 26 side chain, deeply buried in the hydrophobic cavity behind the active site (Jeng *et al.*, 1994).

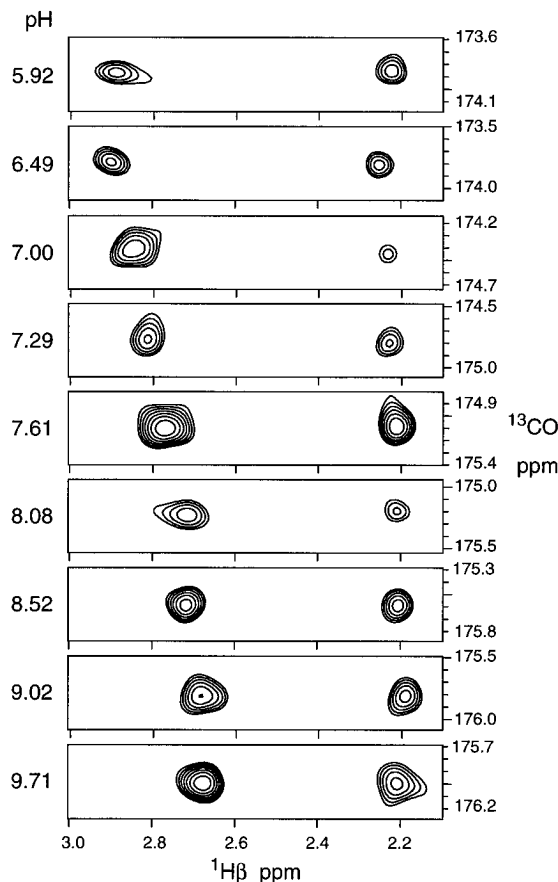


FIGURE 3: Portions of the 500 MHz modified two-dimensional H(CA)CO spectrum of reduced thioredoxin at a number of pH values between 5.92 and 9.71, showing the cross peaks assigned to the Asp 26 C $\beta$ H– $^{13}\text{CO}$  connectivities. These spectra are in general plotted at a lower contour level than that in Figure 1.

All of the other carboxyl side chains and many of the carboxamides (Asn and Gln) are present on the surface of the molecule, where they are in many cases in free rotation even at the C $\alpha$ –C $\beta$  bond (Chandrasekhar *et al.*, 1994; Jeng *et al.*, 1994). This would give rise to relatively narrow line widths for the carboxyl carbons. For Asp 26, however, the side chain is fixed in the interior of the molecule, so the correlation time is strictly controlled by the overall tumbling of the molecule. It is significant that the Asp 26 cross peaks have a tendency to disappear in older samples and at the extremes of the pH range, where a small amount of aggregation (at pH < 6) or unfolding (at pH > 9.5) is more likely to occur. The concomitant increase in average correlation time is then sufficient to broaden the  $^{13}\text{CO}$  resonance beyond detection. A similar lowering of the cross-peak intensity is observed for those carboxamide residues that the solution structure of Trx-(SH) $_2$  indicates are buried, for example, Asn 59 and Gln 98.

The cross peaks corresponding to the Asp 26 C $\beta$ H– $^{13}\text{CO}$  are shown in Figure 3 for all pHs except 5.7 and 10.0, plotted at a lower contour level than for Figure 2. The pH-dependent behavior of the  $^{13}\text{CO}$  and  $^1\text{H}\beta$  resonances is shown in Figure 4 and the pK $_a$ s are shown in Table 2. The magnitude of the shift in the  $^{13}\text{C}$  chemical shift, 2.2 ppm, is comparable to those observed for the titrations of aspartates in ribonuclease H1 (Oda *et al.*, 1994). It is immediately obvious that the pK $_a$  for the transition in chemical shift between pH 5.7 and pH 10 is quite similar for the  $^{13}\text{CO}$  chemical shifts and for those of the H $\beta$ 2 resonances [stereospecific assignments

