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Differences in hydrogen exchange behavior between the oxidized and reduced forms of *Escherichia coli* thioredoxin

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Abstract

Amide proton exchange of thioredoxin is used to monitor the structural effects of reduction of its single disulfide. An effective 3–5-proton difference between the oxidized and reduced protein form is observed early in proton out-exchange of the whole protein, which is independent of temperature in the range of 5–45 °C, indicating that redox-sensitive changes are probably not due to low-energy structural fluctuations. Medium resolution hydrogen exchange experiments have localized the redox-sensitive amide protons to two parts of the sequence that are distant from each other in the three-dimensional structure: the active-site turn and the first β-strand. The sum of the proton differences observed in the peptides from these regions is equal to that of the whole protein, indicating that all redox-sensitive hydrogen exchange effects are observed in the peptide experiments. A model combining structural changes within the protein matrix with changes in the surface hydration properties is proposed as a mechanism for the communication between distant sites within the protein. Sound velocity and density measurements of reduced and oxidized thioredoxin are presented in the accompanying paper (Kaminsky, S.M. & Richards, F.M., 1992, *Protein Sci. 1*, 22–30).

Keywords: *Escherichia coli*; hydrogen exchange; thioredoxin

The role of protein dynamics in function is poorly understood. Changes in equilibrium structures have been established in a number of protein systems in response to ligand binding, both those with single active sites and those subject to allosteric control (Perutz, 1989). In some cases information is clearly transferred between different parts of a protein over substantial distances. Even where equilibrium structural changes are very small, changes in dynamic behavior can be seen. Amide proton exchange is one method of revealing such changes. This paper examines such effects in the thioredoxin from *Escherichia coli*, a small protein of 11,700 Da.

Thioredoxins are ubiquitous proteins that have diverse functions in cells, several depending on redox transfer and disulfide interchange reactions. In addition to its redox functions, some members of this class appear in structural roles in phages f1 and M13 and as a subunit of T7 phage DNA polymerase. The structure of the reduced form is required for these latter functions although the redox property is not otherwise involved. General reviews of thioredoxin properties and functions have been published by Holmgren (1985, 1989).

The crystal structure of the oxidized form of the *E. coli* protein has been determined to 1.68 Å (Katti et al., 1990). The protein consists of a single twisted β-sheet of five strands, three parallel and two antiparallel, surrounded by four α-helices and one 3/10 helix (Kinemage 1). The active site containing the sole disulfide bridge is on the surface of the protein at the carboxyl end of the β-turn (between the central β_2-strand and the α_2-helix) and forms the start of an α-helix (α_2). The reduced form of the protein has not been crystallized. However, the solution structure has been determined by NMR (Dyson et al., 1990).

When thioredoxin is reduced, the temperature of thermal denaturation drops by about 12 °C and the tryptophan fluorescence increases up to sevenfold (Holmgren, 1972); this is indicative of large changes in protein stability and/or structural conformation. However, certain

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physical properties such as those measured by circular dichroism (CD) (Reutimann et al., 1981; Brown et al., 1987) and optical rotary dispersion (Stryer et al., 1967) spectra remain essentially unchanged and the nuclear magnetic resonance (NMR)-reduced structure is very similar to the X-ray structure of the oxidized form. Because the disulfide loop is both very small (four residues) and found in an external location, it is curious that its redox state has such a marked effect on stability.

Amide proton hydrogen exchange experiments were used to determine the magnitude and location of structural changes in thioredoxin upon reduction of the disulfide. The hydrogen exchange technique has been successful in the identification of structural changes due to ligand binding (Louie et al., 1988; Mallikarachchi et al., 1989), changes in redox potential (Patel & Canuel, 1976), and subunit association (Lennick & Allewell, 1981; Rosa & Richards, 1982). It is generally recognized that the amide proton exchange rates are indicative of the stability of the region about the amide, despite current controversies over the mechanism of the exchange process (Englander et al., 1988). Amide protons in proteins frequently exchange much more slowly (up to 10^9-fold) than they do when fully exposed to solvent. Structural changes are required to permit the exchange process to take place, and these will frequently involve the breaking of hydrogen bonds in the native structures.

Experiments were conducted on the intact protein in each redox state to determine the overall differences in exchange behavior between the two forms of the protein as a function of temperature. Medium resolution hydrogen exchange experiments (Rosa & Richards, 1979, 1981, 1982; Englander et al., 1985) were then used to localize the affected areas in the structure. It was found that significant changes occur in two regions of the protein, at the active site, residues 32–35, and within residues 1–13 at the amino terminal end. All changes that occurred upon reduction of thioredoxin as determined in the whole protein experiments were observed in the peptide experiments because the numbers of amide protons with differing exchange behavior were identical in each experiment. The results are compared to two published studies of redox-sensitive changes in thioredoxin determined by NMR (Dyson et al., 1988; Hiraoki et al., 1988).

The two regions that exhibit changes upon reduction are nonadjacent either in linear sequence or three-dimensional space (Kinemage 1). There are two possible pathways for communication between these sites; one is directly through the intervening structure and the other is around the surface of the protein mediated by the solvent layer. The results presented here and in the following paper support the latter of these options.

Communication between nonadjacent sites in a protein has been reported for distances as great as 25 Å, as seen in staphylococcal nuclease (Wilde et al., 1988; Pourmotabbed et al., 1990). Although no mechanism for these effects has been established, the present data suggest that hydration layers may play an important role as an intermediary in signal transduction between distant regions of proteins. It is suggested that technological limitations on our ability to study surface water directly have resulted in an underestimation of the importance of this factor to hydrogen exchange rates, as well as to other properties of proteins.

Results

Thioredoxin was radiolabeled at all amide positions to high specific activity with tritiated water. The tritium was allowed to out-exchange for a set time interval under defined conditions of temperature and pH followed by rapid quenching of exchange. The protein was analyzed for total radiolabel or subjected to protease digestion, chromatographic separation of the peptides, and subsequent determination of remaining tritium.

Exchange in intact thioredoxin

The out-exchange profiles of amide tritons from oxidized and reduced thioredoxin are shown in Figure 1 as log-log plots of $H(t)$ versus time for each of the four temperatures studied (5 °C, 15 °C, 30 °C, and 45 °C). Of the 108 thioredoxin residues 36 main-chain amide protons do not appear to form hydrogen bonds to other atoms in the protein (as determined from the X-ray structure) of the oxidized protein, and five residues are prolines that do not have amide protons. For redox-sensitive changes only those amide protons that are internally hydrogen bonded were expected to be observable. There are a total of 67 in thioredoxin, 48 of which are observed in the earliest time point of the 5 °C experiment. A greater level of radioactivity was retained in the oxidized form of thioredoxin, representing an excess over the reduced form of 2–5 mol of protons per mol of protein. As seen in Figure 1, these differences appear in the early phases of the exchange profile and are not dependent upon temperature in the range of 5–45 °C.

