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Escherichia coli Thioredoxin Folds into Two Compact Forms of Different Stability to Urea Denaturation†

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ABSTRACT: The urea-induced denaturation of *Escherichia coli* thioredoxin and thioredoxin variants has been examined by electrophoresis on urea gradient slab gels by the method of Creighton [Creighton, T. (1986) *Methods Enzymol. 131*, 156-172]. Thioredoxin has only two cysteine residues, and these form a redox-active disulfide at the active site. Oxidized thioredoxin-S_2 and reduced thioredoxin-$(SH)_2$ each show two folded isomers with a large difference in stability to urea denaturation. The difference in stability is greater for the isomers of oxidized than for the isomers of reduced thioredoxin. At 2 °C, the urea concentrations at the denaturation midpoint are ~8 and 4.3 M for the oxidized isomers and 4.8 and 3.7 M for the reduced isomers. The difference between the gel patterns of samples applied in native versus denaturing buffer, and at 2 and 25 °C, is characteristic for the involvement of a *cis*-proline–*trans*-proline isomerization. The data very strongly suggest that the two folded forms of different stabilities correspond to the cis and trans isomers of the highly conserved Pro 76 peptide bond, which is cis in the crystal structure of oxidized thioredoxin. Urea gel experiments with the mutant thioredoxin P76A, with alanine substituted for proline at position 76, corroborate this interpretation. The electrophoretic banding pattern diagnostic for an involvement of proline isomerization in urea denaturation is not observed for oxidized P76A. In broad estimates of $\Delta G°$ for the native–denatured transition, the difference in $\Delta G°$ (no urea) between the putative cis and trans isomers of the Ile 75–Pro 76 peptide bond is ~3 kcal/mol for oxidized thioredoxin and ~1.5 kcal/mol for reduced thioredoxin. Since cis oxidized thioredoxin is much more stable than trans, folded oxidized thioredoxin is essentially all cis. In folded reduced thioredoxin, cis and trans interconvert slowly, on the minute time scale at 2 and 25 °C. In the absence of urea, the folded reduced thioredoxin is less than a few percent trans. Three additional mutants with additions or substitutions at the active site also show electrophoresis banding patterns consistent with a difference in stability between cis and trans isomers.

Escherichia coli thioredoxin is a small, soluble protein involved in diverse redox and regulatory reactions. It participates in oxidation/reduction reactions with several types of reductases (Holmgren, 1985; Tsang & Schiff, 1976; Fuchs & Carlson, 1983) and can serve as a protein disulfide reductase (Holmgren, 1979; Scheibe, 1984). It has high sequence homology to protein disulfide isomerase (Edman et al., 1985), and folding studies suggest that thioredoxin facilitates protein folding by disulfide interchange (Pigiet & Schuster, 1986). Thioredoxin is also required in the replication cycle of some bacteriophages. DNA polymerase activity of bacteriophage T7 gene 5 protein requires prior complex formation with host thioredoxin (Modrich & Richardson, 1975; Nordström et al., 1981; Huber et al., 1986). Coat assembly in the filamentous phages M-13 and f1 also requires the presence of host thioredoxin (Russel & Model, 1985, 1986; Lim et al., 1985). In both phage systems it appears that reduced thioredoxin is the active species of the protein.

E coli thioredoxin contains 108 amino acids and has a molecular weight of 11 700. It has only two cysteine residues, at positions 32 and 35, which form the redox active S–S at the active site. *E. coli* thioredoxin has high sequence homology to other prokaryotic thioredoxins (Eklund et al., 1984; Gleason, 1986; Clement-Metral et al., 1988). Completely conserved residues include the active site sequence Trp-Cys-Gly-Pro-Cys, as well as Pro 76, which is in a cis imide bond in the crystal structure of the oxidized protein. The disulfide bond in oxi-

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dized thioredoxin closes a 14-member ring, the strain of which has been suggested to contribute to the reactivity of the disulfide (Holmgren, 1985).

After reduction of this bond with dithiothreitol (DTT)[1] thioredoxin remains folded but is substantially less stable than the oxidized form (Kelley et al., 1987). Solution measurements indicate that conformational differences between oxidized and reduced thioredoxin are highly localized. While tryptophan fluorescence emission is strongly quenched in oxidized but not reduced thioredoxin, only small changes in circular dichroism (CD) spectra are observed (Brown et al., 1987). The crystal structure has been determined for oxidized thioredoxin (Holmgren et al., 1975; Eklund et al., 1984) but not for reduced thioredoxin, which has not been successfully crystallized. The structural basis for the marked difference in stability between oxidized thioredoxin (thioredoxin-S_2) and reduced thioredoxin [thioredoxin-$(SH)_2$] is not obvious from examination of the oxidized structure. A central, twisted β-sheet of five strands is packed by four α-helices. The disulfide bridge does not apparently stabilize long-range interactions since the cysteines are only separated by two residues and located in a reverse turn joining the α_2 helix and β_2 strand. The S–S group is essentially buried, with a small patch of surface area accessible to solvent on one side of the disulfide bridge.[2] Brown et al. (1987) have shown by CD difference spectroscopy that reduction of thioredoxin gives a 6% increase in α-helix and 3% decrease in β-sheet. They suggest that the apparent increase in α-helical content at the expense of β-structure upon conversion of thioredoxin-S_2 to thioredoxin-$(SH)_2$ may arise from a relaxation of the α_2 helix packing and a subsequent increase in the mobility of the α_2 as a unit. ^{1}H NMR spectra of oxidized and reduced thioredoxin have been reported (Dyson et al., 1988; Hiraoki et al., 1988). The largest changes in chemical shifts are observed for protons in residues of the active site and of the β_2 strand immediately preceding the active site.

