Relationship between Electrostatics and Redox Function in Human Thioredoxin: Characterization of pH Titration Shifts Using Two-Dimensional Homo- and Heteronuclear NMR†

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ABSTRACT: The electrostatic behavior of potentially titrating groups in reduced human thioredoxin was investigated using two-dimensional (2D) 1H and 15N nuclear magnetic resonance (NMR) spectroscopy. A total of 241 chemical shift titration curves were measured over the pH range of 2.1–10.6 from homonuclear 1H–1H Hartmann-Hahn (HOHAHA) and heteronuclear 1H–15N Overbodenhausen correlation spectra. Nonlinear least-squares fits of the data to simple relationships derived from the Henderson-Hasselbalch equation led to the determination of pKₐs for certain isolated ionizable groups, including the single histidine residue at position 43 (pKₐ = 5.5 ± 0.1) and a number of aspartic and glutamic acid carboxylate groups. Many of the titration curves demonstrate complex behavior due to the effects of interacting titrating groups, the long range of electrostatic interactions through the protein interior, and, perhaps, pH-induced conformational changes on the chemical shifts. Unambiguous assignment of the pKₐs for most of the 38 potentially ionizing groups of human thioredoxin could therefore not be made. In addition, there was no clear evidence that Asp-26 titrates in a manner corresponding to that observed in the Escherichia coli protein [Dyson, H. J., Tennant, L. L., & Holmgren, A. (1991) Biochemistry 30, 4262–4268]. The pKₐs of the active site cysteines were measured, however, with Cys-32 having an anomalously low value of 6.3 ± 0.1 and that of Cys-35 between 7.5 and 8.6. These pKₐs are in agreement with proposed mechanisms for redox catalysis of thioredoxin and previously measured pKₐs within the active site of E. coli thioredoxin [Kallis, G. B., & Holmgren, A. (1980) J. Biol. Chem. 255, 10261–10265]. The stabilization of a thiolate anion at physiological pH can be explained by the interaction of the S' of Cys-32 with the amide of Cys-35 observed in the previously determined high-resolution solution structure of reduced human thioredoxin [Forman-Kay, J. D., Clore, G. M., Wingfield, P. T., & Gronenborn, A. M. (1991) Biochemistry 30, 2685–2698].

Determination of the ionization constants or pKₐs of histidines were among the earliest applications of nuclear magnetic resonance (NMR) spectroscopy to the understanding of the behavior of proteins. Because of the important role of electrostatic interactions in many of the functions of proteins, the ability to assign a pKₐ to a specific ionizable group in the molecule (based on chemical shift changes as a function of pH) can aid in the development of mechanistic descriptions of catalysis, binding, and other behavior. Two-dimensional 1H NMR has been used to determine pKₐs of a majority of the aspartate and glutamate side-chain carboxylates of bull seminal inhibitor II A (Ebina & Wüthrich, 1984) and, more recently, the pKₐs for all of the ionizable groups within the pH stability range of mouse epidermal growth factor (Khoda et al., 1991). It is therefore of considerable interest to apply this approach to a protein whose biological activity relies on chemistry involving the ionization of titrating groups.

Such one protein is thioredoxin, a small ubiquitous redox-active disulfide/dithiol catalytic, which has been shown to have a variety of functions in the thiol chemistry of the cell (Holmgren, 1989). The proposed mechanism of action of the protein involves the nucleophilic attack by the Cys-32 thiolate anion on a disulfide-containing substrate, producing a mixed-disulfide intermediate, which is further attacked by the thiolate of Cys-35 to yield a reduced substrate (Kallis & Holmgren, 1980). In this work, both 2D 1H–1H homonuclear and heteronuclear 15N–1H experiments have been performed on reduced human thioredoxin, relying on the previous 1H (Forman-Kay et al., 1989) and 15N (Forman-Kay et al., 1990) resonance assignments and the solution structure determination (Forman-Kay et al., 1991a), to probe the ionization constants of titrating groups in the active site, as well as in the rest of the protein. The present study is aimed at providing clues for a global understanding of the electrostatic behavior of the active form of this redox catalyst and suggests that pH-dependent structural changes and long-range electrostatic interactions due to buried charges can result in complications to a simplistic analysis of chemical shift titration curves.