Thioredoxin proteolysis and peptide identification

The medium resolution hydrogen exchange experiments depend upon obtaining significant proteolysis of thioredoxin under acid conditions followed by rapid separation of the cleavage products. Attempts to proteolyze oxidized thioredoxin with acid proteases from *Endothia parasitica*, *Rhizopus chinensis*, *Aspergillus satoi*, and *Staphylococcus aureus* were unsuccessful as determined by the disappearance of the thioredoxin protein band on a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. Proteases were tried individually and in combination at several values of pH (2–4) and urea concentration (0–4 M). Pepsin in 4 M urea at pH 2.3 yielded significant digestion of

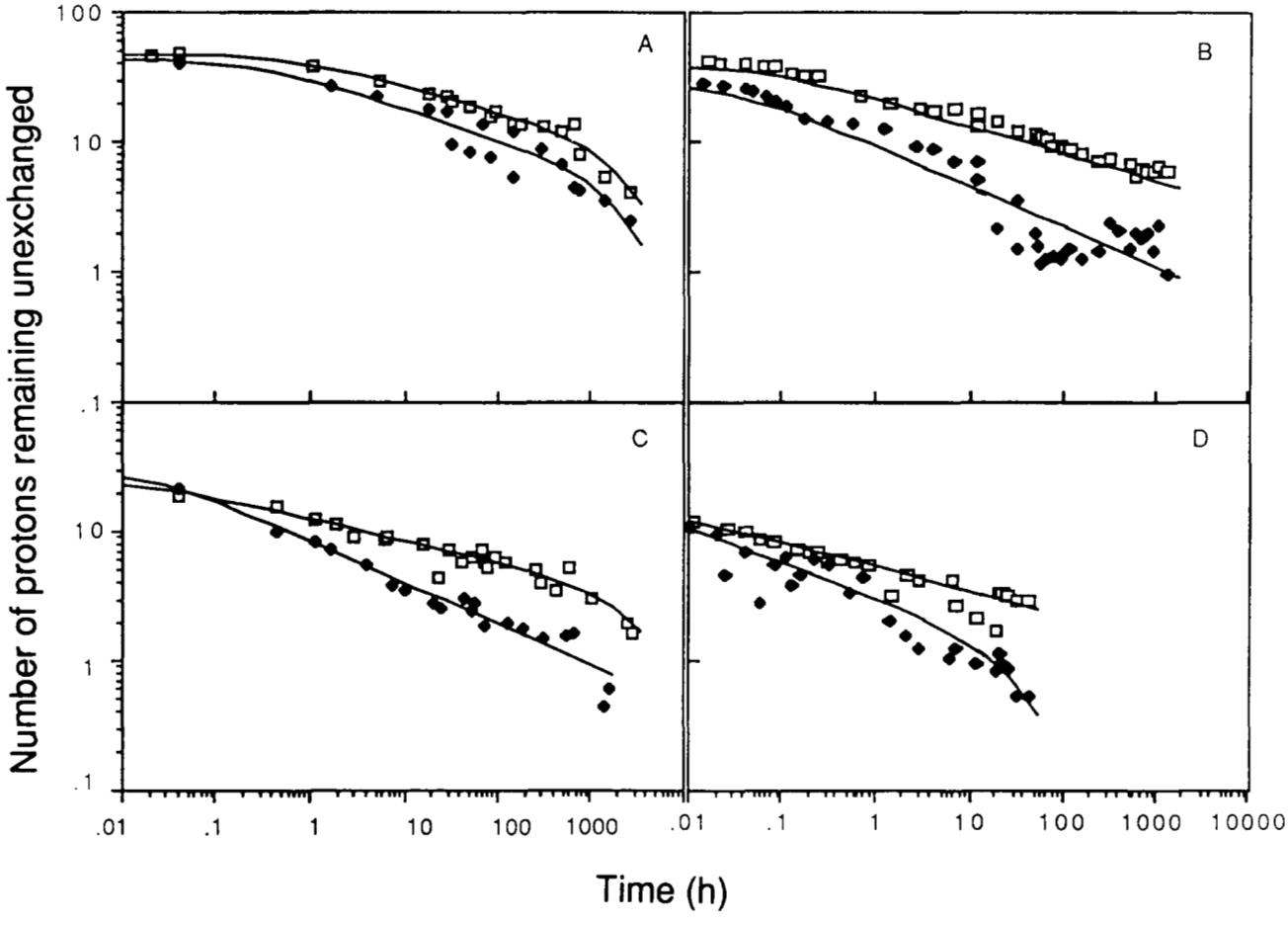

Fig. 1. The log of the number of moles of protons remaining unexchanged per mole of protein plotted against the log of time for out-exchange. Experiments performed at 5 °C, 15 °C, 30 °C, and 45 °C are given in panels A, B, C, and D, respectively. The data for the oxidized protein are represented by open squares and the reduced protein by solid diamonds. The lines are the nonlinear least-squares best fit curves as determined by the procedure outlined in the text.

the protein. The separation of the products by reverse-phase chromatography successfully isolated peptides from all regions of the protein except β_4-sheet residues (Fig. 2). A summary of the peptide origins of the peaks used in this experiment along with the identity of the residue that is on the amino side of each proteolytic cut is given in Table 1, list A. The quality of fit parameter (see Table 1) for the match between the measured compositions of chromatographic peaks to that predicted for the proposed sequence indicates the fits were good for all peaks. These represent the best fits for any sequences in the protein. N-terminal amino acids corroborated the compositional analysis and the proposed cut sites were evaluated for the probability of hydrolysis by pepsin due to its kinetic specificity (Englander et al., 1985).

Exchange in peptides

The parameters required for the calculation of the loss factors for out-exchange of tritium during the analytical steps are given in Table 2. Also listed is the number of amide protons on the peptide (number of residues in the peptide minus the number of proline residues). Only those protons that meet the following criteria were expected to be observable: (1) those that are internally hydrogen bonded as determined by the recent high resolution structure of thioredoxin (Katti et al., 1990), and (2) those that were neither the N-terminal nor penultimate residues (both of which have large intrinsic rate constants and therefore completely exchange with solutions during the analytical procedures). The number of protons remaining unexchanged in a peptide is given by the ratio of $^3H/^{14}C$ on the high performance liquid chromatography (HPLC)-separated peaks, where the ^{14}C is used to determine peptide concentration. Corrections for losses due to the analytical steps are then made and the time course of out-exchange for each identified peptide is shown in Figure 3A. Loss analysis was not done for HPLC peaks containing two peptides because the relative concentration could not be determined.