Because of its stability, moderate size, and its prokaryotic origin, which facilitates isotopic labeling and expression of mutant proteins, *E. coli* thioredoxin is developing as a model system for the study of protein folding and dynamics. We report here studies of the urea-induced unfolding of oxidized and reduced thioredoxin monitored by urea gradient polyacrylamide gels. Folded oxidized thioredoxin and folded reduced thioredoxin each consist of two forms with differing stability to urea denaturation. Their gel behavior strongly suggests that these two forms correspond to the cis and trans isomers of Pro 76. In this model, folded reduced thioredoxin with the cis peptide bond at Ile 75–Pro 76 interconverts on the minute time scale with the less stable trans form. Oxidized thioredoxin may also fold with either the cis or trans isomer at Pro 76, but since the cis isomer is far more stable that the trans isomer, folded oxidized thioredoxin is essentially all cis. These conclusions are corroborated by the gel behavior of the thioredoxin P76A, with an alanine for proline substitution at residue 76. Oxidized P76A shows only one urea denaturation transition under all conditions. Estimated differences in free energy between the cis and trans wild-type isomers at 2 °C are ~1.5 kcal/mol for reduced thioredoxin and ~3 kcal/mol for the oxidized thioredoxin. Three additional variants of thioredoxin purified from genetically engineered strains of *E. coli* were also studied. In one of the "mutant" proteins Cys 32 is replaced by serine, and in another both Cys 32 and Cys 35 are replaced by serines (Russel & Model, 1986). The third has arginine inserted between residues 33 and 34 in the 14-member disulfide ring (C.-J. Lim, F. Gleason, and J. Fuchs, unpublished results).

Materials and Methods

High-level overexpression of wild-type thioredoxin was obtained with *E. coli* strain JF521 [Δ(*lac pro*) *thi*, *sup*E, *met*E46, *trx*A704, *srl*300::*Tn*10, *rec*A, F′, *lac*I^q, *lac*ZΔM15] containing a derivative of plasmid pTK10 carrying the thioredoxin gene *trx*A. The plasmid pTK10 is a derivative of pTL14 (Sung et al., 1987) containing a 1.3-kb *Mlu*I fragment with the kanamycin gene from pBS8 (Spratt et al., 1986). Plasmid pCJF4 containing *trx*A gene (Lim et al., 1985) was cut with *Hin*cII and *Hpa*II, and the fragment containing *trx*A was inserted into the pTK10 via the *Sma*I and *Acc*I sites.

The gene *trx*A76 encoding thioredoxin with proline 76 replaced by alanine (P76A) was obtained from the strain RKAR120 kindly provided by Drs. R. Kelley and F. M. Richards (1987). Gene *trx*A76 was cut with *Hin*cII and *Bam*HI and transferred into pTK10, which had been cut with *Sma*I and *Bam*HI, to generate pTKP76A. This plasmid was transferred into strain JF531 [*thr*1, *ara*14, *leu*B6, Δ(*gpt-pro*A)62, *lac*Y, *tsx*33, *sup*E44, *gal*K2, *his*, *rfb*D, *xyl*5, *mtl*1, *arg*E, *thi*, *lon*1, *trx*A704, *htp*165::*Tn*10, F′, *lac*I^q, *lac*Z, ΔM15] which is *lon* protease deficient. The mutation was confirmed by resequencing of the gene in our expression system.

Thioredoxins with serine for cysteine at position 32 (C32S) and at positions 32 and 35 (C32,35S) were isolated from *E. coli* strains A291 and A294, kindley provided to us by Dr. M. Russel. Russel & Model (1986) constructed these by transformation of parent strain A179 with plasmid pPMR18, which was derived from pBR325 and contains genes *trx*A3 or *trx*A7, encoding C32S or C32,35S, respectively.

Thioredoxin with an arginine inserted between residues 33 and 34 (33R34) was isolated from *E. coli* strain JF521 carrying plasmid pTK33R34. The arginine residue was inserted into the 14-member ring of the active site by cutting the gene encoding wild-type thioredoxin with *Ava*II, filling in the single-stranded regions with DNA polymerase (Klenow), and ligating the blunt ends (C.-J. Lim, F. Gleason, and J. Fuchs, unpublished results). The 33R34 mutant was constructed in pCJF4 and transferred into pTK10 via *Eco*RI and *Hin*dIII sites to generate pTK33R34.

The protein purification protocol was developed from the procedure of Laurent et al. (1964). After sonication of the *E. coli* cells in extraction buffer (20 mM phosphate, 3 mM EDTA, pH 7), the crude cell extract was filtered on Sephadex G-75 (4.5 × 100 cm column) equilibrated with extraction buffer. Fractions containing thioredoxin were applied to DEAE-cellulose equilibrated with the same buffer. Thioredoxin was eluted with a linear gradient of 0–500 mM KCl in extraction buffer. Fractions containing thioredoxin were dialyzed against 0.5% ammonium bicarbonate, pH 7.9, and lyophilized. Thioredoxin-containing fractions were identified from their enzymatic activity in a coupled NADPH–thioredoxin reductase–thioredoxin–DTNB assay, by following the rate of increase of absorbance at 412 nm [method 1 of Luthman and Holmgren (1982)]. Inactive thioredoxin variants were identified by comigration with wild-type thioredoxin on native gels and confirmed by immunoblot with anti-thioredoxin antibodies and by amino acid analysis. The criteria for purity was electrophoretic homogeneity of oxidized proteins on overloaded, native gels at pHs above and below the isoelectric point of 4.5 and on isoelectric focusing plates. Overloaded,

[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid.

[2] Our comments about the structure of oxidized thioredoxin are based on unpublished coordinates provided by Dr. Hans Eklund.

native gels were run at pH 7, 8.7, and 2.5. Isoelectric focusing was carried out on LKB ampholine PAG plates, pH 4.0–6.5. Single bands were observed in both systems under all conditions examined (data not shown).

Polyacrylamide gels with a horizontal gradient of 0–10 M urea and a reverse 15–10% acrylamide gradient (Creighton, 1979) were prepared in a multiple (4X) gel casting stand. Solutions containing urea (ultrapure grade from Bethesda Research Laboratory) were deionized on Amberlite MB-3 before use. A linear gradient of the following two solutions was poured: (1) 0.375 M Tris, pH 8.7, 15% acrylamide, and 0.5% bis(acrylamide); (2) 0.375 M Tris, pH 8.7, 10% acrylamide, 0.34% bis(acrylamide), and 10 M urea. The tray buffer was 20 mM Tris–150 mM glycine, pH 8.7. A 100-μL sample was applied in a band across each gel, as described below. As a control, banding patterns after electrophoresis on gels at pH 7 and 2.5 were determined. For the pH 7 experiments, the gels were prepared in the same way as for those at pH 8.7, but either 50 mM phosphate or 50 mM Tris–acetate was used instead of Tris-HCl. For the pH 2.5 gels the buffer was acetic acid.