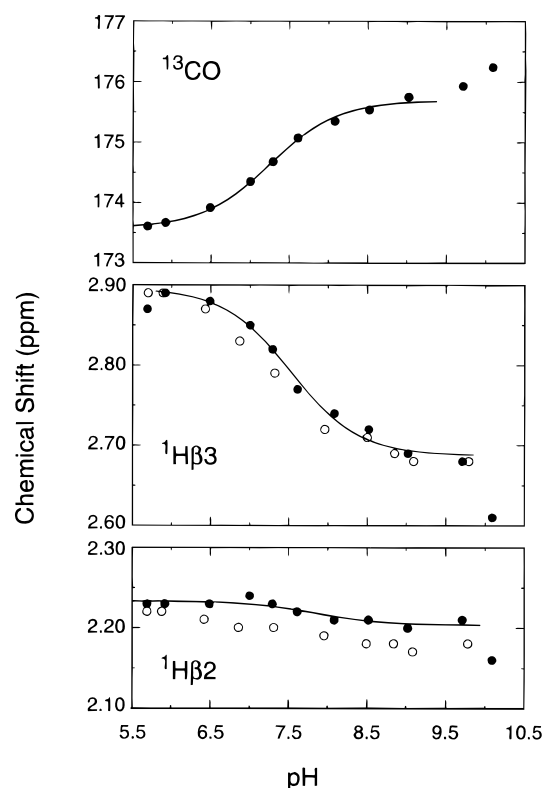


FIGURE 4: Plot of the chemical shift of the resonances shown in Figure 2 as a function of pH. Solid lines are curves fitted to the data (●) using the method of least squares to these points. Also included are data points obtained previously from  $^1\text{H}$  NMR spectra (○) (Dyson *et al.*, 1991). The points at the high-pH extremum appear to be influenced by another pH transition and were not included in the fit.

Table 2: Measured  $pK_a$  Values for the Asp 26 Carboxyl Group

atom	$pK_a$	$\Delta\delta$ (ppm)	source
Asp 26 $^{13}\text{C}$	7.3	2.2	this work
Asp 26 $^1\text{H}\beta_2$	(7.8) <sup>a</sup>	(0.03) <sup>a</sup>	this work
	7.3	0.04	Dyson <i>et al.</i> , 1991
Asp 26 $^1\text{H}\beta_3$	7.5	0.21	this work
	7.3	0.22	Dyson <i>et al.</i> , 1991

<sup>a</sup> The  $pK_a$  value obtained for Asp 26  $^1\text{H}\beta_2$  is not well determined due to the small size of the chemical shift change with pH ( $\Delta\delta$ ).

according to Chandrasekhar *et al.* (1994)]. The  $\text{H}\beta_3$  resonance undergoes very little change over this pH range. At pH values greater than 9 there appears to be another pH-dependent process occurring. The magnitude and abruptness of the change indicates that it is probably not related to the cysteine  $pK_a$  at 9.5. There are two possible explanations for this—changes due to general unfolding of the molecule or deprotonation of another nearby group such as the Lys 57  $\epsilon$ -amino group. Also included in Figure 4 are the data points from the previous  $^1\text{H}$  NMR study of reduced thioredoxin (Dyson *et al.*, 1991). The chemical shift values at any given pH differ slightly between the two data sets, possibly due to the  $10^\circ$  temperature difference between the two sets of measurements. However, the pH-dependent behavior of the resonances in the  $^1\text{H}$  COSY spectra closely parallels that observed for the  $^{13}\text{C}$ – $^1\text{H}$  HSQC measurements, further evidence that the low-intensity cross peaks observed in the latter experiment are indeed those of Asp 26. Most significantly, Figure 4 shows a pronounced single titration in the  $^{13}\text{CO}$  for Asp 26, with a  $pK_a$  of 7.3. There is no sign

of a titration at  $pK_a \geq 9$  as suggested by Wilson *et al.* (1995).

A number of attempts have been made to localize the  $pK_a$ s of the titrating groups in the active site of reduced thioredoxin. Early chemical modification studies indicated that the  $pK_a$  of one of the cysteine thiols in reduced thioredoxin was  $\sim 6.8$  and the other  $\sim 9$  (Kallis & Holmgren, 1980). A later NMR study (Dyson *et al.*, 1991) interpreted a large volume of  $^1\text{H}$  titration data in terms of only two  $pK_a$ s in the active site region, although it was obvious that three titrating groups (Asp 26, Cys 32, and Cys 35) were present. This approach was justified by the inference that two of the  $pK_a$ s (Asp 26 and Cys 32) appeared to have very similar  $pK_a$ s, in the vicinity of 7. A higher  $pK_a$  of 8.4 was found for Cys 35, closer to normal values for cysteine  $pK_a$ s in proteins. However, the pH range over which these measurements were taken was smaller, at least at the high-pH range, than later studies: it appears that the  $pK_a$  obtained for Cys 35 was underdetermined. Later work using specifically (Wilson *et al.*, 1995) and semispecifically labeled thioredoxin (Jeng *et al.*, 1995) indicated that the higher  $pK_a$  in fact more closely approaches 9.5, as originally found by Kallis and Holmgren (1981). A Raman study (Li *et al.*, 1993) gave  $pK_a$ s of 7.1 and 7.9 for the two cysteine thiols, with the behavior of the complex Raman SH stretching band indicating that the two cysteine  $pK_a$ s were below pH 8.2. The same work indicated that all of the carboxyl groups in the molecule were completely deprotonated below pH 8.