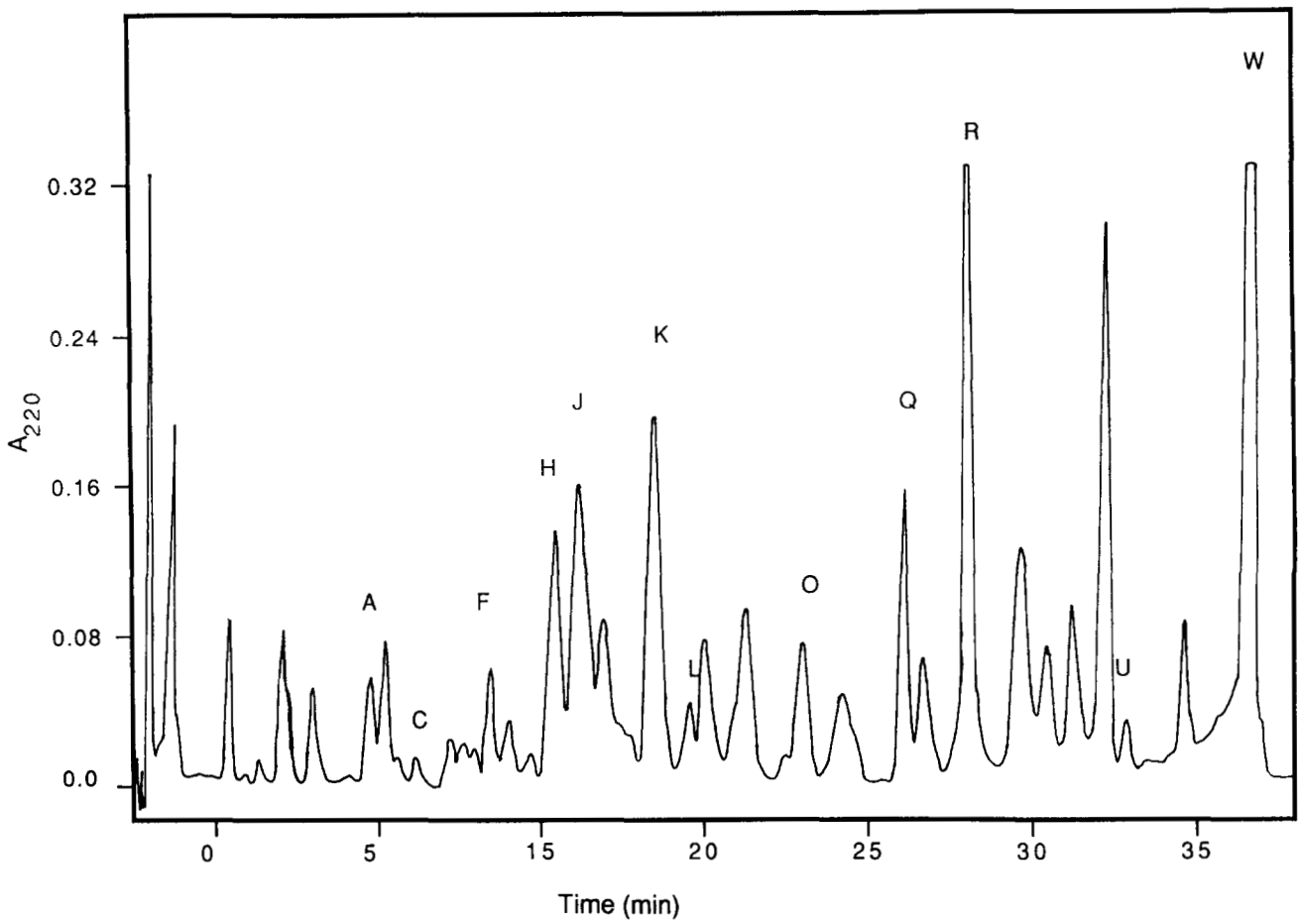

Fig. 2. The chromatographic separation of peptides derived from thioredoxin digested with pepsin for 1 h at pH 2.3 in 4 M urea. Peptides were detected by UV absorbance at 220 nm. The peaks analyzed are identified by letter.

The hydrogen exchange data for a peptide include the sum of a number of exponential terms equal to the number of residues. In order to determine the rate constants, proper modeling of these results would involve fitting more parameters than the number of data points allow. The Wilcoxon rank sum test (Alder & Roessler, 1977) provided a test of significant difference for paired data as parametric analysis was not possible. Only two peaks that contained single peptides, R and W, had a significant difference between the oxidized and reduced forms of the protein, at the 95% confidence level. All peaks in the HPLC chromatogram were analyzed for significant differences in exchange rates. It was expected that regions other than those represented by the identified peptides might exhibit changes upon reduction. Four additional peaks were found to have significant differences again at the 95% confidence level and were subjected to further compositional analysis on a second HPLC column. One of these peaks contained more than five peptides; therefore no information could be derived from it. The remaining three peaks identified as J, K, and U consisted of two peptides each and are identified in Table 1, list B. The relative concentrations within each pair varied somewhat with each experiment. The level of remaining tritium was inadequate to permit a double column separation procedure. The exchange curves for these three unresolved peaks are given in Figure 3B. The ordinate for this figure is given as the ratio of tritium to ^{14}C counts, due to the inability to establish the absolute number of protons per peptide remaining. For each pair of peptides, at least one fragment maps to the same region of thioredoxin as either peptide R or W. It is therefore certain that one peptide exhibits changes in exchange rates upon reduction of thioredoxin, but it cannot be established if these changes are due to one or both peptides.