The pKₐ of a free cysteine is 8.3 in aqueous solution (Cantor & Schimmel, 1980), but, since most cysteine residues in proteins are at least partially buried due to their hydrophobic nature, their apparent pKₐ could be much higher. The active site cysteine sulfhydryls in human thioredoxin are both around 20% surface accessible in the NMR solution structure (Forman-Kay et al., 1991a), suggesting that their pKₐs might be shifted to higher pH values. Chemical modification and fluorescence studies of reduced Escherichia coli thioredoxin, however, have indicated that Cys-32 has an anomalously low pKₐ (~6.7), stabilizing the thiolate at physiological pH (Kallis

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1 Abbreviations: COSY, two-dimensional correlated spectroscopy; DTT, dithiothreitol; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; NMR, nuclear magnetic resonance.

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Values for the pKₐ's of a number of isolated carboxyls of aspartic and glutamic acids, as well as for Cys-32, Cys-35, and His-43, were obtained. Although much of the titration data for human thioredoxin are extremely complex, the pKₐ values of the active site cysteines (Cys-32 and Cys-35) support the proposed mechanism of thioredoxin catalysis by initial nucleophilic attack of the Cys-32 thiolate anion.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation for NMR Experiments.** Natural abundance and uniformly (>95%) ¹⁵N-labeled human thioredoxin was purified as described previously (Forman-Kay et al., 1990, 1991a). For simple 1D ¹H NMR experiments, 0.2 mM samples of natural abundance human thioredoxin with 0.5 mM dithiothreitol (DTT) and 20 mM sodium phosphate buffer in D₂O were used. Five samples were prepared at pH values of 4.0, 5.0, 5.5, 7.0, and 8.5, sealed, and argon blanketed for 30 min. Approximately 80% of the molecules in this sample retained the N-terminal methionine, due to inefficient posttranslational processing (Forman-Kay et al., 1990), leading to a numbering scheme beginning with Met-1. To maintain the protein in a reduced state for the longer 2D experiments in H₂O, over 200-fold excess of fully deuterated DTT was added to the protein solutions under high pH (pH >8) conditions, and samples were incubated at 37 °C for at least 15 min. The solutions were then dialyzed overnight into argon-purged 50 mM sodium phosphate buffer at pH ~6 with trace amounts of DTT. The protein solutions were lyophilized and redissolved in 90% H₂O/10% D₂O with 10 mM deuterated DTT, resulting in ~75–100 mM sodium phosphate. The pH was adjusted by the addition of small amounts of concentrated HCl or NaOH. Measurements of the pH of the sample were not corrected for deuterium isotope effects and were taken before and immediately after the NMR experiment, with the latter measurement considered the most accurate. These values differed by less than 0.1 pH units for most measurements. Solutions of approximately 2 mM reduced human thioredoxin were used for NMR experiments at pH values of 5.6–10.6, while lower protein concentrations of around 0.2 mM were used for pH values of 2.1–5.2, necessitated by the reduced solubility of thioredoxin at low pH values. The protein samples used for the 2D heteronuclear studies lacked most of the N-terminal methionine due to different bacterial growth conditions, leading to molecules predominantly having an N-terminal Val-2 (Forman-Kay et al., 1990).

**NMR Spectroscopy.** All experiments were recorded on a Bruker AM600 spectrometer equipped with digital phase shifters and a "reverse"-mode proton probe and processed on an Aspect 3000 computer. The titration curve for the C⁴⁺ proton of the only histidine, His-43, was obtained by simple 1D experiments in D₂O at 40 °C. Amide ¹H and ¹⁵N chemical shifts for all residues in the protein were measured from a 2D ¹H-¹⁵N Overbodenhausen correlation experiment recorded in H₂O at 40 °C (Bodenhausen & Ruben, 1980; Bax et al., 1990). Presaturation was used to suppress the water signal. Figure 1 shows four ¹H-¹⁵N Overbodenhausen experiments of reduced human thioredoxin, two each from samples at ~0.2 and ~2 mM, over the complete pH titration range. The experiments at low protein concentration were recorded in about 6 h, with 1024 t₁ increments of 2K data points collected, while for high concentration samples 40 min was sufficient. Homonuclear Hartmann-Hahn (HOHAHA) experiments using a WALTZ17 mixing sequence (Bax, 1989) between 1.5-ms trim pulses were performed at 40 °C in H₂O, using the time-proportional incrementation method (Redfield & Kuntz, 1975; Bodenhausen et al., 1980; Marion & Wüthrich,
1983) to obtain pure phase absorption peaks. Experiments were recorded in 9 h for high concentration samples and 19 h for those at low concentration, with mixing times of 37–43 ms and 512 t1 increments of 2K data points. Water suppression involved a 90° "flip-back" pulse, a 100-μs recovery delay, and an on-resonance jump–return pulse sequence following the WALTZ17 mixing sequence (Bax et al., 1987). Figure 2 illustrates two HOHAHA spectra, at pH values of 5.2 and 7.6.

