Proton-Transfer Effects in the Active-Site Region of *Escherichia coli* Thioredoxin Using Two-Dimensional ^1H NMR†

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ABSTRACT: A series of two-dimensional (2D) correlated ^1H NMR spectra of reduced and oxidized *Escherichia coli* thioredoxin have been used to probe the effects of pH in the vicinity of the active site, -Cys_{32}-Gly-Pro-Cys_{35}-, using the complete proton resonance assignments available for thioredoxin. In either oxidation state, the majority of residues of the thioredoxin molecule remain unchanged between pH 5.7 and pH 10, as indicated by the identical chemical shifts of the $C^\alpha H$, $C^\beta H$, and other protons. In reduced thioredoxin, a fairly widespread region around the active-site dithiol is affected by the titration of a group or groups with pK_a approximately 7.1–7.4 in ^2H_2O. Another titration, with pK_a approximately 8.4, affects a smaller region of the protein. Oxidized thioredoxin contains a disulfide and no free thiol groups; nevertheless, the proton resonances of many groups in the active-site region were observed to titrate with a pK_a of 7.5, probably as a result of an abnormally high pK_a value for the carboxyl group of the buried Asp-26 residue. For reduced thioredoxin, the results indicate that Asp-26 is titrating in this pH range, as well as both thiol groups. The new results are strongly suggestive that the mechanism of thioredoxin-catalyzed protein disulfide reduction may be critically dependent on proton transfer as well as electron transfer within the active site.

Thioredoxin is a small protein found in prokaryotic and eukaryotic cells, containing a conserved redox-active disulfide/dithiol with the sequence Cys_{32}-Gly-Pro-Cys_{35} (Holmgren, 1985). Many functions of thioredoxin are known, in particular as a protein disulfide oxidoreductase (Gleason & Holmgren, 1988; Buchanan, 1986; Holmgren, 1989). On the basis of chemical modification studies, Kallis and Holmgren (1980) postulated a mechanism of action of *Escherichia coli* thioredoxin involving a mixed-disulfide intermediate, as a result of nucleophilic attack on the substrate by the thioredoxin Cys-32 thiolate. Tryptophan fluorescence studies (Holmgren, 1972; Reutimann et al., 1981) indicated that a group of pK_a 6.5–6.7 was titrating in the active-site region of reduced thioredoxin. A similar pK_a was obtained for Cys-32 from the titration study in the presence of iodoacetate or iodoacetamide (Kallis & Holmgren, 1980), while a second sulfhydryl titration occurred at pH >8.5. At pH 7.5, only Cys-32 was alkylated in native thioredoxin (Kallis & Holmgren, 1980). An interaction with a positively charged group, possibly the ϵ-amino group of Lys-36, was invoked to explain the abnormally low pK_a value of the Cys-32 thiol. The nuclear magnetic resonance (NMR)[1] structure of reduced thioredoxin (Dyson et al., 1990) and the X-ray crystal structure of oxidized thioredoxin (Holmgren et al., 1975; Katti et al., 1990), as well as recent mutagenesis studies (Gleason et al., 1990), have shown that the Lys-36 side chain is probably not an essential component of the active site. However, the sulfhydryl group of Cys-35 is in close proximity to the ϵ-amino group of Lys-57 and the side chain carboxyl group of Asp-26 (Dyson et al., 1990), suggesting that these groups may participate in proton-transfer reactions associated with formation or reduction of the active-site disulfide.

The availability of complete proton resonance assignments for both reduced and oxidized thioredoxin (Dyson et al., 1989) allows for the first time a direct site-specific measurement of the pK_a's of the amino acid side chains in the active-site region. To elucidate the role of possible pH-dependent structural changes in the active-site region, and their role in the reaction mechanism of thioredoxin, two-dimensional NMR spectra of reduced and oxidized thioredoxin were accumulated at a number of pH values between 5.7 and 10.0. The spectra were analyzed by using the complete proton resonance assignments available for thioredoxin (Dyson et al., 1989), and the results were interpreted in terms of alternative mechanistic hypotheses for thioredoxin.

Materials and Methods

Sample Preparation for NMR Experiments. Samples of reduced and oxidized thioredoxin were prepared as described previously (Dyson et al., 1988, 1989). The protein was reduced by the addition of dithiothreitol to a concentration of 10 mM, and the sample was desalted at a given pH in ^2H_2O by passage through a short column of Sephadex G25 equilibrated with 0.1 M potassium phosphate buffer under argon. pH values in ^2H_2O are uncorrected meter readings, and the values quoted are those obtained after the completion of the NMR experiment. For pH values above 9, the desalted solutions were not well buffered by phosphate, but the pH could be adjusted upward by the addition of small amounts of 1 M NaO^2H in ^2H_2O. No spectroscopy was attempted at pH values below 5.7, since thioredoxin aggregates at lower pHs, as indicated by an increase in resonance line width (Holmgren & Roberts, 1976). No dimerization or other aggregation phenomena were observed in the present experiments. Resonance line widths remained constant throughout the pH range used (see Figure 1).

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[1] Abbreviations: NMR, nuclear magnetic resonance; 2QF COSY, double quantum filtered two-dimensional correlated spectroscopy.