The two peptides from peak K were both derived from segments near or overlapping with R and W. Peptide K2 was from the β_1-strand; although it had a relatively poor compositional match it contained histidine, which is only found in this region of thioredoxin. K1 was a very good fit to the segment 25–27, the carboxyl end of the β_2-strand, but only the amide proton of residue 27 would have been detected in this experiment due to the fast exchange rates of the other two positions. It is possible that

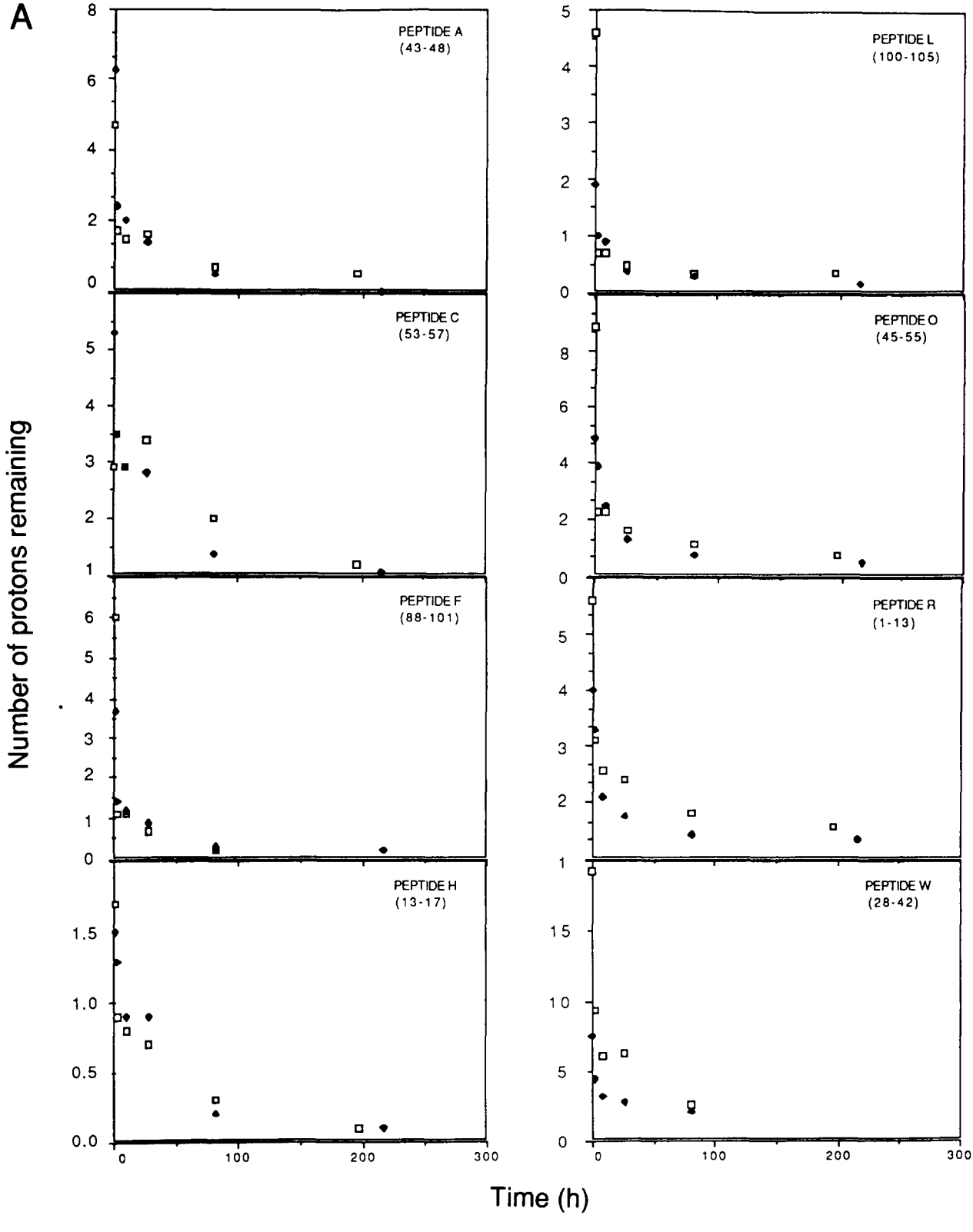

Fig. 3. Hydrogen exchange maps of some of the peptides shown in Figure 2. **A**: Those HPLC peaks that contained a single peptide. The ordinate represents the number of protons per peptide as corrected by the loss correction term outlined in the text. **B**: The maps for HPLC peaks with coeluting mixtures of peptides. The ordinate for B is the ratio of disintegrations per minute (DPM) ^{3}H to DPM ^{14}C because conversions to number of protons remaining require knowledge of the absolute amount of each peptide, which is not possible for these peaks. Open squares represent data for the oxidized protein, solid diamonds are for the reduced protein. Solid squares indicate that the data point is identical for each of the two redox states. (*Part B appears on facing page.*)

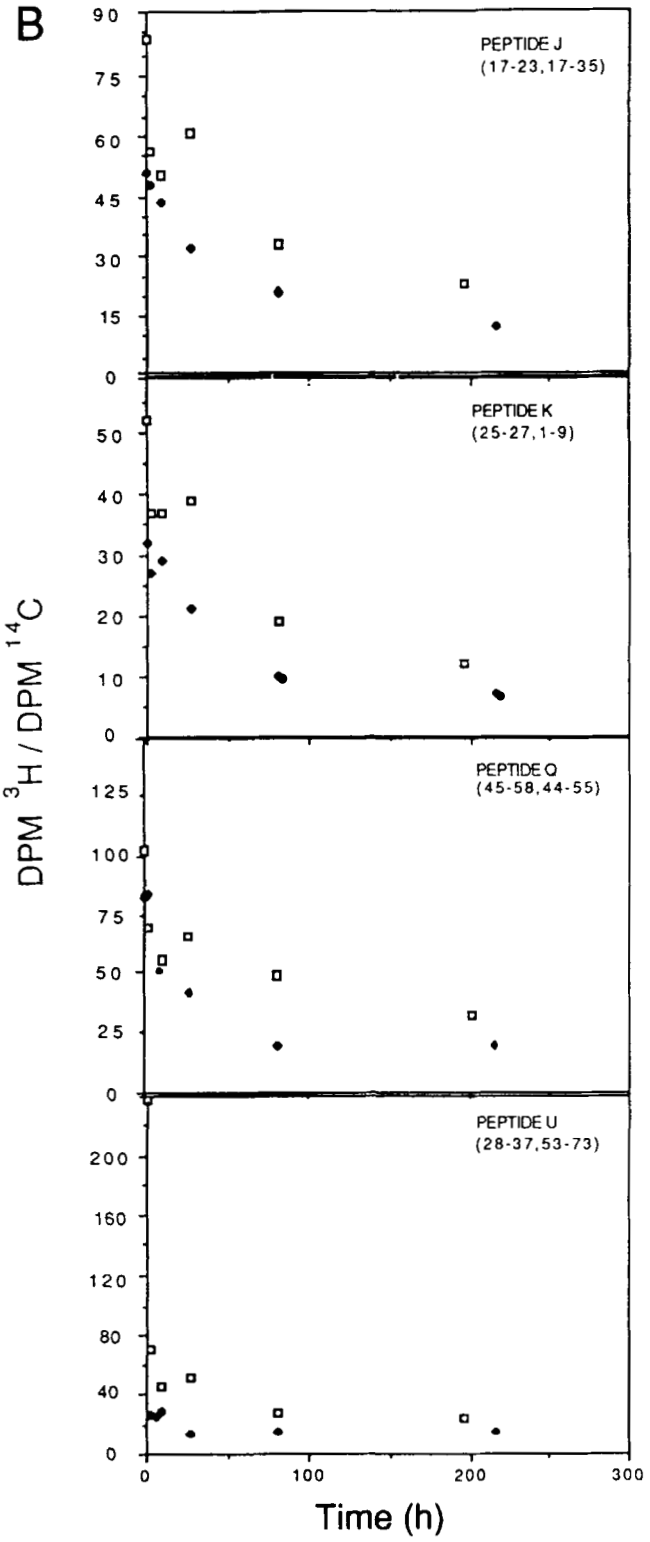

Fig. 3B.

only one of the peptides was responsible for the observed exchange rate dependence on redox state of the protein.