**pH Titrations.** Heteronuclear $^{15}$N–$^{1}$H correlation experiments were performed at 26 different pH values between 2.1 and 10.6, the latter value already giving rise to some denaturation. These values were 2.1, 2.2, 2.4, 2.5, 3.2, 3.6, 3.8, 4.3, 4.6, 5.0, 5.2, 5.6, 6.1, 6.4, 6.8, 7.2, 7.5, 7.8, 8.1, 8.4, 8.9, 9.4, 9.8, 10.0, 10.0, and 10.6. HOHAHA experiments were recorded at 23 different pH values of 2.8, 3.2, 3.3, 3.8, 3.80, 4.1, 4.6, 4.7, 4.9, 5.2, 5.5, 6.2, 6.3, 6.6, 6.7, 7.0, 7.1, 7.6, 7.7, 8.1, 8.2, 8.7, 9.1, and 9.7. The primary goal in recording NH–aliphatic correlations from H2O HOHAHA spectra was to obtain C–H and C–H chemical shifts for cysteine and aspartic acid residues, especially Asp-26, and C–H shifts for glutamic acid residues. The resonance of the downfield C–Hs of Asp-Ala overlapped the C–Hs of Met-37 at both pH 5.5 and 7.0 in previous spectra of the aliphatic–aliphatic region, making the more generally useful D2O HOHAHA or COSY-type experiments inappropriate for extracting the pKt value for this important residue. Proton chemical shifts were calibrated with respect to the internal $^{1}$H2O resonance. The chemical shift of $^{1}$H2O as a function of pH at 40 °C was measured in a series of aqueous solutions of 4 mM sodium trimethylsilylproprionate-d4 (taken as 0.00 ppm) at eight different pH values. A very small shift of approximately 0.02 ppm was observed between pH 4 and 6, and the proton chemical shift titration curve data were corrected by this amount. Because of the large number of pH values investigated and the resulting smooth titration curves, the resonances in all spectra could be easily assigned by comparison with completely assigned spectra recorded at pH 5.5 and 7.0 (Forman-Kay et al., 1989, 1990).

**Calculations of pKt Values.** The large number of pH titration curves were analyzed by multiple fits to a simple model derived from the Henderson–Hasselbalch equation:

$$\text{pH} = \text{pKt} + \log \left( \frac{[\text{conjugate base}]}{[\text{conjugate acid}]} \right) = \text{pKt} + \log \left( \frac{\theta}{1 - \theta} \right) \quad (1)$$

where θ represents the fractional concentration of the conjugate base. Assuming a rapid equilibrium between protonated and unprotonated forms,

$$\delta = \delta_{\text{base}} \theta + \delta_{\text{acid}} (1 - \theta) \quad \text{and} \quad \theta = (\delta - \delta_{\text{acid}})/ (\delta_{\text{base}} - \delta_{\text{acid}}) \quad (2)$$

where δ is the chemical shift of a resonance as a function of pH, and δacid and δbase represent the chemical shift values at the low and high extremes of pH, respectively. Substituting the relationship for θ as a function of chemical shift into the Henderson–Hasselbalch equation ultimately leads to

$$\delta = \left[ \delta_{\text{acid}} - \delta_{\text{base}} \right] 10^{(pH - pKt)} / [1 + 10^{(pH - pKt)}] \quad (3)$$

The program FACSIMILE (Chance et al., 1977; Clore, 1983) was used to perform nonlinear least-squares fits of the data to this relationship, with the following three variable parameters: the chemical shifts at the low and high extremes of pH and the pKt. Data from all chemical shift titration curves of resonances which monitored the titrating group were included, in order to increase the confidence level of the pKt determination. For situations where many titrating groups could potentially influence one another, fits were made to more than one pKt (2–7) and up to 50 titration curves were used. A noninteracting model derived as a simple extension of the above relationship was nonetheless sufficient to fit these curves. The resulting general equation for a fit of n different pKt values, $pKt_1$–$pKt_n$, can be written as

$$\delta = \frac{\sum_{i=0}^{n} [\delta_{i} 10^{-\sum_{j=1}^{n}[pKt_i + pH]}]}{\sum_{i=0}^{n} [10^{-\sum_{j=1}^{n}[pKt_i + pH]}]} \quad (4)$$
where $\delta_o = \delta_{\text{acid}}$, $\delta_n = \delta_{\text{base}}$, and other $\delta_i$ values are intermediate chemical shifts between the $pK_a$ values. Even with this simple model, however, there were many variable parameters, including the individual $pK_a$s and the chemical shifts at the intermediate pH values for the titration curve of each resonance. Tests to see if a fit of three $pK_a$s to active site titration curves could
amide 'HN Thr-30

action between the pKas and led us to apply a noninteracting model in all cases.