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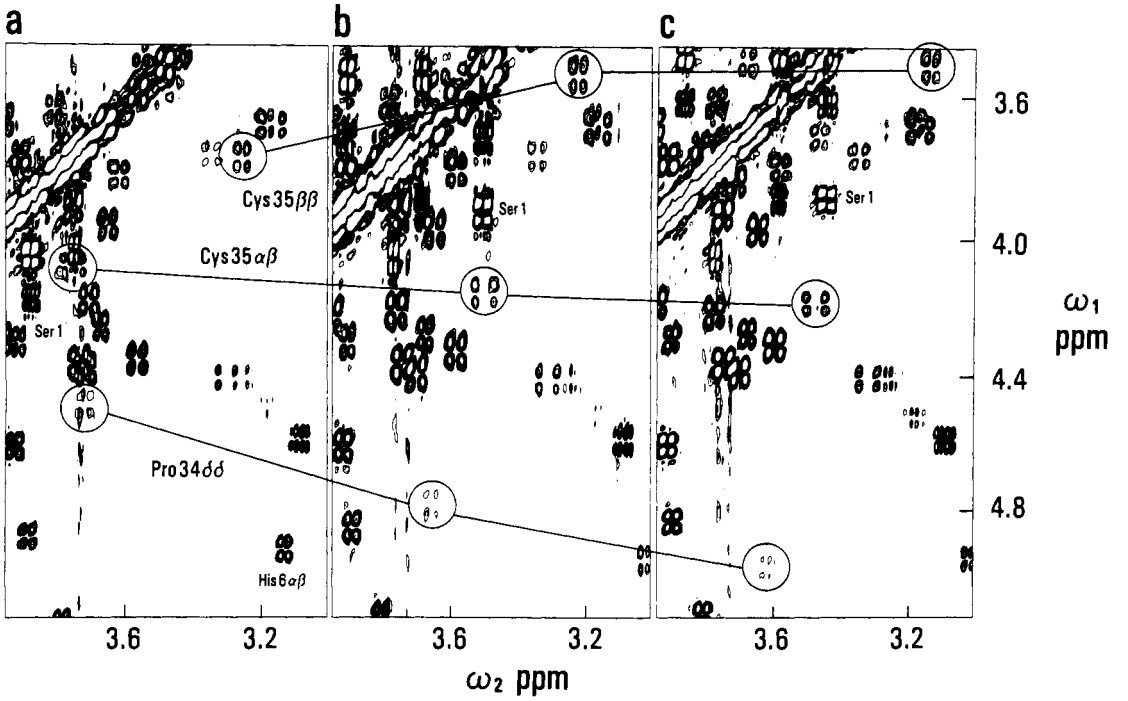

FIGURE 1: Portions of representative 2QF COSY spectra from the titration of reduced thioredoxin: (a) pH 5.89; (b) pH 7.96; (c) pH 9.08. Cross-peaks which move significantly in this range are indicated.

NMR Measurements. All NMR measurements were made on a Bruker AM500 spectrometer equipped with digital phase-shifting hardware. Chemical shifts are referred to an internal standard of dioxane. At each pH, a double quantum filtered phase-sensitive COSY spectrum [2QF-COSY (Rance et al., 1983)] was acquired with 4K complex data points, with a spectral width of 5000 Hz, and with 500 t_1 points. Spectra were Fourier-transformed by using a Sun workstation fitted with a Sky Warrior array processor and the software of Dr. Dennis Hare. Phase-shifted sine-bell window functions were used in both dimensions.

Titration Data Treatment. Chemical shift data as a function of pH were fitted by the method of least-squares to a titration curve of the form

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

where δ represents the chemical shift value, δ_{HA} and δ_A are the chemical shift values for a given proton in the species present at the low- and high-pH limits, and n is the number of protons transferred with the given pK_a. In all the calculations used in this study, the n value was fixed at 1. The curve fits and pK_a values quoted were obtained by using the program TempleGraph (Temple University) on the Sun workstation. For oxidized thioredoxin, all of the titration data for protons in the active-site region fitted satisfactorily to the single-proton titration (eq 1).

Where the data for reduced thioredoxin showed evidence of two titrations, they were at first crudely fitted by using separate single-pK_a titration curves. A second round of fits was made by using the following two-pK_a titration algorithm:

$$\delta = \{1/([H^+]^2 + [H^+]K_1 + K_1K_2)\} \times (\delta_{H2A}[H^+]^2 + \delta_{HA}K_1[H^+] + \delta_AK_1K_2) \quad (2)$$

where δ_{H2A}, δ_{HA}, and δ_A are the chemical shift values for a given proton in the low-, intermediate-, and high-pH species and K_1 and K_2 are the acid dissociation constants for the first and second proton transfers, respectively. In this simplified model, the number of protons transferred in each step is kept at 1, and no attempt is made to include more than one proton-transfer pathway.

An estimate of the average pK_a values for the apparent two-proton titration of reduced thioredoxin was obtained by a simultaneous least-squares fit using the same double-titration algorithm (eq 2) to the titration data for 35 protons in the active-site region. These were chosen on the basis of the total chemical shift change during the titration being in excess of 0.06 ppm. Consensus pK_a values were obtained from this calculation, and, for each of the data sets, final values of δ_{H2A}, δ_{HA}, and δ_A were derived.

RESULTS

The 2QF COSY spectrum at each pH was assigned as completely as possible by reference to the published complete proton assignments (Dyson et al., 1989). For a spectrum in ^2H_2O, this allows assignment of the majority of the C$^\alpha$H and C$^\beta$H resonances, the C$^\gamma$H$_3$ resonances of all of the valine residues and some of the isoleucines, and all of the aromatic resonances. The C$^\alpha$H resonances of Gly-21 and Thr-11 and the C$^\alpha$H and C$^\beta$H resonances of Ile-75 could not be assigned by this method, since resonance overlap precludes observation of a COSY cross-peak for Gly-21 (C$^\alpha$H resonances coincident) or Thr-11 (C$^\alpha$H and C$^\beta$H resonances coincident) and the $^3J_{\alpha\beta}$ coupling constant of Ile-75 is so small that a COSY cross-peak is unobservable. Only one C$^\alpha$H–C$^\beta$H COSY cross-peak is generally observed for most of the residues, especially for those in the β-sheet; in some cases, the C$^\beta$H–C$^\beta$H cross-peak could be resolved to give the assignment of the other C$^\beta$H resonance.