For the electrophoresis experiments shown in Figures 2, 4, 5, and 6, samples loaded in the native state, denoted N, contained 0.25 mg of protein dissolved in 100 μL of gel buffer with 25% glycerol. Samples loaded in the denatured state, denoted D, contained 0.25 mg of protein dissolved in 100 μL of gel buffer that was 10 M in urea and were allowed to stand for 60 min. Electrophoresis running time was 90 min. For the experiment in Figure 1, the protein concentration was increased to 1 mg/100 μL, and the electrophoresis running time was 180 min.

Electrophoresis was carried out at a constant current of 20 mA/gel, at 2, 25, or 38 °C. A constant gel temperature was maintained by an external circulating water bath. For the 2 °C runs, the electrophoresis apparatus and circulating water bath were operated in a freezer room. The temperature of the gel was determined by a thermister, with the probe inserted directly into the gel. After electrophoresis, protein bands were visualized by staining for 1 h with 0.2% Coomassie Blue in 50% methanol and 10% acetic acid. No difference in banding pattern is observed when the staining step is preceded by fixation with 10% trichloroacetic acid in 25% 2-propanol. The banding patterns of the gels show no dependence on protein concentration over a 20-fold concentration range (0.1–2 mg/100 μL of tray buffer). Gels loaded with lower protein concentrations show sharper bands at high and low urea concentrations (that is, the N and D horizontal bands outside the transition) but essentially the same S-shaped bands in the transition zone. The concentration of 0.25 mg/100 μL was chosen for publication because the gels are easily visualized.

Thioredoxin is isolated in the oxidized thioredoxin-S_2 form. Samples of reduced thioredoxin-$(SH)_2$ were prepared by addition of 0.5 mg of DTT to thioredoxin-S_2 at 0.25 mg/100 μL of buffer. We determined by the quantitative Ellman's assay (Riddles et al., 1983) that, at pH 8.7, a 200-fold excess of DTT is necessary for full reduction of the wild-type thioredoxin cystine to 2 equiv of cysteinethiol. For this determination, DTT was added to thioredoxin samples and then removed prior to the assay by rapid filtration, under nitrogen, on Sephadex G-25. The Ellman's assay was performed immediately after filtration and 3 h after filtration during which the samples were exposed to air. No difference was observed in the extent of reduction; that is, no air oxidation was detectable during that time. This question is relevant since our samples do not contain EDTA to sequester the divalent cations that catalyze air oxidation. EDTA was omitted to make all data comparable to those of the CD experiments, which were run in the absence of EDTA because it interferes with the CD signal. Special care was taken to exclude the addition of divalent cations in the buffers. A number of additional control experiments were carried out to show that air oxidation of reduced samples does not occur during the course of electrophoresis. Electrophoreses with DTT in the sample buffer, with DTT in the sample and upper tray buffers, or with DTT in the sample buffer and preelectrophoresis with thioglycolate show no difference in banding patterns. We routinely used DTT in the sample buffer, although numerous gels were run under the other two conditions.

The banding patterns are highly reproducible, indicating a similarly reproducible urea gradient. The linearity of the horizontal gradient was verified by pouring gels with 1 mg/mL bovine serum albumin added to the 10 M urea solution. The resulting albumin gradient was determined after Coomassie blue staining with a scanning densitometer. The urea concentration at the midpoint of the unfolding transition was determined by locating the position of the band where the mobility was half the difference between the folded and unfolded forms and calculating the corresponding urea concentration from the width of the gel. Estimates of $\Delta G°$ were made by extrapolation from the transition region to 0 M urea as described in Hollecker and Creighton (1982).

Circular dichroism spectra were recorded on a Jasco J41C spectropolarimeter using a jacketed cell maintained at 25 °C, with a 1-mm path length. Protein samples in 0.375 M Tris, pH 8.7, and variable concentrations of urea were prepared by dilution of stock solutions to a final protein concentration of 0.5 mg/mL. The reduced samples contain a 200-fold excess of DTT. The samples were equilibrated for 15 min before spectra from 205 to 300 nm were measured. The fraction of unfolded protein at each concentration of urea was determined from the percent change in ellipticity at 219 nm relative to the initial and final values at 0 and 10 M urea (Pace, 1986).

Results

The urea-induced cooperative denaturation transition of globular proteins is conveniently monitored by electrophoresis of a band of protein through slab gels containing a urea gradient perpendicular to the direction of migration (Creighton, 1979, 1980, 1986). The extended conformation of unfolded protein reduces the electrophoretic mobility of denatured species compared to that of the more compact native species. This provides a clear visual distinction between folded and unfolded forms as a function of urea concentration. Urea gradient gels of oxidized versus reduced thioredoxin show striking differences (Figure 1). The urea gradient is 0–10 M from left to right. At 25 °C, oxidized thioredoxin unfolds at a urea concentration of ~8 M, with very diffuse staining in the transition region. The blurred appearance of the Coomassie blue band in this region indicates that the native to denatured transition (N $\Leftrightarrow$ D) at urea concentrations around the unfolding midpoint is slow on the electrophoresis time scale (Creighton, 1979). Reduced thioredoxin, in contrast, has two continuous S-shaped bands at 25 °C, one with a midpoint of denaturation around 4.2 M urea and the other with a midpoint around 5.5 M urea. The presence of two bands indicates that there are two folded forms of reduced thioredoxin with significantly different stabilities in urea.