RESULTS AND DISCUSSION

The 'H and 'N chemical shifts for backbone and side-chain amide groups of reduced human thioredoxin which showed significant titration shifts (greater than ~0.05 ppm in the 'H dimension or 0.5 ppm in the 'N dimension) over the entire pH range were measured from 'H-'N Overbodenhausen correlation experiments. All aliphatic 'H chemical shifts of aspartic and glutamic acids, of the five cysteines, and of residues near the active site region of thioredoxin (Ser-28-Trp-31) were tabulated from the NH-aliphatic cross peaks in HOHAHA experiments. The large amount of data collected can be grouped as follows: 'H chemical shift titration curves were obtained for 86 backbone amides and partial data for six pairs of side-chain amino NH2 groups (26 pH values) and for 26 C0Hs, 10 degenerate C1Hs, 16 pairs of nondegenerate C0Hs, two degenerate C1Hs, partial data for five pairs of nondegenerate C1Hs, and the single N0'H of Trp-31 (23 pH values), yielding a total of 175 titration curves. One-dimensional data at four pH values and 2D data from the HOHAHA at six different pH values resulted in a titration curve for the His-43 C0'H of both the N-met and N-Val forms of the protein, covering the interval from pH 4.0 to 8.5. 'N chemical shifts from 60 backbone amides, three side-chain NH2 groups, and the one N01'H of Trp-31 were also measured at 26 pH values, leading to 64 additional titration curves. For residues having duplicated resonances due to the N-Met(N) heterogeneity, chemical shifts were measured for both peaks where possible. For the amide groups, the increase in the exchange rate at higher pH values resulted in the loss of some resonances even before reaching the denaturation pH. For most aspartic and glutamic acid residues, chemical shifts of aliphatic protons were only measured at pH values of 7.1 or less.

A qualitative assessment of the amide data reveals that the largest titration shifts occur in the active site region, from residues Thr-30 to Cys-35 and between residues Asp-61 and Gln-63. The largest titration shifts for aliphatic protons are also located within the active site between Ser-28 and Cys-35 and at residue Asp-61. A summary of these observed values is listed in Table 1. The observation that the amide proton and nitrogen chemical shifts are the most sensitive to changes in the electronic environment due to changes in the pH may be explained by the fact that the amide N-H bond is much easier to polarize than the C-H bond. Since they are more sensitive, leading to greater chemical shift changes over the pH range, the data measured from these 'N-'H correlation experiments have smaller relative errors and yield a more accurate picture of the electrostatic behavior of the protein. The unfortunate aspect of this sensitivity, though, is that due to the apparently low dielectric within the protein core, changes in partial charges can affect resonances over long distances.

For each of the 38 potentially titrating groups in reduced human thioredoxin, a set of titration curves for resonances of nuclei close in space to the chemical group was chosen to be included in the fit to generate its ionization constant. The potentially titrating groups include seven aspartic (16, 20, 26, 58, 60, 61, 64) and 10 glutamic (6, 13, 47, 56, 68, 70, 88, 95, 98) acids, one C-terminal carbonylate, five cysteines (32, 35, 62, 69, 73), one tyrosine (49), one histidine (43), 12 lysines (3, 8, 21, 36, 39, 48, 72, 81, 82, 85, 94, 96), and one N-terminal amino group. The location of these titrating groups on a ribbon drawing representation of the structure of reduced thioredoxin is shown in Figure 3. In the case of some of the surface-exposed and isolated carbonylate groups, the data provide clear answers. For lysine residues and the single tyrosine, however, no accurate ionization constants could be obtained, due to their high pKa close to the denaturation pH, as well as the loss of signal due to rapidly exchanging amide protons. There was no evidence, however, for any anomalous low pKa of these groups. For many of the other groups, the interaction of other nearby titrating species and the effects of long-range electrostatic interactions from partially buried charges led to complications in the determination of the pKA or an inability to assign any pKa value unambiguously. Very small chemical shift changes over the titration range also prevented our determination of pKa values for a number of residues. Many of these difficulties were especially apparent in the attempt to understand the titrating behavior of groups within the active site, including Asp-26, which has attracted considerable attention regarding its potential role in redox catalysis by thioredoxin (Langsetmo et al., 1990, 1991a,b; Dyson et al., 1991).