The most obvious feature of the titration of both oxidation states of the protein is that the majority of the resonances resolvable in the COSY spectrum are invariant in chemical shift between pH 5.7 and pH 10, implying that most of the molecule is unchanged in structure in this range. This in itself is an unusual result, and gives further evidence of the extraordinary stability of the thioredoxin tertiary structure, comparable with its stability to high temperature (Reutimann et al., 1981; Hiraoki et al., 1988). For reduced thioredoxin, approximately 20% of the residues in the molecule show evidence of change in this pH range. For oxidized thioredoxin, a smaller, but significant, proportion of the molecule is in-

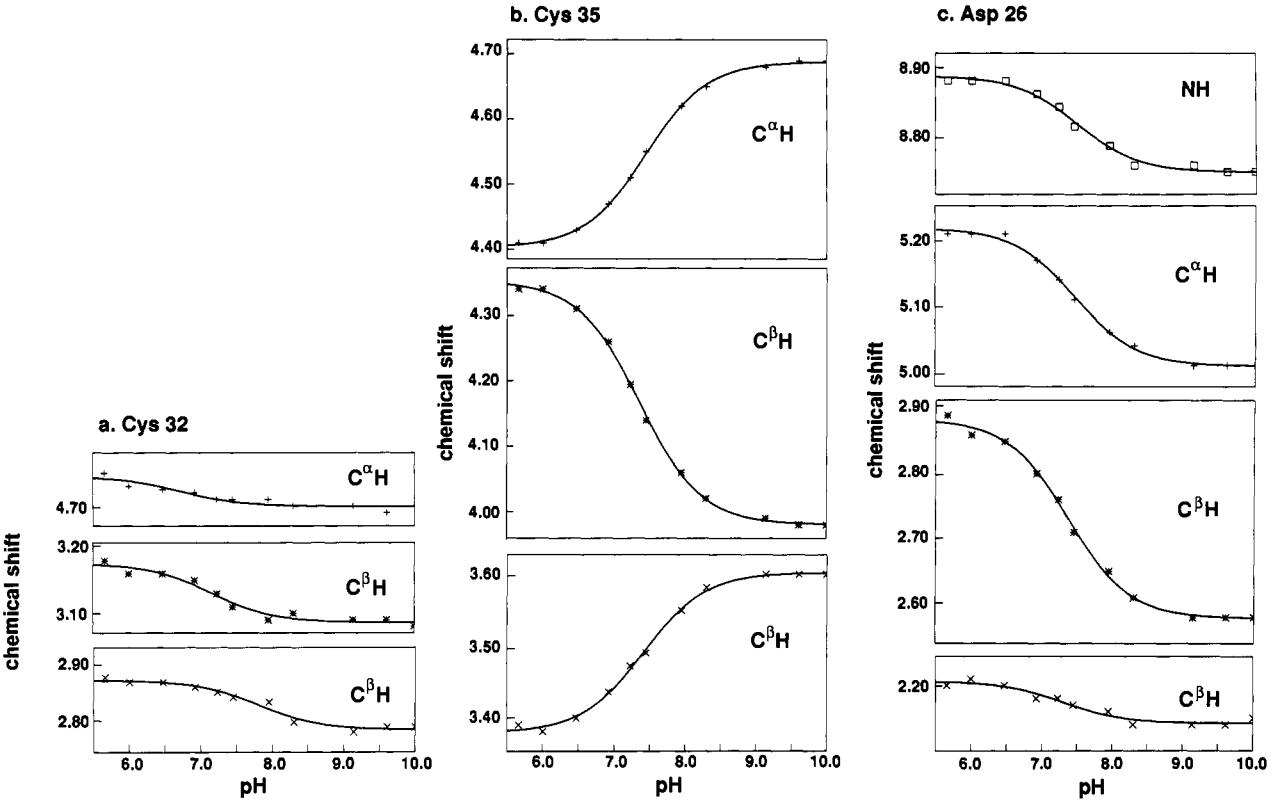

FIGURE 2: Titration data for oxidized thioredoxin: (a) $C^{\alpha}H$ and $C^{\beta}H$ resonances of Cys-32; (b) $C^{\alpha}H$ and $C^{\beta}H$ resonances of Cys-35; (c) NH, $C^{\alpha}H$, and $C^{\beta}H$ resonances of Asp-26. Solid lines were fitted by the method of least-squares, using eq 1.

fluenced by the pH changes. Possible titrating groups include the N-terminal amino group, the imidazole ring of His-6 for both forms of the protein, and the thiol groups of Cys-32 and Cys-35 for reduced thioredoxin. It is also possible that other groups, such as the side chains of Asp, Glu, Lys, or Arg, may titrate in this range with abnormal pK_a's.

N-Terminal Amino Group of Ser-1. The $C^{\alpha}H$ and $C^{\beta}H$ resonances of Ser-1 show marked pH-dependence, with average pK_a values of 7.35 for reduced thioredoxin and 7.34 for oxidized thioredoxin (Table I). No other resonances are influenced by this titration, consistent with the structure of reduced thioredoxin (Dyson et al., 1990), which suggests that the N-terminal three to four residues are disordered. Nevertheless, the pK_a value is low for an α-amino group.