One of the two peptides from peak J was a subset of the other. The exchange rate change upon reduction of

Table 1. *Identification of peptides followed in hydrogen exchange experiments*

Peptide name	Assigned sequence	Residual[a]	N-terminal amino acid	Kinetic specificity[b]
List A				
A	43–48	0.093	Asp	Asp, Tyr
C	53–57	0.193	Leu	Leu, Leu
F	88–101	0.078		Ala, Phe
H	13–17	0.190	Asp[c]	Asp, Lys
L	100–105	0.178		Lys, Asn
O	45–55	0.125		Ile, Ala
R	1–13	0.190		Ser,[d] Thr
W	28–42	0.260	[e]	Trp, Asp
Q1	45–58	0.055		Ile, Asn
Q2	44–55	0.234	Glu	Glu, Ala
List B				
J_1	17–23	0.106	Leu	Leu, Leu
J_2	17–35	0.304	Leu	Leu, Lys
K_1	25–27	0.064	[f]	Val, Trp
K_2	1–9	0.454[d]		Ser,[d] Asp
U_1	28–37	0.170	[e]	Trp, Ile
U_2	53–73	0.249	Leu	Leu, Gly

[a] Sum of the magnitudes of the differences between observed and expected amino acid content (mol fraction).

[b] The residue types of the carboxyl side of the peptide bonds broken in formation of the given peptide. The first residue listed in the measured or estimated N-terminus of the peptide itself. The second residue is the N-terminus produced by cleavage at the C-terminus of the peptide. The kinetic specificity of pepsin is such that bonds with C-terminal Leu or Phe are found in about 30% of the cleavages.

[c] This was a poorly resolved peak in the Dansyl-amino acid separation.

[d] One end of this peptide is the N-terminus of the protein.

[e] Trp is likely the N-terminal residue; however, it does not survive acid hydrolysis.

[f] If the amino acid analysis is correct, the N-terminal residue is valine. The valine-X bond is hard to hydrolyze.

thioredoxin for this peak was thus unambiguously determined to be within the region of the larger peptide, β_2, and the start of α_2.

Peak U contained two peptides: U1 and U2. U2 mapped to a region that is already represented in part by peptides O and C; each had no difference in exchange behavior upon reduction. The carboxyl end of this peptide uniquely covered the 3/10 helix segment from residues 63 to 73. U1 mapped to the active site β-turn region and was thus most likely to be the source of the difference in hydrogen exchange rates upon reduction for peak U.

Peak Q represented two peptides that shared most of the same sequence, 45–58 (Q2) and 44–55 (Q1), respectively. The statistical analysis showed that differences in exchange behavior of this peptide with thioredoxin redox state are only significant at the $\alpha > 0.05$ level. Given that peptides from peaks O, A, and C together accounted for the entire region of the peptides from peak Q and that these peptides show no significant differences suggests that there is no significant difference for peak Q. The exchange curves for peak Q are given in Figure 3B.

Table 2. *Peptide characteristics needed to correct for losses during analysis*

Peptide	F_{digest} [a]	F_{HPLC} [a]	H_0 [b]	H_t [c]	L [d]
A	0.45	0.85	6	2.2	2.8
C	0.44	0.75	5	1.7	3.0
F	0.45	0.85	14	5.4	2.6
H	0.45	0.71	5	1.7	2.9
L	0.49	0.76	6	2.3	2.6
O	0.47	0.82	11	4.1	2.7
R	0.50	0.80	14	5.3	2.6
W	0.46	0.80	15	4.8	3.1

[a] The sum of the fractional loss of all amide protons for the indicated peptide during digestion and HPLC separation. The calculation of this parameter is described in the text.

[b] The total number of amide protons on the peptide.

[c] H_t is the number of remaining protons after analysis. It is equal to $\Sigma_i(f_i^{digest} \times f_i^{HPLC})$, where the sum is over all i amino acids in the peptide.

[d] Loss factor = H_0/H_t.

Peptides encompassing every amide along the backbone except the β_4-strand (74–87) were identified and analyzed for differences in amide proton exchange behavior upon reduction of thioredoxin. Because most cleavage sites are found intact within at least one of the other peptides, information on exchange rates of the N-terminal and penultimate residues, which would normally be lost in the analytical steps, has thus been recorded. Every peak in the HPLC separation was examined; therefore a peptide representing all or part of the β_4-strand would have been likely to be in one of the peaks that was redox insensitive. One possibility is that an HPLC peak containing β_4 had a coeluting peptide whose redox-dependent hydrogen exchange effects compensate in hydrogen exchange behavior for those that would be observed for the peptide from β_4. Another possibility for β_4 to be redox sensitive would be that it is uniquely present in the one peak that had statistically significant changes in exchange behavior and which contained five components. However, it is unlikely that any part of the sequence will appear in only one peak due to incomplete pepsin proteolysis. It is more probable that the changes localized to the active site and first β-strand are the only changes that occur in the protein upon reduction.

Discussion

Amide proton exchange profiles from whole thioredoxin demonstrate that overall about 3–5 protons increase in exchange rate upon reduction of the intact protein. From thermal denaturation data the lowered T_m of the reduced form of the protein indicates a decrease in stability upon reduction possibly due to fewer internal hydrogen bonds that normally increase the energy barriers for hydrogen exchange for the affected amide protons. Three possible models for the changes observed are: (1) there are only 3–5 amide protons that are affected by reduction; (2) all amides are slightly affected resulting in an effective 3–5-proton shift in exchange rates; or (3) the protein undergoes a conformational change, and the hydrogen exchange behavior of all amide protons is affected. The last option is not likely given the similarity of CD spectra between the two redox forms as well as the NMR data, which indicate that most of the protein is not affected by reduction (Dyson et al., 1988, 1989; Hiraoki et al., 1988). In addition, the relaxation curves (Hvidt & Nielsen, 1966) derived from the whole exchange data (not shown) indicate that there are no large changes in conformation and therefore the rank order of exchange for each redox form is similar. To determine location of redox-sensitive amide protons within the protein and thus distinguish between the first two models, medium resolution hydrogen exchange experiments were performed.