Both bands of reduced thioredoxin are more distinct than the band observed for oxidized thioredoxin. Also, the reduced thioredoxin band at higher urea concentration (right side of the gel) is more diffuse than the one at lower urea concen-

Table I: Urea Denaturation of Oxidized and Reduced Thioredoxin and of Thioredoxin Variants[a]

	2 °C N (gel)		2 °C D (gel)		25 °C N (gel)		25 °C D (gel)		25 °C (CD)	
	$[U]_m$[b]	$\Delta G°$[c]	$[U]_m$	$\Delta G°$	$[U]_m$	$\Delta G°$	$[U]_m$	$\Delta G°$	$[U]_m$	$\Delta G°$
trx-S_2										
cis	~8	9.5[d]			~8	9.5			7.4	10
trans			4.3	6.5						
trx-$(SH)_2$										
cis	4.8	6.0			5.5	6.5	5.3	5.5	4.4	6.5
trans	3.7	4.5	3.6	4.0	4.2	4.5	4.2	4.0		
C32S										
cis	4.7	4.5			5.3	6.0	5.2	6.5		
trans	3.1	2.5	2.9	4.0	4.1	4.0	4.0	4.0		
C32,35S										
cis	5.4	8.0			5.9	7.0	6.1	7.0		
trans	3.8	5.0	3.5	5.0						
33R34-S_2										
cis					6.2	7.0	5.9	6.5		
trans			2.6	3.0						
P76A-S_2										
trans	3.5	4.0	3.5	4.5	5.2	8.0	5.3	6.0		

[a] Urea concentrations at the midpoint of the urea-induced N ⇔ D transition, $[U]_m$ (in molarity), and estimated free energies, $\Delta G°$ (in kcal/mol), in the absence of urea were estimated from urea gradient gels or from circular dichroism (CD) unfolding data. Values for species proposed to be cis or trans at Ile 75–Pro 76 are given for oxidized (S_2) and for reduced ($(SH)_2$) forms. [b] Urea concentrations at the midpoint of the unfolding transition, $[U]_m$, were estimated from urea gradient gels as described under Materials and Methods. [c] Free energies, $\Delta G°$, in the absence of urea were estimated from urea gradient gels by the method of Hollecker and Creighton (1982) and from the CD unfolding data in Figure 3, as described in Pace (1986). [d] The value of $\Delta G°$ for trx-S_2 cis at 2 °C was estimated by assuming a transition curve similar to the trans curve (D), with a midpoint of 8 M urea.

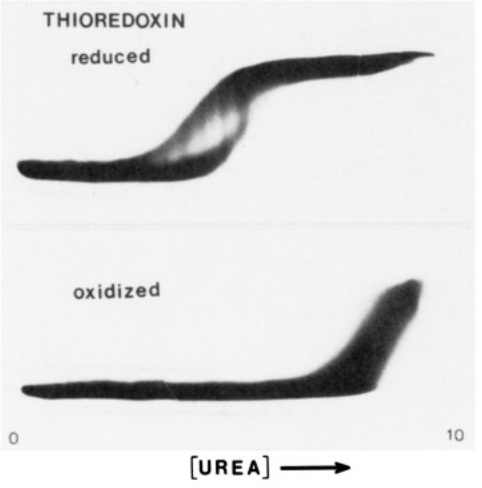

FIGURE 1: Urea gradient gels of oxidized and reduced thioredoxin at 25 °C. The gels are cast with a horizontal urea gradient, from 0 to 10 M urea, left to right. Protein was applied in a band across the top of the gel, and vertical electrophoresis is toward the anode. Thioredoxin was applied either after reduction with dithiothreitol (upper gel) or in the oxidized state (lower gel). The N ⇔ D transition produces an S-shaped band since the electrophoretic mobility of the protein decreases as the protein unfolds. Reduced thioredoxin shows two distinct transitions, at 4.4 and at 5.7 M urea. Oxidized thioredoxin shows only one transition at ~8 M urea.

tration. This indicates that N ⇔ D interconversion rates for both folded forms of reduced thioredoxin are faster than those for the oxidized protein at urea concentrations close to the unfolding midpoint and that the reduced form with lower stability interconverts more rapidly than the one with higher stability. Significantly, after electrophoresis at 38 °C, both oxidized and reduced thioredoxin gels show bands at about the same urea concentrations as those at 25 °C, but all three bands (two for reduced and one for oxidized) are sharpened and more continuous (data not shown). This is expected if the diffuse staining patterns at lower temperature are the result of slow interconversion of native and denatured forms.

The urea-induced N ⇔ D transition of proteins can be further probed by comparison of gels obtained at 2 versus 25 °C because the *cis*-proline–*trans*-proline isomerization is slow on the electrophoresis time scale at 2 °C but fast at 25 °C (Creighton, 1980). Further, comparison of banding patterns for gels loaded with unfolded versus folded protein may also distinguish between different models for the kinetics and thermodynamics of urea-induced unfolding (Creighton, 1980). Therefore, a standard set of four electrophoresis conditions was used for each protein. For each set, protein was applied to the gel in either native N or denaturing D buffer at both 2 and 25 °C.

Figure 2 shows the set of gels for oxidized and reduced thioredoxin. At 2 °C, but not at 25 °C, when denatured (D) oxidized thioredoxin is applied to the gel, an N ⇔ D transition at 4.3 M urea is observed in addition to the transition at ~8 M urea (Figure 2D). The ~8 M urea transition under the conditions of Figure 2D is routinely observed as a blurred band as in Figure 2A–C. It is much more faint since most of the material is in the band showing the 4.3 M urea transition. This behavior of oxidized thioredoxin was previously reported by Wilson et al. (1986). In contrast, at 2 °C as well as 25 °C, reduced thioredoxin shows the same bands, regardless of whether loaded as N or D. A significant difference among the reduced gels, however, is that for D (Figure 2F,H) the bands for the transitions at lower urea concentration are more intense than those for N (Figure 2E,G).

The banding patterns after electrophoresis at pH 7 and 2.5 were also determined (data not shown). Gels run at pH 7 are the same as those run at pH 8.7. At pH 2.5, both oxidized and reduced thioredoxins are greatly destabilized. Oxidized thioredoxin shows one S-shaped band at about 3.2 M urea at pH 2.5. Reduced thioredoxin runs as a single horizontal band with a mobility corresponding to the unfolded form. That is, at pH 2.5, reduced thioredoxin is acid denatured even in the absence of urea.