Imidazole Group of His-6. The imidazole group of the single histidine, His-6, apparently titrates with an average pK_a of 5.97 in reduced thioredoxin and 6.04 in oxidized thioredoxin (Table I). These are typical values for a solvent-exposed histidine and are virtually identical with the previously determined pK_a value of 5.98 for oxidized thioredoxin, obtained from one-dimensional ^1H NMR spectroscopy (Holmgren & Roberts, 1976). The His-6 titration influences mainly the $C^{\delta}H$ and $C^{\epsilon}H$ resonances of the histidine, but small shifts are observed for the $C^{\alpha}H$ and $C^{\beta}H$ resonances, as well as the $C^{\alpha}H$ resonances of Ile-5, Val-55, and Ala-56 (Table I). The latter residues are situated close to His-6 in the neighboring β-strand, according to the structures of both oxidized and reduced thioredoxin (Katti et al., 1990; Dyson et al., 1990).

Active-Site Region. Apart from the localized effects near His-6 and at the N-terminus, the only pH changes evident in the thioredoxin NMR spectra are for residues in the region of the active site. The changes in chemical shift with pH are significant, as shown in Figure 1, and are distributed over a number of residues near the active site.

If the cysteine thiol groups are the only titrating groups in the active-site region, then the oxidized form of the protein, which contains a disulfide, should show no titration behavior in this region. This is not the case. The resonances of a number of residues in this area show marked changes in chemical shift (Table II), including the $C^{\alpha}H$ and $C^{\beta}H$ resonances of both cysteines (Figure 2). All of the proton chemical shift titrations of oxidized thioredoxin fit well to a single-pK_a titration curve (eq 1).

By contrast, for reduced thioredoxin, only a few of the resonances show good fits to single-pK_a titration curves. Most of the resonances show the effects of two or more pK_a's (Figure 3). The data for all protons which exhibit a significant change in chemical shift with pH were fitted by using the method of least-squares to one- and two-pK_a titration curves (eq 1 and 2). The pK_a values and associated chemical shift differences, $\Delta\delta$, obtained from these calculations are shown in Table II. The apparent two-proton titration observed for many of the resonances in this region of reduced thioredoxin was fitted by

Table I: Behavior of Protons Influenced by Ser-1 and His-6 Titrations[a]

		reduced		oxidized	
residue	proton	pK_a	$\Delta\delta$	pK_a	$\Delta\delta$
	(a) Protons Influenced by Ser-1				
Ser-1	$C^{\alpha}H$	7.32	0.61	7.32	0.61
	$C^{\beta}H$	7.51	0.18	7.44	0.18
	$C^{\beta}H$	7.23	0.45	7.26	0.46
	(b) Protons Influenced by His-6				
Ile-5	$C^{\alpha}H$	5.90	0.19	6.49	0.17
His-6	$C^{\alpha}H$	5.67	0.10	*a*	*a*
	$C^{\beta}H$[b]	5.86	0.26	6.25	0.18
	$C^{\delta}H$	5.94	0.32	5.86	0.35
	$C^{\epsilon}H$	6.11	0.92	6.17	0.86
Val-55	$C^{\alpha}H$	6.26	0.05	*a*	*a*
Ala-56	$C^{\alpha}H$	5.75	0.12	*a*	*a*

[a] Only protons which exhibit a $\Delta\delta > 0.04$ ppm between the high- and low-pH spectra are included in this table. [b] The $C^{\beta}H$ proton resonances of His-6 are coincident.

Table II: Behavior of Protons in the Active-Site Region[a]