Redox-sensitive exchange rates were found on peptides derived from the first β-strand and the region around the active site disulfide including the carboxyl end of β_2, the β-turn, and the start of the α_2-helix (see Fig. 4 and Kinemage 1). The sum of the changes seen in peptides R and W is approximately the 3–5-proton difference between the two redox forms of whole thioredoxin. This implies that all the changes observed in whole protein experiments can be accounted for by these two regions, which is further evidence that no changes are likely to occur at β_4, the only region not identified in the peptide experiments.

Previous NMR studies by Dyson et al. (1988) and Hiraoki et al. (1988) found structural changes at the active site turn as well. The Dyson paper indicated that other changes not observed in this study occurred in the α_3, β_3, and β_4. These effects were much less than those at the active site and were not seen in the Hiraoki study either. For the other redox-sensitive regions reported by Dyson, proton assignment was not presented and therefore accuracy by comparison to the fully assigned proton spectrum (LeMaster & Richards, 1988) could not be done. The Hiraoki paper contains several contradictory proton assignments as compared to the LeMaster data. Given all these inconsistencies, no conclusions are drawn from the NMR data except the changes at the active site.

The path of propagation of the effects of reduction from the active site to the nonadjacent β_1-strand (about 10 Å) is not apparent from this data. It is expected that intervening residues of β_3 would be redox sensitive as well, but the data from peptides C, Q, and O appear to contradict this assumption. The probability at the 95% confidence level that the exchange rates of the two redox states are the same is greater than 10% for each of these peptides.

With the data presented here, along with the hydrogen-

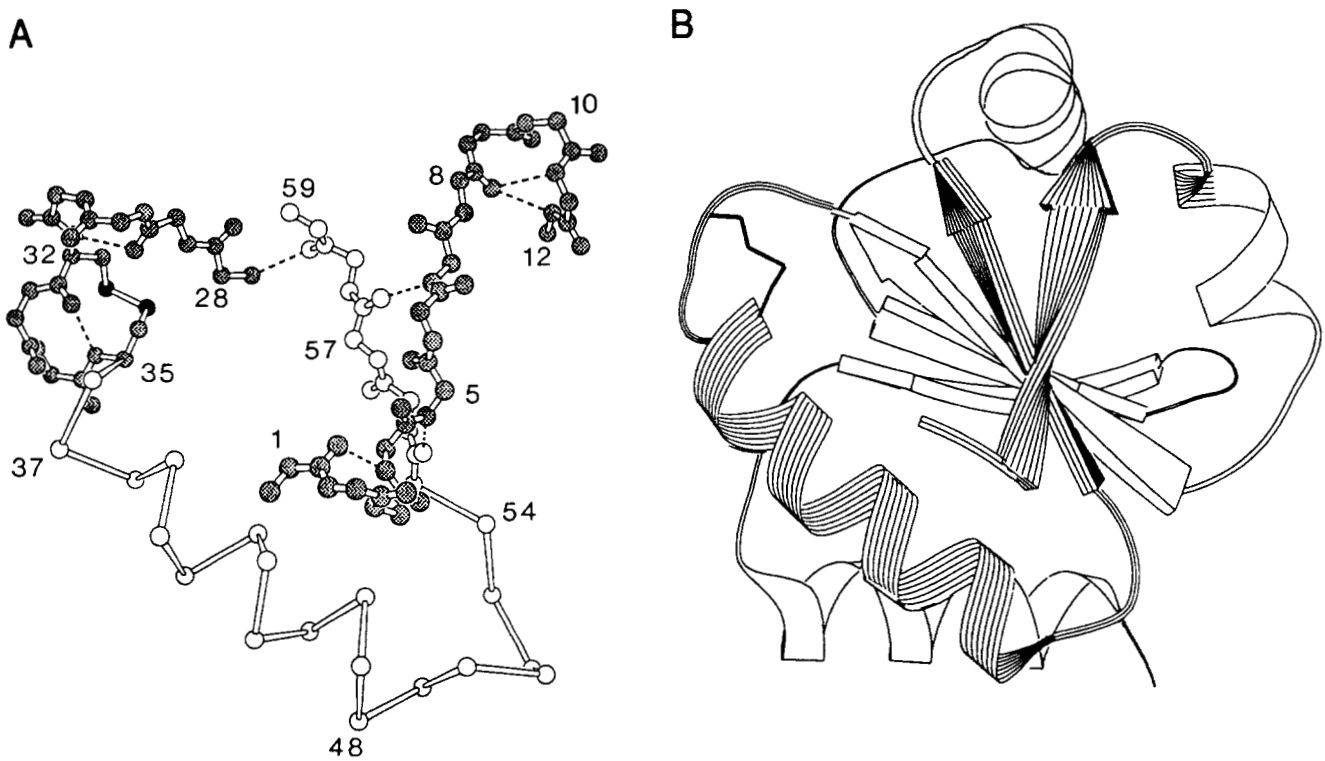

Fig. 4. A portion of the X-ray structure of *E. coli* thioredoxin determined by Katti et al. (1990). **A**: A ball-and-stick model of the redox-sensitive peptides 1–13 and 28–35 identified in this study, and a segment of the rest of the chain. All main-chain atoms are shown for the two peptides (cross-hatched circles) and segment 55–59 (open circles) to whose carbonyl groups they are hydrogen bonded. The α-carbon chain of the helical segment connecting 35 to 55 is also shown. Residue numbers are given at selected α-carbon atoms. The hydrogen bonds are shown as dashed lines. All NH groups not involved in the bonds shown are either bonded to solvent molecules or are too far from potential protein acceptors to be considered partners in acceptable hydrogen bonds. The sulfur atoms of the Cys 32–Cys 35 disulfide are shown as filled black circles. **B**: A ribbon diagram of the whole thioredoxin molecule in the same viewing direction as A. The parts of the chain shown in detail in A are shaded for identification in the ribbon diagram.

bonding map from the refined crystal structure presented in Figure 4 and Kinemage 1, it is proposed that reduction of the disulfide results in the splaying of the ends of the β_2- and β_3-strands, thus lowering the energy barrier to solvent accessibility of the amide positions of residues 28–35. If this is the case, it would result in a faster exchange rate of only one potentially observable amide proton, Trp 28, involved in the hydrogen bonding between the two strands. This amide occurs in an observable sequence position only in J, which is too complex a mixture to permit confident interpretation of kinetics, although the redox shift does appear statistically significant. There would be no expected differences in exchange rates for amide protons of the β_3-strand because the first proton associated with an amide position, 58, of β_3 is hydrogen bonded to the carbonyl of residue 26, which is two residues further from the end of the sheet. Upon reduction there was an apparent increase in flexibility of the active site turn as indicated by the faster exchange rates of the amide protons in peptides that mapped to this region. The only observable protons at this site are the amides of Trp 31, Cys 32, and Cys 35.