The concentrations of urea measured from the gels at the midpoints of denaturation are given in Table I. The values in Table I are averages from two to eight replicate gels. Approximate $\Delta G°$ values in the absence of urea may be estimated from the gels by assuming a simple linear extrapolation (Creighton, 1979; Hollecker & Creighton, 1982). These estimates are also given in Table I.

The fraction of denatured protein as a function of increasing urea concentraton may also be determined from the changes in circular dichroism spectra (Pace, 1986). Figure 3 shows

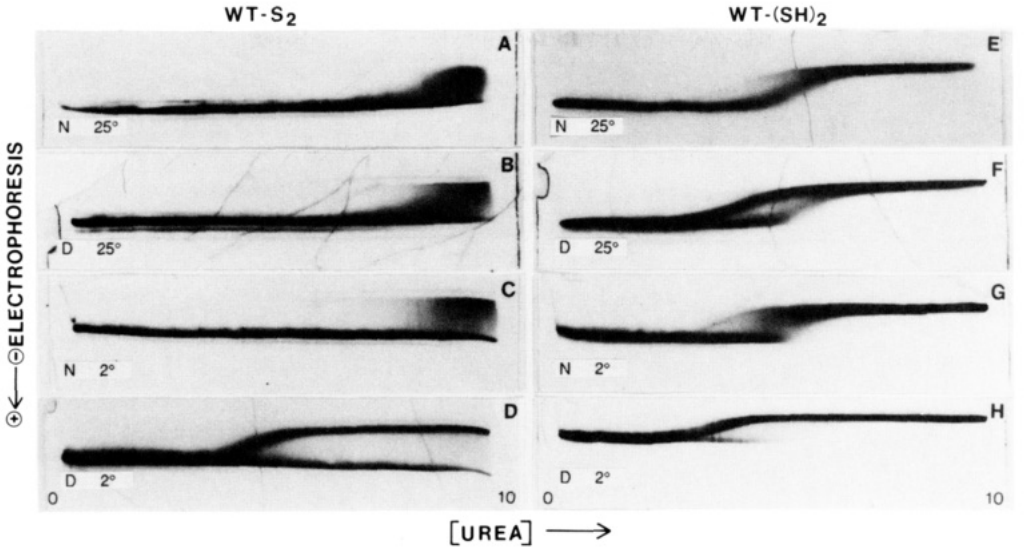

FIGURE 2: Urea gradient gels of wild-type thioredoxin. Electrophoresis was carried out at 25 or 2 °C with samples applied in the native (N) form or the denatured (D) form, as indicated for each gel. Thioredoxin was either oxidized (gels A–D) or reduced with DTT (gels E–H).

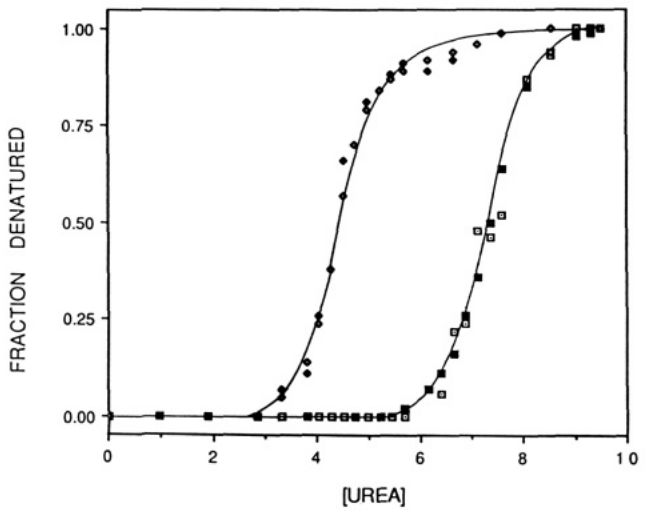

FIGURE 3: Urea denaturation of reduced (◇, ◆) and oxidized (□, ■) thioredoxin measured by circular dichroism. The open and filled symbols represent values obtained in duplicate experiments. The fraction denatured as a function of urea concentration (molar) was determined from the percent ellipticity change at 219 nm relative to the ellipticity difference between native and fully denatured thioredoxin.

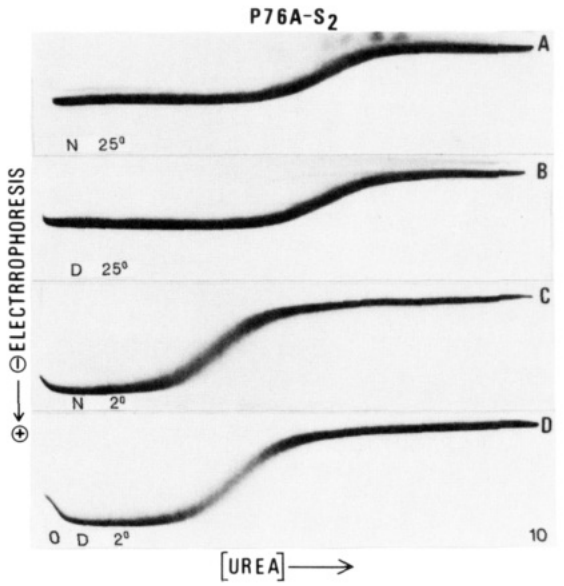

FIGURE 4: Urea gradient gels of oxidized thioredoxin mutant P76A. Electrophoresis was carried out at 25 or 2 °C with samples applied in the native (N) form or the denatured (D) form, as indicated for each gel.

the CD data for urea denaturation of oxidized and reduced thioredoxin. Values of $\Delta G°$ and urea concentrations at the transition midpoint obtained from the these data are given in Table I.

A thioredoxin variant having proline 76 replaced with alanine (P76A) was isolated from the gene *trx*A76 kindly supplied to us by R. Kelley and F. Richards (1987). The gene was cut and ligated into our expression system, and its sequence was confirmed. Urea gradient gels for oxidized P76A are shown in Figure 4. Further characterization of P76A is being carried out and will be reported later. Oxidized P76A is much less stable than the wild type, with urea midpoints of 3.5 M at 2 °C and 5.2 M at 25 °C. At both temperatures, there is a single band for N and D samples.