		reduced								oxidized	
		1-proton[b]		2-proton[c]				total fit[d]		1-proton[b]	
residue	H	pK_a	$\Delta\delta$	pK_{a1}	$\Delta\delta_1$	pK_{a2}	$\Delta\delta_2$	$\Delta\delta_1$	$\Delta\delta_2$	pK_a	$\Delta\delta$
Val-25	$C^\alpha H$	7.2	0.10	[6.9	0.04	7.4	0.06][e]	0.09	0.01	7.2	0.11
	$C^\beta H$		0							7.3	0.04
	$C^\gamma H$	6.5	0.03								0
	$C^\gamma H$	7.7	0.01							7.3	0.01
Asp-26	NH^g									7.5	0.15
	$C^\alpha H$	7.6	0.09	6.7	0.05	8.4	0.05	0.06	0.04	7.5	0.21
	$C^\beta H$	7.3	0.22	7.2	0.18	8.7	0.04	0.17	0.05	7.4	0.30
	$C^\beta H$	7.3	0.04							7.3	0.07
Trp-28	NH^g										0
	$C^\alpha H$	7.3	0.23	6.9	0.15	8.4	0.09	0.16	0.07	7.4	0.14
	$C^\beta H$	7.1	0.13	[5.9	0.03	7.0	0.14][e]	0.13	0	7.5	0.25
	$C^\beta H$	*h*		7.6	0.16	8.3	0.13	0.13	0.11	7.5	0.05
	C4	7.5	0.08	7.0	0.05	8.1	0.04	0.06	0.03	8.2	0.01
	C5	8.3	0.06							7.8	0.04
	C6	7.5	0.04							7.5	0.04
Ala-29	NH^g									7.2	0.16
	$C^\alpha H$	7.3	0.07	6.9	0.05	8.4	0.03	0.05	0.03	8.0	0.04
	$C^\beta H$	7.2	0.16	7.0	0.14	8.4	0.04	0.14	0.04	7.2	0.10
Cys-32	$C^\alpha H$	7.7	0.12	7.2	0.07	8.3	0.06	0.07	0.06	7.2	0.02
	$C^\beta H$	6.9	0.18	[6.8	0.08	7.0	0.10][e]	0.20	0.02	7.3	0.08
	$C^\beta H$	7.6	0.23	6.8	0.13	8.4	0.15	0.14	0.12	7.4	0.11
Gly-33	$C^\alpha H$	7.5	0.10	7.1	0.06	8.2	0.05	0.07	0.04		0
	$C^\alpha H$		0								0
Pro-34	$C^\alpha H$	7.6	0.09	7.0	0.03	7.9	0.06	0.06	0.04		0
	$C^\beta H$	7.8	0.09	[7.9	0.12	9.4	0.03][e]	0.06	0.07		0
	$C^\delta H$	7.5	0.10	[6.6	0.02	7.6	0.18][e]	0.07	0.03		0
	$C^\delta H$	7.8	0.44	6.9	0.23	8.4	0.30	0.25	0.28		0
Cys-35	$C^\alpha H$	7.2	0.10	6.7	0.07	8.3	0.04	0.08	0.03	7.4	0.29
	$C^\beta H$	7.1	0.29	7.1	0.27	8.2	0.02	0.28	0.01	7.4	0.24
	$C^\beta H$	8.5	0.13	[			][f]	0.01	0.14	7.4	0.37
Ile-38	$C^\alpha H$	7.3	0.18	7.1	0.14	8.4	0.05	0.14	0.05	7.6	0.16
	$C^\gamma H_3$	7.5	0.07								0
Ala-39	$C^\alpha H$	7.3	0.07	6.9	0.05	8.4	0.03	0.05	0.03	7.6	0.09
Lys-57	$C^\alpha H$	7.7	0.03							7.5	0.05
	$C^\beta H$	7.5	0.10	6.8	0.06	8.4	0.06	0.06	0.04	7.4	0.11
	$C^\beta H$	7.0	0.10	[6.0	0.09	6.6	0.16][e]	0.11	0.01		0
Leu-58	NH^g									7.5	0.33
	$C^\alpha H$	6.8	0.06	[			][f]	0.08	0.02	6.9	0.05
	$C^\beta H$	7.6	0.09	[7.9	0.10	8.1	0.02][e]	0.06	0.04	7.5	0.13
Gly-74	$C^\alpha H$	7.2	0.08	[			][f]	0.07	0.01	7.5	0.10
	$C^\alpha H$	7.0	0.03								0
Pro-76	$C^\alpha H$	7.2	0.17	7.2	0.16	7.9	0	0.16	0.01	7.4	0.31
	$C^\beta H$	7.3	0.25	7.1	0.20	8.5	0.06	0.20	0.06	7.4	0.32
	$C^\beta H$	7.3	0.04								0
	$C^\gamma H$	7.4	0.10	[			][f]			7.3	0.15
	$C^\delta H$	7.3	0.19	[			][f]	0.16	0.04	7.4	0.10
	$C^\delta H$	7.3	0.11	[6.6	0.07	8.3	0.03][e]	0.08	0.04	7.9	0.06
Leu-78	$C^\alpha H$	6.8	0.04								0
	$C^\beta H$	7.4	0.09	6.7	0.02	7.6	0.07	0.07	0.02	7.4	0.13
Lys-90	$C^\beta H$	6.5	0.05								0
	$C^\beta H$	7.6	0.11	6.8	0.05	8.1	0.08	0.07	0.05		0
Val-91	$C^\alpha H$	7.3	0.06	7.0	0.05	8.4	0.02	0.04	0.02		0
	$C^\beta H$	7.1	0.04							7.4	0.04
	$C^\gamma H$	7.3	0.06	6.6	0.04	8.2	0.03	0.04	0.02		0
Gly-92	$C^\alpha H$	6.9	0.04								0
	$C^\alpha H$	7.9	0.03							7.9	0.05
Ala-93	$C^\alpha H$	7.9	0.05							7.5	0.06

[a] Only protons which exhibit a $\Delta\delta > 0.04$ ppm between the high- and low-pH spectra are included in this table. [b] Using eq 1. [c] Using eq 2. [d] Final differences in chemical shift derived for each of the 35 protons by a simultaneous least-squares fit to all of the data (calculated by using eq 2), giving consensus pK_a values of 7.06 and 8.43. Only those protons exhibiting pK_a's in the vicinity of 7 and 8.5 and $\Delta\delta_{total} > 0.06$ ppm (excluding Ser-1) were included in the final least-squares calculation. [e] Values in brackets were derived from data sets where the least-squares calculation using eq 2 was underdetermined, resulting in pK_a and $\Delta\delta$ values which are not reliable. [f] For these protons, the fit using eq 2 did not converge. [g] The backbone amide protons of these residues (as well as of some other residues which are not influenced by pH) are persistent in ^2H_2O for both oxidized and reduced thioredoxin. The absence of data in this table and in Figure 3 for the persistent amide protons of reduced thioredoxin does not reflect a difference in amide proton exchange rates between the two forms. The samples used for the spectra of reduced thioredoxin had been prepared in ^2H_2O for a longer time than those used for the spectra of oxidized thioredoxin. Hence, the data for the persistent amide protons of reduced thioredoxin were incomplete, and no calculations were made. [h] A downfield shift of this resonance at low pH was apparently reversed in the high-pK_a titration; a single pK_a could not be calculated.