Given the lack of temperature dependence of the intact protein experiments, it is unlikely that the reduction-induced changes at the active site and the first β-strand are related to normal thermal fluctuations in the range of 5–45 °C, as expected for the breaking of a covalent bond such as disulfide reduction.

Of the first 13 amide protons, only 3 are internally hydrogen bonded to other backbone positions (Fig. 4; Kinemage 1), mainly the β_3-strand and α_1-helix, 7 interact with solvent, and 3 are bonded to side chains (2 in the same peptide, 1 to α_3). Given that no differences in the exchange rates of β_3-amide protons were observed in this study, it is most likely that changes in the β_1 region were due to those amides that were seen to be hydrogen bonded to solvent. These results imply that there were changes in the hydration layer that would have affected the exchange rates of those β_1 protons. The increase in exchange rates for the β_1-strand might be due to changes in water accessibility on the exterior of the protein upon reduction of the disulfide. Some possibly relevant water positions are shown in Kinemage 2.

Because effects that are transmitted to regions far away

along the primary sequence do not appear to be through the protein matrix, the possibility that changes are propagated through the solvent, mediated by alteration of the hydration properties, was explored. In addition, the changes in flexibility around the active site upon reduction should result in changes in compressibility. Both of these properties have been studied by sound velocity and density measurements and are discussed in the following paper.

Methods and materials

Chemicals

All chemicals were Baker Analyzed reagents or Fisher Certified chemicals unless indicated otherwise.

Equipment

HPLC was performed with two model 510 pumps and a model 680 controller from Waters Associates. Detection of peptides was by a Gilson Medical Instruments Holochrome variable wavelength monitor connected to a Spectra Physics integrator (model 4290) fitted with a homemade event marker consisting of a voltage follower to an RC time delay throwing a minirelay inline with a thermal pen. A Gilson model FC203 fraction collector was connected to the UV monitor for peak detection and the integrator for event marking, and to the HPLC controller for precise start of collection. A refrigerated circulating bath provided temperature control of both the jacketed column and the loading loop, which was embedded in an insulated brass block.

Thioredoxin purification

Thioredoxin was purified to homogeneity from the overproducing strain MG1655/pDL59, kindly provided by D.M. LeMaster, as described (LeMaster & Richards, 1988). Purified thioredoxin was stored frozen in 50 mM ammonium bicarbonate or as a lyophilized powder. Thioredoxin concentrations were determined by absorbance at 280 nm using an extinction coefficient of 13.7 $mM^{-1}\,cm^{-1}$ (Reutimann et al., 1981).

Uniformly ^{14}C-labeled thioredoxin was prepared by adapting MG1655/pDL59 to grow on glucose as the sole carbon source. Once adapted, an overnight culture was diluted 1:100 into 20 mL of M9 (Maniatis et al., 1982) supplemented with 0.12% glucose (0.5 mCi [U-^{14}C]glucose, Amersham), 2 mM $MgSO_4$, 0.1 mg/mL ampicillin, and incubated at 30 °C until a Klett reading of 150 was obtained. Thioredoxin was induced by shifting the temperature to 43 °C and continuing growth for 3 h. Metabolically released $^{14}CO_2$ was trapped in two sequential traps containing 2 M sodium hydroxide. After induction, cells were harvested and lysed in 20 mL of 50 mM ammonium bicarbonate, 2 mM EDTA, 10 mg/mL lysozyme (Sigma Chemical Co.), and two cycles of freeze–thawing in dry ice/isopropanol. Unbroken cells and debris were removed by centrifugation (34,000 × *g*, 15 min at 4 °C). The supernatant was loaded onto a 7.5-mL DE52 (Whatman Inc.) column equilibrated in 50 mM ammonium bicarbonate buffer. After washing with 25 mL of the same buffer, thioredoxin was eluted from the column with 0.5 M ammonium bicarbonate. The high salt eluate was lyophilized, resuspended in water, and transferred into 50 mM ammonium bicarbonate by passage through a Trisacryl GF05 (LKB Biochemicals) column (12 × 0.9 cm). The protein was relyophilized, dissolved in 2 mL of water, and loaded onto a 2.15 × 15.0-cm DEAE-5PW semipreparative column (Waters Associates) equilibrated with 50 mM ammonium bicarbonate at 8 mL/min. After washing the column for 10 min with this buffer, the protein was eluted with a 50-min linear gradient to 0.5 M ammonium bicarbonate. The resulting protein appeared as a single band on an SDS polyacrylamide gel. The ^{14}C-thioredoxin had a specific activity of 13.2 μCi/mg.

Thioredoxin tritiation

Thioredoxin (1.4 mg + 34 μg ^{14}C-thioredoxin) dissolved in 1 mL 50 mM sodium phosphate buffer (pH 7) was filter sterilized (0.45 μm filters) into a sterile vacuum hydrolysis tube (Kontes Glass) and lyophilized. For equilibration with tritiated water, the sample tube was connected to an apparatus similar to that used by Rosa and Richards (1979) and 1 mL of 27 Ci/mol tritiated water was vacuum transferred to the sample tube. The protein solution was heated to 60 °C for 2.5 h (conditions determined by proton NMR for complete exchange with ^2H-H_2O). The ^3H-H_2O was removed by lyophilization and trapped in the storage tube for subsequent experiments. In order to remove all tritons with fast exchange rates (buffer and side-chain bound) the sample tube was removed from the tritiation apparatus, cooled on ice, and the protein was dissolved in 1 mL of water at 0 °C for 45 s, followed by quick freezing in dry ice/isopropanol and relyophilized. The tritiated thioredoxin was then stored in vacuo at −20 °C.

Out-exchange experiments—Intact thioredoxin

Whole thioredoxin studies were initiated by dissolution of the tritiated protein in 3 mL of degassed, sterile-filtered 50 mM sodium phosphate buffer (pH 7) with or without 2 mM dithiothreitol (DTT) (Pierce Chemical Co.), and 2 mM EDTA for reduced or oxidized experiments, respectively. The same solution was used for the peptide studies, but the volume was 275 μL. All solutions and glassware were preequilibrated to the temperature of the corresponding experiment. Whole protein exchange experiments were done over long time periods. To maintain

reducing conditions, 2 μL of 1 M DTT were added each day to the reduced thioredoxin sample. Time zero of the experiment was defined as that moment when all thioredoxin dissolved. Normally this occurred within 15 s of addition of the solvent. A 50-μL aliquot was removed with a precooled (0 °C) gas-tight (Hamilton Co.) syringe. The aliquots were analyzed immediately or frozen by immersion into dry ice/isopropanol depending on frequency of sampling.