Unambiguous results were obtained for the one histidine and for some aspartic and glutamic acid carbonylate groups. The titration curve observed for the C0'H of His-43 (Figure 4) exhibits simple Henderson-Hasselbalch behavior and yields

<table>
<thead>
<tr>
<th>residue</th>
<th>'HN (±0.5 ppm)</th>
<th>'NH (±0.5 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr-30</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>Asp-61</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>Cys-35</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>Trp-31</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Gln-63</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Cys-32</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Cys-62</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Asp-61</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Ser-28</td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 3: Ribbon drawing representation of the solution structure of reduced human thioredoxin illustrating the location of the titrating groups. The structure is from Forman-Kay et al. (1991a), and the ribbon drawing was created with the program MOLSCRIPT (Kraulis, 1991).
Individual fits yielded the same pK, value as those involving a pKa of 5.5 including all resonances of the residue. In all cases, the in-or more solvent-exposed surface (Forman-Kay et al., 1991a).

Multiple titration curves. Both Asp16 and Asp-20 have 80% least-squares best fit to the data derived from resonances of both forms, demonstrating a pK, of 5.5 ± 0.1.

The pKa of Glu-13 seems to be 4.8 while they may be influencing each other, due to their location through the protein interior makes assignment of any resulting changes resulting from the titrations of interest. In order to extract the major pK, from these complicated curves, a simplified approach was taken. Data from titration curves of both amide and aliphatic resonances of a titrating residue were simultaneously fit to a Henderson–Hasselbalch curve (eq 3).
Although the experimental curves showed deviations from these simple fits, the results provide qualitative information about the pKₐ's of certain ionizable groups of human thioredoxin. Asp-64 appears to have a pKₐ of 3.1 ± 0.2, although it is close to a cluster of other aspartic acid residues including Asp-58, Asp-60, and Asp-61 and has a relatively low surface accessibility of 20%, giving rise to titration curves which are influenced by multiple pKₐ's. Glu-68 and Glu-88 exhibit a midpoint of their titration curves at 4.2 ± 0.2 and 3.9 ± 0.2, respectively, but they clearly show the effects of other lower pKₐ's. With the large number of negatively charged residues in human thioredoxin, this is not surprising. These pKₐ's of carboxyl groups are also near the values of 4.0 for aspartic acid and 4.4 for glutamic acid side chains seen in denatured proteins (Nozaki & Tanford, 1967).

In an effort to analyze the large amount of data which were collected and to derive more meaningful results regarding the electrostatic behavior of thioredoxin, the ionizable residues within the molecule were grouped into potentially interacting groups. The experimental data were simultaneously fit to ionization constants for each of the involved tittering side chains, with two to seven pKₐ's using eq 4. These calculations included titration curves of many resonances, which appeared to be monitoring not only the pKₐ's of interest in the interacting group but other pKₐ's as well. The results of these multiple fits, though, did not yield pKₐ's which could be unambiguously assigned to single side-chain groups, although in some cases general trends could be inferred.