using a simultaneous least-squares fit to the two-pK_a titration (eq 2). Data for 35 protons, for each of which the total chemical shift change was in excess of 0.06, were used in this calculation; values of 7.06 and 8.43 were obtained for the two pK_a's, with a final rmsd for all 350 data points of 7×10^{-3}. For each proton, chemical shift differences $\Delta\delta$ associated with the two pK_a's obtained from the latter calculation are included in Table II for comparison with the numbers obtained from

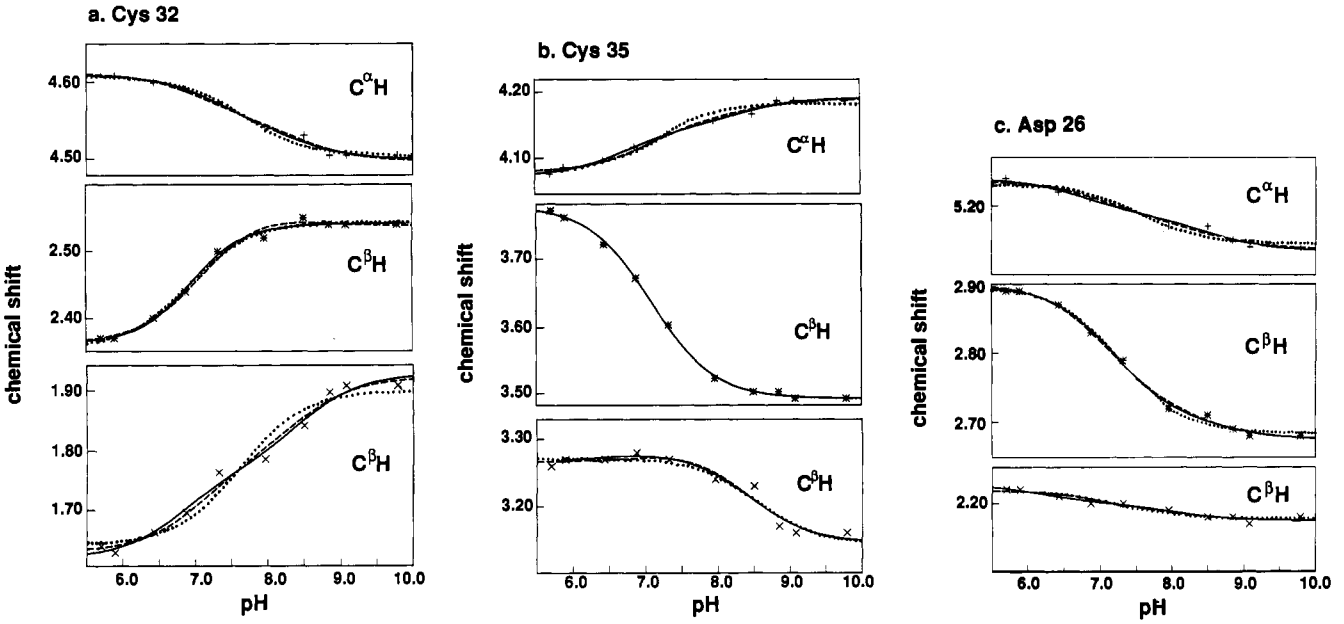

FIGURE 3: Reduced thioredoxin titration data: (a) $C^{\alpha}H$ and $C^{\beta}H$ resonances of Cys-32; (b) $C^{\alpha}H$ and $C^{\beta}H$ resonances of Cys-35; (c) $C^{\alpha}H$ and $C^{\beta}H$ resonances of Asp-26. Dotted lines were fitted by the method of least-squares, using eq 1, solid lines by using eq 2, and dashed lines represent the fits calculated by using the averaged values of pK_1 and pK_2. The absence of data for the NH of Asp-26, as appears in Figure 2, does not reflect a difference in the exchange rates between oxidized and reduced thioredoxin (see footnote *g* to Table II).

the individual calculations.

Discussion

In the absence of any significant differences in the majority of the proton resonances on changing the pH by over 4 units, the changes observed for the residues in the vicinity of the active site obviously reflect the influence of the titration of local groups. The behavior of reduced thioredoxin indicates that at least two groups are titrating in the active-site region. Even the $C^{\alpha}H$ and $C^{\beta}H$ resonances of the cysteines themselves cannot be fitted well to a single-pK_a titration curve (Figure 3). The data obtained for individual protons can be fitted by using a least-squares method, with varying degrees of success (see Table II), to a two-pK_a titration curve. In a few cases, the least-squares fit using eq 2 was unable to converge because the data constitute a well-determined single-pK_a titration (for example, for Val-25 $C^{\beta}H$ and Trp-28 $C^{\beta}H$). The majority of the difficulties with the least-squares fits could be ascribed to underdetermination of the parameters to be fitted by the data. There are five unknowns to be fitted for each proton resonance (chemical shifts for three species plus two pK_a's). Only 10 COSY spectra were recorded for the reduced thioredoxin titration (11 for the oxidized titration), giving only 10 (or 11) data points for each resonance. Hence, a further least-squares calculation was performed by using the data for 35 protons simultaneously. For this calculation, the number of data points is 350, while the number of unknowns is proportionally smaller (35×3 chemical shifts plus 2 pK_a's = 107), giving better statistical definition of the fitted parameters. No fits using three pK_a's were attempted, although, as discussed below, there are strong indications that three titratable groups are involved, two of which have a similar pK_a.