Whole protein samples were separated from tritiated water on a Sephadex G-10 column (0.9 × 19.0 cm) connected to the previously described HPLC and equilibrated in 50 mM sodium phosphate at pH 2.8 at 1 ± 1 °C with a flow rate of 0.5 mL/min. The protein peak was detected by absorbance at 220 nm and collected as 6- or 12-drop fractions (approximately 130 or 260 μL, respectively) directly into scintillation vials. The samples were counted after 24 h (to prevent interference from static and chemiluminescence) at an efficiency of approximately 42%.

The number of protons in the protein remaining unexchanged was determined from the specific activity. The quantity of protein was determined from the integrated area of the UV absorbance peak. The kinetic exchange data were fit to the equation for $H(t)$, the number of remaining protons at time t,

$$H(t) = b(1 + at)^{-n}e^{-ct} \quad (1)$$

introduced by Knox and Rosenberg (1980), which was determined here to be a good model for the amide proton exchange of thioredoxin. A Gauss–Newton nonlinear least-squares procedure (Fraser & Suzuki, 1973; Delfino, pers. comm.) was used to determine the parameters a, b, n, and c; the variable t in the equation is time.

Out-exchange experiments— Peptide fragment analysis

At the appropriate time intervals 50 μL of the protein solution was removed with a pipette tip and placed in a tube containing enough 1 M phosphate buffer and urea to bring the final solution to 4 M urea and pH 2.3. The tube and the pipette tip were precooled to 0 °C to minimize loss of amide tritons. An aliquot of a stock solution of pepsin was added. The proteolytic reaction was kept on ice for exactly 1 h and then pipetted into the loading loop of the HPLC, which was maintained at 0 °C.

For proteolysis of the tritiated thioredoxin, porcine pepsin (Sigma) was prepared before each experiment by dissolving the lyophilized enzyme and transferring into 50 mM sodium acetate, pH 4.5, on a 0.9 × 10-cm Trisacryl GF05 column at 5 °C. This served to remove autolysis products. Pepsin was added to the thioredoxin–urea solution to a final concentration of 0.85 mg/mL.

Peptide chromatography and identification

Peptides were separated by the HPLC apparatus described above with a 0.39 × 30-cm C-18 reverse-phase μ-Bondapak column (Waters Associates) and 150 mM sodium phosphate with 5% acetonitrile at pH 2.8 (buffer A) and acetonitrile as buffer B at a constant flow rate of 1.1 mL/min. Peptides were eluted as follows: 96% buffer A at injection; 4–17% buffer B, 18.5 min, using convex ramp 5; 17–30% buffer B by 32 min with a linear gradient; 30–55% buffer B by concave gradient (curve 8) by 36 min; 55–100% buffer B by concave gradient (curve 8) by 76 min. All peaks were eluted by 40 min. Samples were collected directly into vials with 5 mL of scintillation fluid. Each vial was capped, shaken vigorously, and allowed to settle for 24 h before being loaded into the scintillation counter.

To determine peptide identity, peptides from a nonradioactive identical digest and separation were transferred to trifluoroacetic acid (TFA): acetonitrile on a second reverse-phase column and analyzed by standard amino acid analysis at the Protein Chemistry Facility at Yale. In some cases coeluting peptides were separated on the second column and each identified as single peptide peaks. Compositions were matched to sequence using a computer program (Dumont et al., 1985) that compared observed frequencies of amino acids from the hydrolyzed peptide to those predicted by all possible segments along the thioredoxin sequence. For some peptides the best fit sequence was confirmed by N-terminal identification using the derivatization method of Allen (1981) and HPLC analysis similar to that of Wiedmeier et al. (1982).

Scintillation counting

For quantitation of radioactive isotopes, HPLC fractions were collected directly into 5 mL of Optifluor scintillant (Packard Instruments) and counted in a Packard model 2000CA scintillation counter in automatic efficiency control, except for double label experiments. Scintillation counting of double-labeled samples was accomplished by the method of Attri and Minton (1987), with two energy windows on the scintillation counter set to 0–15 keV and 15–156 keV.

Calculation of tritium losses

To determine the amount of tritium originally present on each peptide as it existed before fragmentation of the protein, the losses of the analytical procedures were calculated from the intrinsic rate constants for the fully solvent-exposed amide protons at each position (Molday et al., 1972; Englander et al., 1985). Due to the addition of different components (urea, acetonitrile) that affect hydrogen exchange rates, the losses during the digestion and chromatography steps were calculated separately. (1)

For the digestion step, the presence of 4 M urea required a correction of the rate constants by a factor determined from the data given by Lofthus et al. (1986). (2) Tritium loss during the chromatographic separation was determined using the procedure of Englander et al. (1985). This accounts for effects on exchange rates due to the presence of an increasing concentration of organic solvents in the elution buffer. The method calculates a parameter (t_{cor}), which is the equivalent time for exchange in aqueous solvent as that observed in the presence of organic solvents. The relationship is given in this equation

$$t_{cor} = (1 - e^{-k_{SF}t})/(SF_0 k_{SF}) \quad (2)$$

where SF_0 is the slowing factor as defined by Englander et al. (1985) and is a function of the type and concentration of organic solvent, k_{SF} (0.022 min^{-1} here) is the rate of increase of the slowing factor during chromatographic separation, and t is the peptide retention time. The gradient used in the separation step was reasonably approximated as linear, a necessary assumption for this calculation.

The loss for a peptide is the sum of the losses for each amide, separately determined by the t_{cor} and the intrinsic rate constant applicable to each one. The product of the fraction of amide protons remaining during the peptide separation step and the digestion step resulted in the number of protons remaining, H_t, after the complete analysis. The ratio of the number of amides on a peptide, H_0, to the number H_t is the loss factor given here

$$L = H_0/H_t. \quad (3)$$

When the number of amide protons remaining at the end of the experiment was multiplied by this loss factor it resulted in the number of amide protons remaining on the peptide just after the exchange-out incubation. This correction does not affect the relationship between the two curves for a given peptide derived from thioredoxin under different redox states, because the value of L is the same for the analysis of both redox experiments. For the peptides in this experiment L ranged from 2.6 to 3.1.

Statistical analysis

To determine whether the exchange for a given peptide was significantly different between the two redox forms of the protein, the nonparametric Wilcoxon rank sum test was used (Roessler, 1977).

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