The question of interpretation of these data hinges on the identification of the titrations of three groups, Asp 26, Cys 32, and Cys 35. The  $^{13}\text{C}$  NMR indicates two  $pK_a$ s for the cysteines of 7.5 and 9.5 (Jeng *et al.*, 1995), while the  $pK_a$  of Asp 26 is shown by the present work to be 7.5. The simplest explanation is that of Jeng *et al.* (1995): the two cysteine  $pK_a$ s are 7.5 and 9.5 and the Asp 26  $pK_a$  is also 7.5. By contrast, the interpretation of Wilson *et al.* (1995) is more complex. The higher of the two  $pK_a$ s seen in the  $^{13}\text{C}$  experiments on the cysteines is ascribed to the titration of Asp 26. Even without the direct evidence presented in this paper on the Asp 26  $pK_a$ , several facts argue against this interpretation. First, while proton chemical shifts can be influenced by a number of titrating groups (Dyson *et al.*, 1991), the influence of titrating groups other than the immediately adjacent one on the  $^{13}\text{C}$  chemical shift of the adjacent carbon atom should be small: the major influence on the  $^{13}\text{C}\beta$  carbon chemical shifts of the cysteines should therefore be the titrations of the cysteine thiols themselves. Second, a significantly greater change in  $^{13}\text{C}\beta$  chemical shift is seen for the  $pK_a$  9.5 transition for Cys 32 than for Cys 35, exactly the opposite of what would be expected from the relative proximity of the two cysteines to the Asp 26 side chain. Third, the structures of  $\text{Trx}-(\text{SH})_2$  and  $\text{Trx}-\text{S}_2$  are the same (Jeng *et al.*, 1994): only the most subtle changes in dynamics (Stone *et al.*, 1993) and hydrogen exchange (Jeng & Dyson, 1995) reveal any differences between the two forms at all. It is therefore at least plausible that the pH-dependent behavior of Asp 26 should be the same in the two forms of the protein, even though the charge balance in the active site is more negative at high pH in  $\text{Trx}-(\text{SH})_2$  than in  $\text{Trx}-\text{S}_2$ . In addition, the same differences in dynamics and hydrogen exchange indicate that the backbone mobility in the region of the active site is actually significantly greater in  $\text{Trx}-(\text{SH})_2$ : we would therefore expect the Asp 26  $pK_a$ , if different from that of  $\text{Trx}-\text{S}_2$ , to be removed toward the lower

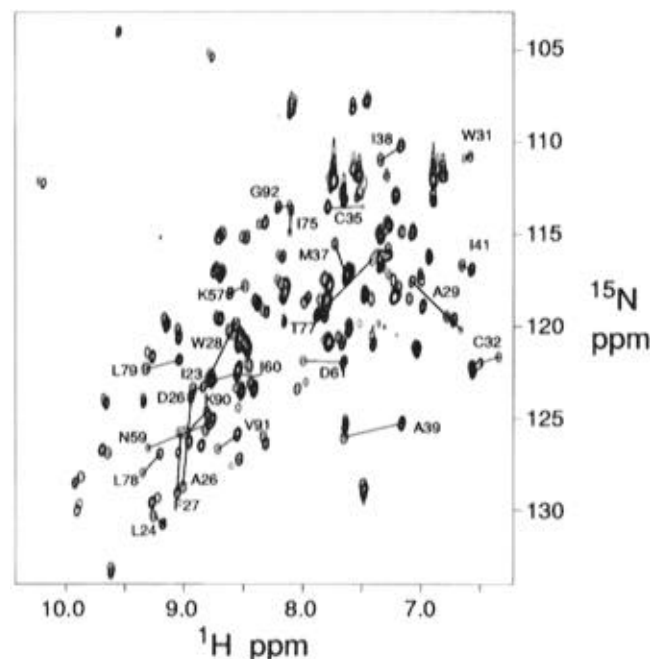


FIGURE 5: Superposition of the 500 MHz  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectra of wild-type Trx-(SH)<sub>2</sub> (black) at pH 6.49, 298 K, and D26A mutant Trx-(SH)<sub>2</sub> (red) at pH 6.51, 298 K, showing the extent of the changes in the chemical shifts in the vicinity of the active site as a result of the mutation.

values more characteristic of solvent-exposed carboxyl groups, rather than higher by 2 units in Trx-(SH)<sub>2</sub>, as suggested by Wilson *et al.* (1993).