For reduced thioredoxin, the Cys-32 and Cys-35 thiol groups provide obvious candidates for the titrating groups whose effects are seen in the NMR spectra. The identity of the group which titrates with pK_a 7.5 in oxidized thioredoxin is not so obvious. It cannot be the histidine imidazole group ($pK_a \sim 6$), and is unlikely to be the N-terminal amino group (far distant from the active site). Both the oxidized X-ray (Holmgren et al., 1975; Katti et al., 1990) and reduced NMR structures (Dyson et al., 1990) of thioredoxin show the close approach of Lys-57 and Asp-26 side chains to the disulfide bond and to the cysteine thiol groups, respectively, and to Pro-76. An abnormal pK_a for one of these groups could be responsible for the pH-dependence of the chemical shifts of the protons in this region. Neither the extent of the shift in pK_a from normal values nor the large pH-dependent chemical shift changes for protons in the vicinity are surprising, given the hydrophobic environment of these charged groups. The magnitude of some of the chemical shift changes seen for some amino acid residues, such as Pro-34 and Trp-28, suggests that the titration may be associated with a significant local conformational change, perhaps including the aromatic ring of Trp-28. Such a conformational change has been inferred from fluorescence measurements of reduced thioredoxin as a function of pH (Holmgren, 1972).

Chemical shift changes close to the ϵ-amino group of Lys-57 are difficult to observe for two reasons: first, long side chains are relatively mobile, so that effects may be averaged and end up negligible; second, the cross-peaks in the 2QF COSY spectrum corresponding to the scalar connectivities at the end of the lysine side chain are overlapped and low in intensity due to multiple couplings. It is therefore very difficult to pinpoint any effect of an abnormally titrating Lys-57, and the possibility that the ϵ-amino group of this residue is responsible for the observed titration behavior of oxidized thioredoxin cannot be excluded. A greater body of evidence points toward the Asp-26 carboxyl group. In the titration of oxidized thioredoxin, the largest $\Delta\delta$ is seen for the resonances of Asp-26, Cys-35, and Pro-76; of these, only the Asp-26 carboxyl is capable of titration, since the Cys-35 sulfur participates in a disulfide bond. This conclusion is supported by studies with a mutant thioredoxin (Asp-26 → Ala) (Langsetmo et al., 1990). From the relative electrophoretic mobilities of mutant and wild-type proteins, the pK_a of Asp-26 was determined to be 7.5 in oxidized thioredoxin (K. Langsetmo, J. A. Fuchs, and C. Woodward, submitted for publication).

It is possible that the group which titrates in the active-site region of oxidized thioredoxin may also act in a similar manner in reduced thioredoxin. Because of the complexity of the

titration data for reduced thioredoxin and the close spatial proximity of the titrating groups, it is difficult to localize each titration. Three hypotheses can be invoked to explain the observed behavior: (1) three titrating groups (Cys-32 thiol and Asp-26 carboxyl with pK_a 7.1–7.4 and Cys-35 thiol at pK_a 8.4); (2) two groups (Asp-26 titrating at pK_a 7.1–7.4 and one of the cysteine thiols at pK_a 8.4); (3) two groups (Cys-32 and Cys-35 thiols titrating at pK_a 7.1 and 8.4). It should be noted that all of the least-squares fits were performed by using n, the number of protons transferred per titration, fixed at a value of 1, since each of the possible titrating groups can only lose a single proton. This is not inconsistent with the transfer of two protons from different groups with very similar pK_a's.

In order to distinguish among the above alternatives, a number of observations can be made from the data in Table II and Figures 2 and 3. The influence of a titrating group on a given proton is proportional, among other factors, to the distance between them. An estimate of the magnitude of such distances can be obtained from structural studies (Katti et al., 1990; Dyson et al., 1990). This information has been used with caution in the following arguments, since conformational fluctuations can greatly influence interatomic distances in macromolecules. In particular, the active site of thioredoxin appears from ^{1}H NMR studies to be quite solvent-exposed and mobile (Dyson et al., 1989), prompting extra caution.

A number of protons, including those of Gly-33 and Pro-34, are influenced by two titrating groups in reduced thioredoxin, but are not affected by pH in the oxidized titration. The distances between these protons and the Asp-26 carboxyl group are comparable in the oxidized and reduced thioredoxin; the distances to the two cysteine thiols are much smaller. This implies that both Cys-32 and Cys-35 thiols are titrating in reduced thioredoxin; hypothesis 2 is not substantiated by this observation.

A number of protons close to the Asp-26 carboxyl (and relatively distant from the cysteine thiols) show titration behavior in both oxidized and reduced thioredoxin. These include Val-25 C$^\alpha$H, Leu-58 C$^\alpha$H and C$^\beta$H, and Leu-78 C$^\beta$H. In these cases, not only are the distances of the protons from the Asp-26 carboxyl comparable between the two forms of the protein, but the titration behavior is also very similar (Table II). This observation implies that Asp-26 titrates in reduced thioredoxin, and is incompatible with hypothesis 3 above. Thus, we conclude that it is likely that all three groups, the two cysteine thiols and the Asp-26 carboxyl, contribute to the pH-dependence of the residues in the active-site region of reduced thioredoxin. This conclusion is supported by an examination of the extent of the effect of the titrations at pK_a $\sim$7 and $\sim$8. The titration with the lower pK_a apparently affects a larger region of the protein than the titration with the higher pK_a, consistent with the involvement of a greater number of titrating groups. In order to confirm that all three groups are titrating, and to identify the pK_a's of the individual groups, further experiments, including work with mutant proteins, will be necessary.