The set of ionizable groups on which most of our effort was focused comprised those within the active site (Figure 3). This contains an overabundance of potentially charged residues, including two Cys-32 and Cys-35 sulfhydryls, Glu-56, Asp-26, Asp-58, Asp-60, and Asp-61 carboxylates, Lys-36 and Lys-39 NH₂ groups, and the His-43 imidazole ring. In addition, many of these side chains are relatively surface inaccessible, especially Asp-26, Glu-56, Asp-58, and the two active site cysteines. Multiple calculations were performed, fitting different groups of chemical shift curves over various pH regions to equations involving anywhere from one to seven pKₐ's. All of the results exhibited midpoints of titration at pH 6.1–6.4, corresponding to the pKₐ value of 6.3 ± 0.1 extracted from a simple fit of eq 3 to only Cys-32 and Cys-35 resonances. A fit involving 28 titration curves from active-site resonances (Ser-28 CH, COH, COH; Ala-29 NH, 15N, COH, COH; Thr-30 NH, 15N; Trp-31 NH, 15N, N=H, 15N(N); Cys-32 NH, 15N, COH, COH, COH; Cys-35 NH, 15N, COH, COH, COH; Cys-35 NH, 15N, COH, COH, COH; Lys-36 NH, 15N; Met-37 NH, 15N; Thr-74 NH) revealed multiple pKₐ's of 3.0 ± 0.1, 4.1 ± 0.1, 6.3 ± 0.1, and 8.2 ± 0.3, showing the influence of a number of ionizable groups in this region. The resulting best fit curves to the Cys-32 and Cys-35 titration curves are shown as solid lines in Figure 7. Another fit involving only the resonances of the two active site cysteine residues (Cys-32 NH, 15N, COH, COH, Cys-35 NH, 15N, C=O) is also illustrated, with a dashed line. This fit yielded similar pKₐ's of 2.4 ± 0.2, 4.2 ± 0.4, 6.3 ± 0.1, and 8.4 ± 1.3. The titration observed at pH 8.2–8.4 could tentatively be assigned to the Cys-35 sulphydryl. This pKₐ value was not very well determined, however, since variation between many different fits were noticed, clustering around 7.5–8.6, but even exhibiting values of 7.1–9.2 in others. This pKₐ does correspond, though, to one around 8.5–9.0 measured in previous biochemical and NMR studies of E. coli thioredoxin (Kallis & Holmgren, 1980; Dyson et al., 1991). More specific assignments of the low and high pKₐ's could not be made beyond attributing them to one or more of the three aspartic acids and one or both of the two lysines. The average percentage of the chemical shift changes for the seven Cys-32 and Cys-35 resonances plotted in Figure 7 attributable to each of the pKₐ's is as follows: pKₐ 2.4 (16%), pKₐ 4.1 (12%), pKₐ 6.3 (63%), and pKₐ 8.4 (8%). The dominance of the Cys-32 titration at pH 6.3 is evident. Note that no pKₐ around 5.5 was detected in the fits, showing that the titration of His-43 is independent of these other ionizations, allowing the determination of its pKₐ solely from analysis of the C=O chemical...
shift curve and effectively eliminating it from consideration in terms of redox function.

The C-terminal carboxylate of thioredoxin was included in a group with the side chain of Glu-103 (Figure 3). Fits to two pKₐs resulted in values of 3.2 ± 0.1 and 4.9 ± 0.1 for the C-terminus and Glu-103, respectively. These tentative assignments were made on the basis of the closer fit of the titration of the Glu-103 C'Hs to the higher pKₐ value and on the basis of reference pKₐ values of 4.4 for glutamic acids and 3.8 for the C-terminus. The α-amino group of the N-terminal Val-2 was analyzed as a separate, noninteracting ionization. A titration of 7.3 ± 0.2 was observed, equal to the reference pKₐ of 7.5 for α-amino groups. The fact that this pKₐ is so close to neutral suggests a possible cause for the dramatic duplication of a third of the backbone resonances of reduced thioredoxin caused by the N-terminal heterogeneity (Forman-Kay et al., 1990). If the pKₐ of the N-terminal Met-1 α-amino group is even slightly shifted from the value observed for Val-2, a localized partial charge difference might explain the duplications in chemical shift for resonances spread over the whole face of the protein. Alternatively, the movement of the charge could also explain these chemical shift changes, even with no difference in pKₐ.

The three additional cysteines of human thioredoxin are also found within close range of each other (Figure 3). No evidence for titrations within this clustered group of cysteines was found, however. The amide proton of Cys-73 exchanges too rapidly to be seen much above pH 7, while the very small shifts for the resonances of Cys-62 and Cys-69 were not sufficiently reliable to extract pKₐ values. These latter two cysteine residues may well remain protonated up to pH values close to the denaturation pH of the protein, since both sulfhydryl groups are less than 10% surface accessible. The accessibility of Cys-73 is greater, though, at approximately 60%.

Another group of interacting charges was studied, comprising the six potential salt bridges identified within the solution structure of reduced human thioredoxin (Forman-Kay et al., 1991a). These are between the carboxylate group of Asp-20 and the N'H₃⁺ groups of Lys-81 or Lys-82, Glu-56 and Lys-36 or Lys-39, Glu-68 and Lys-85, Glu-95 and Lys-94, and Glu-98 and Lys-48 (Figure 3). None of the fits were conclusive in determining whether an interaction between the charges could stabilize the potentially buried charge of Glu-56 or if these interactions led to anomalous pKₐ values. A simple fit to the Glu-56 C'Hs is not convincing due to the extremely small changes in chemical shifts observed across the entire pH range (0.01 and 0.04 ppm).