Russel and Model (1986) have generously supplied us with the *E. coli* strains they constructed carrying genes *trx*A3 and *trx*A7. These genes code for the production of thioredoxin with one or both cysteine residues replaced by serine. Since these cannot form the active site disulfide bridge, the variants may be considered analogues of reduced thioredoxin. Figure 5A–D shows the urea denaturation for thioredoxin with Cys 32 replaced by serine (C32S), leaving Cys 35 as a thiol (SH). Dithiothreitol was added to prevent intermolecular disulfide formation.[3] The 25 °C gel pattern of C32S is very similar to that of reduced wild-type thioredoxin (Figure 5A,B vs Figure 2E,F). Both have two bands representing two N ⇔ D transitions, and no difference is observed for N vs D, except for an intensification of the band at lower urea concentration for D. At 2 °C the situation is more complicated (but highly reproducible). When C32S is applied to the gel as N at 2 °C, two bands similar to wild-type thioredoxin-$(SH)_2$ are observed (Figures 5C and 2G). However, when C32S is applied as D at 2 °C (Figure 5D), three distinct bands are observed at zero urea (far left of the gel). Two of these show unfolding around 3 M urea. Their difference in mobility is retained in the denatured state; i.e., two distinct bands are observed at high

[3] When electrophoresis of C32S is carried out in the absence of DTT, double bands are seen at all urea concentrations, probably due to intermolecular disulfide formation.

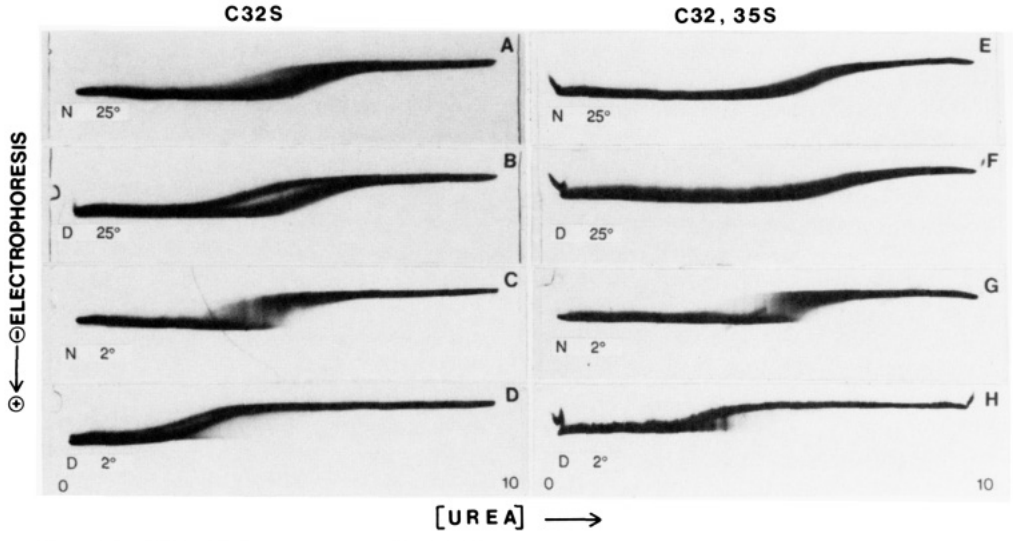

FIGURE 5: Urea gradient gels of thioredoxin mutant proteins C32S and C32,35S. C32S is thioredoxin with Cys 32 replaced by serine (gels A–D). C32,35S is thioredoxin with both Cys 32 and Cys 35 replaced by serine (gels E–H). Electrophoresis was carried out as described in the legend to Figure 4.

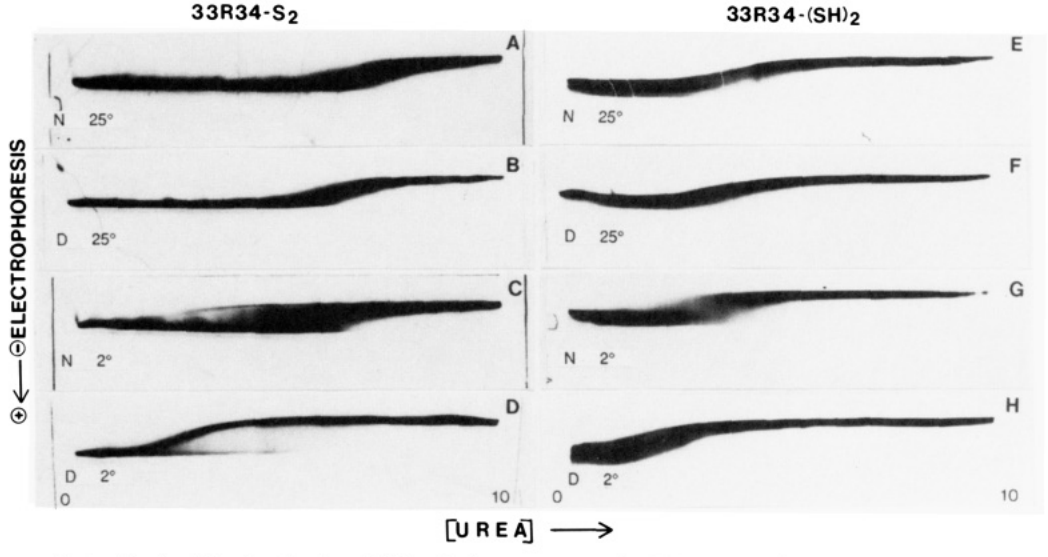

FIGURE 6: Urea gradient gels of oxidized and reduced thioredoxin mutant protein 33R34, thioredoxin with arginine inserted between active site residues 33 and 34. Protein was either oxidized (gels A–D) or reduced with DTT (gels E–H). Electrophoresis was carried out as described in the legend to Figure 2.

urea concentrations (far right of the gel). The third (middle) band at low urea concentrations extends as a spur until about 4 M urea and then unfolds to a form with mobility identical with that of one of the two bands observed a high urea.