Examination of the sequences of thioredoxins from a number of species reveals that Asp-26 is a highly conserved residue (Eklund et al., 1991). The titration behavior of both oxidized and reduced thioredoxin appears to implicate Asp-26 in the mechanism of electron–proton transfer in thioredoxin, consistent with its close proximity to the active site in both oxidized and reduced thioredoxin.

From a mechanistic point of view, the series of experiments described herein appears to have raised more questions than it has answered. The chemical modification data (Kallis & Holmgren, 1980) clearly showed that only Cys-32 is alkylated at neutral pH in thioredoxin (pK_a $\sim$6.7 in H_2O), strongly suggesting that this group constitutes the thiol–disulfide interchange thiolate anion. However, the actual nature of the reduced thioredoxin molecule at the pH optimum of reaction in the absence of substrate remains to be shown. The titration results reported herein are consistent with the mechanism which postulates that the Cys-32 thiol titrates with a lowered pK_a of $\sim$7. However, the NMR structure of reduced thioredoxin (Dyson et al., 1990) shows no groups in close proximity to the Cys-32 thiol which could stabilize the thiolate anion. Other possible mechanisms cannot be excluded; for example, there may be rapid proton exchange between the Cys-32 thiol and another group in the active-site region, rather than strict localization of ionized and protonated titrating groups. Alternatively, if the ionized group at neutral pH were one of the other possible titrating groups in the active site, an induced proton transfer upon binding of the substrate could generate the Cys-32 thiolate anion required for nucleophilic attack and mixed-disulfide formation. Further experiments to define and localize the pH dependence of reduced thioredoxin are in progress, including examination of the behavior of mutant proteins.

Added in Proof

The pK_a of Asp-26 in reduced thioredoxin has also been determined in electrophoretic experiments with wild-type and mutant D26A to be 7.0 (K. Langsetmo, J. A. Fuchs, and C. Woodward, personal communication), a result which is in excellent agreement with our NMR measurements.

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Electrophoretic and Hydrodynamic Properties of Duplex Ribonucleic Acid Molecules Transcribed in Vitro: Evidence That A-Tracts Do Not Generate Curvature in RNA†

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ABSTRACT: We have developed a T7 RNA polymerase based transcription system for the production of fully complementary RNA molecules (i.e., molecules capable of forming blunt-ended duplex species) as the direct products of transcription, thus rendering unnecessary the enzymatic removal of single-stranded ends. A combined gel electrophoretic and hydrodynamic analysis of a 180 bp double-stranded (ds) RNA molecule containing four A_5-tracts in approximate phase coherence with the helix repeat provides no indication that the helix axis is curved, in sharp contrast to DNA molecules containing phased A-tracts. The electrophoretic behavior of dsRNA molecules reveals that their mobilities in nondenaturing acrylamide gels are approximately 10-20% lower than the corresponding mobilities of duplex DNA, in accord with earlier observations in the literature. Furthermore, the relative mobilities are only slightly modulated by gel concentration, the concentration of monovalent salt, or the presence of spermidine and/or Mg^{2+}. The reduced mobilities are not caused by increased contour length, since direct hydrodynamic measurements using transient electric birefringence indicate that the average helix rise, h, of the dsRNA molecules examined in the current study is 2.8 ± 0.1 Å/bp. The reduced electrophoretic mobilities, extrapolated to zero acrylamide concentration, are consistent with the lower residual charge predicted for dsRNA by counterion condensation theory. Finally, birefringence measurements indicate that dsRNA is only marginally stiffer than DNA, with a persistence length of ca. 500-700 Å.

A precise knowledge of RNA structure is important in many areas of molecular biology. For example, such knowledge is prerequisite to a detailed understanding of RNA-protein interactions (Wu & Uhlenbeck, 1987; Malim et al., 1990), RNA-catalyzed processes such as self-splicing of intervening-sequence RNA (Zaug & Cech, 1986; Murphy & Cech, 1989), the self-cleaving of plant satellite RNA (Uhlenbeck, 1987; Symons, 1989), and the processing of tRNAs by ribonuclease P (Guerrier-Takada et al., 1983, 1989), the mechanism of action of the ribonucleoprotein telomerases (Greider & Blackburn, 1989; Shippen-Lentz & Blackburn, 1990), and the mechanisms involved in protein biosynthesis (Moazed et al., 1986; Draper et al., 1988; Sampson et al., 1989; Denman et al., 1989).

An important advance in the study of RNA structure has come about through the development of preparative in vitro transcription systems, thus enabling the efficient production of RNA molecules of virtually any sequence and length for physical studies. One widely used technique is based on the use of RNA polymerase from bacteriophage T7. Plasmids bearing the T7 late promoter can be grown in *Escherichia coli* without interference from bacterial RNA production. Moreover, the T7 RNA polymerase can be overproduced in large amounts (Davanloo et al., 1984), thus facilitating the production in vitro of milligram amounts of RNA transcript. RNA molecules transcribed in vitro have been employed to address a number of issues regarding RNA structure, including the helix repeat of duplex RNA (Tang & Draper, 1990; Bhattacharyya et al., 1990), the conformations of internal loops (Varani et al., 1989), and the local conformation of a plant viroid self-cleaving domain (Heus et al., 1990). Principal methods for studying RNA structure have included either two-dimensional NMR (van den Hoogen et al., 1988; Chou et al., 1989; Puglisi et al., 1990) or chemical protection assays (Moazed et al., 1986; Ehresmann et al., 1987). However, while both approaches provide a detailed picture of local helix structure, they are not sensitive to longer range structure (e.g., branch geometries).

Two experimental approaches that have proven to be useful for the study of long-range structure in DNA should be directly applicable to the study of RNA structure. The first approach,

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