Other potentially buried charges are located with the extended active site region, including Asp-26 and Asp-58 (Figure 3). The solution structure identifies possible stabilizing interactions for Asp-26 with the O'H of Ser-28 and for Asp-58 with the main-chain carboxyls of Thr-30, Asp-60, and Asp-61 (Forman-Kay et al., 1991a). In addition, a study of the structure of bound water in reduced human thioredoxin reveals a water located close to Ser-28 and Asp-26 side chain (Forman-Kay et al., 1991b). The fits of the entire active site do not reveal any specific information about these groups, but the individual fits suggest a pKₐ of 3.1 ± 0.2 for Asp-58, although the effects of a probable interaction with the Asp-60 and Asp-61 pKₐ on this value cannot be ignored. Asp-26 is much more complex, showing multiple pKₐ values within the titration curves for the resonances of this residue (Figure 8), but with overall little change in chemical shift, especially for the C'Ohs (0.06 ppm). A major titration overlaps the active site pKₐ of 6.3, with two inflections in the acidic pH range around 3 and 5 (near the His-43 C'O titration). A fit involving 16 titration curves monitoring the buried Asp-26 and Glu-56 carboxylate groups (Asp-26 NH, 13N, C'O, C'O,H, C'O,H; Ser-28 NH, 13N, C'O,H, C'O,H; Thr-30 C'O,H; Glu-56 NH, 13N, C'O,H, C'O,H; C'O,H, C'O,H) shown as a solid curve, led to pKₐs of 3.2 ± 0.3, 5.0 ± 0.4, 6.4 ± 0.1, and 9.3 ± 0.1. These values agree with those derived from a fit using only the five resonances observed for Asp-26, 3.2 ± 0.9, 5.0 ± 0.4, 6.4 ± 0.1, and 9.3 ± 0.1. These fits are plotted as dashed curves. These patterns observed for the Asp-26 titration curves of human thioredoxin are markedly different from those seen in the NMR study of the reduced E. coli protein (Dyson et al., 1991), where a significant titration shift (~0.2 ppm) of one of the C'Ohs was observed with a pKₐ of 7.2–7.3.

The basically unperturbed chemical shift (±0.06 ppm) of the Asp-26 C'O in reduced human thioredoxin as a function of pH (range: 2.1–10.6) can most readily be explained by assuming that the side-chain carboxyl is always protonated, as perhaps expected in its relatively buried position, or, less likely, that the effects of other ionizable groups or conformational changes are masking the intrinsic titration of the Asp-26 carboxyl. Since the average chemical shift change of the measured C'Ohs of aspartic acid residues in this study is only 0.10 ± 0.07, however, this small chemical shift change may not be a clear diagnostic for a stable protonated state over...
the entire pH range. A similar argument could hold for
the buried Glu-56 being protonated at physiological pH, since
the changes in chemical shift over the measured pH range
(2.8-7.1) for the two $^{13}$C-Hs are only 0.01 and 0.04 ppm. The
average chemical shift change for measured $^{13}$C-Hs of glutamic
acid residues, however, is also not large (0.07 ± 0.06). The
possibility exists that the pK$_a$ of Asp-26 is one of the values
involved in the fits described above (3.3, 5.0, 6.3, or 9.0), but
an equally likely possibility is that these values reflect the
titrations of other groups within the protein monitored by
Asp-26 resonances.

In interpreting any of these titration data, the relationship
between the chemical shift and electrostatics must be ad-
dressed. The analysis of pK$_a$s relies on a macroscopic view
of the system, since at any one time an ionizable group is either
protonated or deprotonated. The chemical shift of a given
resonance results from the averaging of the NMR frequencies
from two different conformational states in rapid equilibrium,
one in which a proton is bound and one in which it is ionized.
The resonance frequency of a nucleus in the individual states
is due to its electronic shielding. The dominant effects on the
chemical shift are the electromegativity and partial charge
on atoms which are covalently bound, since these have an
immediate influence on the polarization of the electrons sur-
rounding the nucleus observed in the NMR experiment. Other
important effects on the chemical shift can be attributed to
the relative position of the nucleus to charges on ionizable
groups and $\pi$-shell aromatic electrons and to the collective
charge potential of all electrons of the protein in the given
conformational state. The changes in chemical shift over the
pH range are caused by a shift in the equilibrium from a
situation favoring the protonated states of titrating groups to
one favoring the deprotonated states. The conformational
change required to accommodate or lose a proton may in some
cases be extremely small or may in certain situations be more
significant. Thus, the change in chemical shift may be at-
tributed primarily to a change in the local charge density near
an ionizable group, especially on a covalently bound atom, or
to a conformational change accompanied by a change in the
overall electric potential.