The urea denaturation for thioredoxin with both Cys 32 and Cys 35 replaced by serine (C32,35S) is shown in Figure 5E–H. C32,35S behaves differently from C32S. At 25 °C, C32,35S N and D give identical single bands with a midpoint at 5.9 M urea (Figure 5E,F). However, at 2 °C, C32,35S N and D gels show two transitions, with midpoints at 3.8 and 5.4 M urea (Figure 5G,H). Significantly, the lower stability band is far more intense for D compared to N.

The genetically engineered thioredoxin variant having an arginine inserted between Gly 33 and Pro 34 in the 14-member ring of the active site, designated 33R34, was also examined. The enzymatic activity of 33R34 is approximately 5% that of wild type in the DTNB reduction assay (C.-J. Lim, F. Gleason, and J. Fuchs, unpublished results). The Arg insertion reduces the stability of both the oxidized and reduced 33R34, compared to that of the wild-type thioredoxin. At 25 °C, oxidized 33R34 gives a single, diffuse band with a midpoint of 6.2 M urea (Figure 6A,B). However, at 2 °C, oxidized 33R34 clearly shows a second, lower stability band, with a midpoint of 2.6 M urea, for both N and D (Figure 6C,D). The band for the lower stability transition is more intense for D (Figure 6D). Reduced 33R34 has multiple bands at low urea; these show unfolding transitions around 2–4 M urea to give multiple unfolded forms (Figure 6E–H).

Discussion

Wild-Type and P76A Thioredoxin. Urea gradient gels are diagnostic for several aspects of urea-induced cooperative unfolding transitions. The existence of multiple species of different stability can be distinguished as individual S-shaped bands. Further, involvement of *cis*-proline–*trans*-proline isomerization in protein folding can be inferred by comparison of gels run at both 2 and 25 °C for samples applied under both native (N) and denaturing (D) conditions (Creighton, 1980). These conditions test for proline isomerization for two reasons. At 25 °C cis–trans interconversion is rapid on the electrophoresis time scale for small proline-containing peptides, which are considered models for D. However, proline isomerization

in model peptides is slow at 2 °C on the electrophoresis time scale; therefore, gels run at 2 °C can resolve cis and trans isomers with different stabilities in urea. Also, protein applied as N is essentially all in the isomeric configuration found in the native protein, but protein equilibrated under D conditions is expected to have a ratio of approximately 30:70 cis:trans at proline-containing peptide bonds. For this reason, the isomeric ratio of proline peptide bonds can be varied by equilibrating the protein as N or D before electrophoresis.

From Figures 1 and 2, several properties of the thioredoxin urea-induced transition can be deduced. First, there are two compact species of native reduced thioredoxin that have indistinguishable molecular volumes but a marked diffrence in stability. These are resolved in urea gradient gels as two sigmoidal bands reflecting two cooperative unfolding transitions at different concentrations of urea (Figures 1 and 2E–H). Both bands merge at urea concentrations below and above the transitions, indicating identical or very similar molecular shapes for both folded forms and for both unfolded forms. Second, oxidized thioredoxin is far more stable than reduced thioredoxin, and native oxidized thioredoxin unfolds at ~8 M urea (Figures 1 and 2A–C). However, a second, lower stability form of oxidized thioredoxin is detected at 2 °C when the protein is denatured prior to electrophoresis (Figure 2D). This observation was previously reported by Wilson et al. (1986). Third, a *cis*-proline–*trans*-proline isomerization is implicated as the conformational process giving two forms of different stability. This follows from the differences observed between N and D samples for oxidized thioredoxin at 2 °C (Figure 2C,D) and for reduced thioredoxin at 2 and 25 °C. For oxidized thioredoxin, the less stable form is observed only when its isomer is kinetically trapped in the D sample at 2 °C because its interconversion with the other isomer is slow at this temperature. For reduced thioredoxin at both 2 and 25 °C, the band representing the transition at lower urea concentrations (left band) is obviously darker than the band for the transition at higher urea (right band) (compare gels E–F and G–H of Figure 2).

The appearance of the lower stability isomer in D for oxidized protein at 2 °C and for reduced protein at both temperatures implies that the predominate configurational isomer in the unfolded protein is different from the one in the folded protein. In D, the predominate proline isomer is trans, and its interconversion with cis is slow at 2 °C. If the difference in stability is due to a proline isomerization, it follows that the major species in N is cis. Oxidized thioredoxin contains a *cis*-proline peptide bond, at position 76 (Eklund, 1984). Pro 76 is highly conserved, and its side chain has extensive contacts with the active site disulfide.[2] Therefore isomerization of the Ile 75–Pro 76 peptide bond is the prime candidate for the temperature-dependent process underlying the urea gel results in Figure 2. This implies that the cis isomers of both oxidized and reduced thioredoxin are more stable than their respective trans isomers.

This interpretation is shown schematically in Figure 7. Folded thioredoxin exists in two forms of different stability in urea. In this model, the more stable folded form, F_C, is in the cis configuration about the proline 76 peptide bond. The less stable folded form, F_T, is trans at the same bond. F_C and F_T denature to their related unfolded form, U_C and U_T, which also interconvert. In Figure 7 the proposed species are superimposed on diagrams of their corresponding urea gradient gel patterns. Native reduced thioredoxin is a mixture of both cis and trans and gives two S-shaped bands (Figure 7A). Native oxidized thioredoxin is essentially all cis and shows only

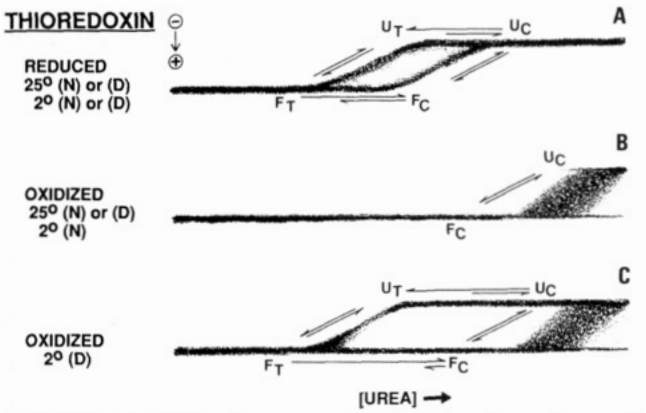

FIGURE 7: Model for the urea gradient gel banding patterns of thioredoxin. The diagram represents our interpretation of the electrophoretic behavior of oxidized and reduced thioredoxin. The diagram shows the species responsible for the gel patterns when the protein is applied to the gel in native (N) or denaturing (D) buffer, at both 2 and 25 °C. The two folded isomers of thioredoxin which show different stabilities to urea denaturation are proposed to correspond to cis and trans isomers of Pro 76 (F_C and F_T in the diagram). As the urea concentration is increased from left to right, F_C and F_T unfold to give their respective unfolded isomers (U_C and U_T). Diagrams A, B, and C represent our interpretation of the gels in Figure 2E–H, Figure 2A–C, and Figure 2D, respectively. See text for further discussion.

one diffuse band (Figure 7B). Unfolded, oxidized thioredoxin is predominantly trans, U_T, and a lower stability band, along with a diffuse, higher stability band is observed at 2 °C (Figure 7C).