The interpretation of Wilson *et al.* (1995) relies on two lines of evidence: changes in cysteine  $pK_a$ s in mutant proteins and Raman spectra that indicate that the cysteine  $pK_a$ s are 7.1 and 7.9 (Li *et al.*, 1993). However, they ignore other Raman data in the same paper that indicate that the titrations of all of the carboxyl groups (presumably including that of Asp 26) are complete below pH 8.0, and the fact that neither of the NMR titration studies (Wilson *et al.*, 1995; Jeng *et al.*, 1995) shows any indication of a  $pK_a$  of 7.9 for the cysteine  $^{13}\text{C}^\beta$  titrations. The major evidence cited by Wilson *et al.* (1995) in favor of a high  $pK_a$  (>9) for the buried Asp 26 carboxyl group is a comparison of the results for wild-type reduced thioredoxin with those obtained for a mutant in which the aspartate side chain is replaced by an alanine (D26A). This mutant has been the subject of a large amount of experimental research (Langsetmo *et al.*, 1990, 1991a,b), and we have also undertaken extensive NMR and biochemical studies of this and other mutants in the active site region (Dyson *et al.*, 1994; H. J. Dyson and A. Holmgren, manuscripts in preparation). Both the study of Wilson *et al.* (1995) and our own measurements indicate that the  $pK_a$ s of the two cysteine thiols in the mutant Trx-(SH)<sub>2</sub> are between 7.5 and 8.0. We have made complete assignments of the  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  NMR spectra of the D26A mutant using a combination of two- and three-dimensional homo- and heteronuclear spectra. These studies reveal that while the majority of the protein is relatively unaffected by the mutation, the structure in the active site region is greatly perturbed, as shown by large chemical shift differences between mutant and wild-type Trx-(SH)<sub>2</sub> at sites far removed in the sequence from the immediate area of the mutation. This is illustrated in Figure 5, which shows a comparison of the wild-type and D26A mutant  $^{15}\text{N}$  HSQC

spectra. A significant rearrangement of the hydrophobic pocket where the Asp 26 side chain resides in the wild-type protein is consistent with the observed increase in thermodynamic stability of the mutant protein (Langsetmo *et al.*, 1991b), although the stability of the protein to pHs lower than 6.0 is decreased (H. J. Dyson, unpublished observations). In view of the structural differences apparent between the mutant and wild-type proteins, it is not valid to extrapolate from the behavior of the mutant to make conclusions about the behavior of the wild-type protein: the  $pK_a$ s of the two cysteine thiols are most probably changed by a rearrangement of the active site structure in the mutant. In addition, the lowering of the  $pK_a$  of Cys 35 from 9.5 in wild-type to 7.5–8 in the mutant would appear to be a logical consequence of the removal of the negatively charged Asp 26 from its local environment.

A change in the  $pK_a$  of Asp 26 to 8.3 is observed in the double mutant C32S/C35S (Dyson *et al.*, 1994). A  $pK_a$  increase upon removal of the two potential negative charges on the cysteines is apparently paradoxical and was another of the justifications of Wilson *et al.* (1995) for their assignment of the  $pK_a$  to the >9.0 titration. By this argument, the high  $pK_a$  of Asp 26 in the wild-type protein is *reduced* in the mutant by the removal of the negative charges. Once again, extrapolation of mutant data to make conclusions about the wild-type protein is simplistic: other changes occur than a simple charge removal—the group that has replaced the charged group must be considered, as well as the structural rearrangements caused by the mutation in the hydrophobic pocket where the Asp 26 carboxyl is situated.

We have shown unequivocally that the Asp 26  $pK_a$  in wild-type reduced thioredoxin closely resembles that observed for the oxidized form of the protein. This is consistent with the high degree of similarity between the two structures (Jeng *et al.*, 1994) and with a mechanism that includes a shared proton between two thiols as a means both of stabilization of the reactive thiolate form of the Cys 32 side chain and of promoting complex formation between reduced thioredoxin and protein substrates (Jeng *et al.*, 1995). Both the oxidative and reductive reactions of thioredoxin are severely impaired in the D26A mutant, mainly in the slowing of reaction rates (A. Holmgren, H. J. Dyson, and I. Slaby, manuscript in preparation). The buried aspartate, with its  $pK_a$  poised at the lower of the two cysteine thiol  $pK_a$ s, is clearly involved in control of the efficient proton transfers at the active site during the reactions of thioredoxin, probably by serving as a proton source and proton sink removed from the exterior solvent after the formation of the hydrophobic complex between thioredoxin and its substrate.

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