There are other difficulties in interpreting these pH titration
curves in terms of the biochemical mechanism of thioredoxin
catalysis. For example, it is possible for more than one group
to ionize within what appears to be a single observed titration
shift. Therefore, it is difficult to separate and individually
assign very close pK$_a$s. The overall results of these experiments,
though, do correlate with the current understanding of thio-
redoxin catalysis as well as with previous measures of pK$_a$s
within the active site (Kallis & Holmgren, 1980; Reutimann
et al., 1981).

Having determined these experimental pK$_a$ values, it would
be instructive to compare our results with those predicted by
electrostatic theory. Computational algorithms for this purpose
are still in a developmental stage, with some limited success
in predicting pK$_a$s of titrating groups in proteins (Bashford
& Karplus, 1990). Their primary application, however, may
not be in predicting pK$_a$s but in aiding the interpretation of
observed electrostatic effects. Application of the program
DelPhi (Honig et al., 1988) to study the anomalously low pK$_a$
observed for Cys-32, as well as other pK$_a$s derived from these
titration experiments, is now under way.

CONCLUDING REMARKS

Using 2D $^1$H and $^{15}$N NMR, a total of 241 chemical shift
titrations occur as a function of pH were measured. Nonlinear
least-squares fits of the data to a simple relationship derived
from the Henderson-Hasselbalch equation allowed unam-
biguous pK$_a$s for certain isolated ionizable groups to be
obtained. These include pK$_a$s for the titration of His-43 and
and a number of aspartic and glutamic acid residues. The values
obtained for the most part were close to the reference pK$_a$s
of the amino acid side chain. The pK$_a$ value of His-43 (5.5
± 0.2), however, is significantly shifted to a lower pH value
than the reference value of 6.3 (Nozaki & Tanford, 1967),
consistent with its limited surface accessibility of about 66%.

The results for titrations within the active site support the
model for thioredoxin redox catalysis by initial attack of the
thiolate anion of Cys-32. The experimentally observed pK$_a$
of Cys-32 is 6.3 ± 0.1, while that of Cys-35 is probably be-
tween 7.5 and 8.6. These values are in agreement with the
previously measured pK$_a$s of active site residues of E. coli
thioredoxin of 6.4-6.8 and 8.5-9.0 from chemical modification
and fluorescence studies (Holmgren, 1972; Kallis & Holmgren,
1980; Reutimann et al., 1981) and 7.1-7.4 and 8.4 from NMR
analysis (Dyson et al., 1991). The anomalously low pK$_a$ for
Cys-32 can be explained by the hydrogen-bonding interaction
between its S$^\prime$ atom and the backbone amide of Cys-35 seen
in the solution structure of reduced human thioredoxin
(Forman-Kay et al., 1991a), stabilizing the Cys-32 thiolate
anion at physiological pH values.

There is no clear evidence for the titration of the Asp-26
carboxylate, and the titration behavior of many of the 38
potentially ionizable groups in thioredoxin, including other
groups within the active site, is extremely complex. The effects
of nearby interacting titrating groups, long-range interactions
through the low dielectric core of the protein, and pH-induced
conformational changes on chemical shifts can lead to com-
plicated titration curves which often do not yield unambiguous
assignments of pK$_a$s to individual ionizable groups. This study
of the pH-induced chemical shift titrations of NMR resonances
of the protein, however, has resulted in the determination of
the pK$_a$ of Cys-32 and, in conjunction with knowledge from
the solution structure of reduced human thioredoxin, has
provided a foundation for further analysis of the role of
electrostatics in the function of this redox catalyst.

SUPPLEMENTARY MATERIAL AVAILABLE

Titration curve data in the form of a table of chemical shifts
as a function of pH for the 241 tabulated resonances (16
pages). Ordering information is given on any current masthead
page.

Registry No. Asp, 56-84-8; Glu, 56-86-0; Cys, 52-90-4; Tyr,
60-18-4; His, 71-00-1; Lys, 56-87-1.

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Calmodulin is a ubiquitous intracellular Ca^{2+}-receptor protein which in the Ca^{2+}-loaded state can interact with and activate a number of protein kinases [see Klee (1988)]. The crystal structure of calmodulin (Babu et al., 1985, 1988; Kretzinger et al., 1986) shows that the protein consists of two Ca^{2+}-binding domains, each containing two E-F hand motifs, separated by an extended central α-helix to form a dumbbell-like shape. The mammalian protein contains two tyrosine residues but no tryptophan. Conformational changes involving...