Native oxidized thioredoxin is essentially all cis because oxidized F_C is far more stable than oxidized F_T and any oxidized thioredoxin in the F_T configuration immediately converts to F_C at 25 °C. In the D form, oxidized U_C equilibrates with oxidized U_T. At 2 °C the $U_C \Leftrightarrow U_T$ interconversion is slow. Therefore, we observe the second, lower stability band, which represents the refolding transition of U_T to F_T, the less stable trans folded form of oxidized thioredoxin. This explains why the transition $F_T \Leftrightarrow U_T$ is observed only for the D sample of oxidized protein at 2 °C (Figure 2D).

For reduced thioredoxin, F_C and F_T have more similar, but clearly distinguishable, stabilities in urea. The $F_T \Leftrightarrow U_T$ transition is visualized as the left band at lower urea concentration on the gels. Similarly, U_C represents the unfolded form in the cis configuration at Pro 76 that interconverts with the higher stability folded form, F_C. Reduced F_C and F_T interconvert slowly on the electrophoresis time scale at both 2 and at 25 °C, which is why both bands are observed at both temperatures (Figure 2E–H). U_C and U_T interconvert slowly compared to either $F \Leftrightarrow U$ reaction, and U_T is favored. This explains the difference in banding patterns between reduced thioredoxin applied under native (N) and denaturing (D) conditions, i.e., the enhancement of the $F_T \Leftrightarrow U_T$ band in D.

While urea gel bands are not reduced to numerical parameters as precisely as experiments where the fraction of denatured protein is determined from average spectroscopic properties, urea gradient gels do have some advantages. The gels monitor individual denaturation transitions of interconverting folded species which have no distinguishable spectroscopic or hydrodynamic differences, provided the interconversions occur on the time scale of minutes or longer. Under the conditions used here, the gels test for the involvement of cis–trans isomerization in folding, as discussed. Urea concentrations at the midpoint of the transition obtained from the gels are reproducible. These are given in Table I. It is interesting to note that the urea midpoint concentrations are

consistently lower at 2 °C, indicating that all forms of the protein are less stable to urea at lower temperatures. Rough estimates of the free energies of stabilization in the absence of urea can also be made for each species by extrapolation of the gel bands, as described by Hollecker and Creighton (1982), but these should be regarded as much less reliable than the estimates of urea denaturation midpoint concentrations. Approximations of $\Delta G°$ at 2 °C, pH 8.7, are ~6 and ~4.5 kcal/mol for the cis and trans isomers of reduced thioredoxin and ~9.5 and ~6.5 kcal/mol for cis and trans isomers of oxidized thioredoxin, respectively (Table I).

The CD data at 25 °C and pH 8.7 for oxidized thioredoxin (Figure 3) indicate simple two-state behavior. The urea midpoint concentration of 7.4 M and the $\Delta G°$ of ~10 kcal/mol compare reasonably well with values obtained from the urea gels of ~8 M urea and ~9.5 kcal/mol (Table I). Kelley et al. (1987) have examined urea and guanidine hydrochloride unfolding of oxidized thioredoxin at 25 °C and pH 7 and reported a $\Delta G°$ of 8.7 kcal/mol and a urea midpoint concentration of 6.7 M. For reduced thioredoxin, the curve is intermediate between two transitions observed on the gradient gels, as expected for the equilibrium CD measurement of two forms that are interconverting on the minute time scale.

Urea gradient gels of oxidized P76A, the mutant thioredoxin with Pro 76 replaced by alanine, support the interpretation illustrated in Figure 7. The P6A gels are shown in Figure 4. Substitution of proline at position 76 eliminates the electrophoretic behavior that is diagnostic for involvement of *cis*-proline–*trans*-proline isomerization, namely, a difference between N and D samples at 2 °C, dramatically illustrated in Figure 2C,D. Thioredoxin variants with amino acid alterations around the active site, C32S, C32,35S, and 33R34, all exhibit variations of this behavior (Figures 5 and 6). For oxidized P76A, there is a single S-shaped band under all conditions (Figure 4).

The observation of a single band for P76A representing the unfolding transition with a urea midpoint around 4 M strongly suggests that this thioredoxin variant is primarily trans at position 75–76, in agreement with Kelley and Richards (1987), who proposed a trans configuration for P76A from a different line of argument. A second striking feature of the P76A gels is that the protein is significantly less stable to urea denaturation at the lower temperature. As mentioned above, a similar trend is observed for all the other thioredoxins studied here, though the magnitude of the change in urea midpoint concentrations is not as great. This suggests the very interesting possibility that thioredoxin has a cold denaturation and that the temperature of maximum stability is significantly higher for P76A than for the other variants, including wild type.

In summary, the existence of two folded thioredoxin species, which differ by being either cis or trans at peptide bond 75–76, is the most reasonable working model to explain the two compact species of differing stability observed in urea gradient gels. The transition at higher urea concentration represents denaturation of a folded cis isomer, and the transition at lower urea concentration represents denaturation of a folded trans isomer. These urea gel experiments are specifically designed to monitor involvement of *cis*-proline–*trans*-proline isomerization in urea denaturation (Creighton, 1980), and wild type thioredoxin consistently demonstrates the characteristic banding pattern for a proline isomerization, namely, a more intense band for the lower stability transition when applied in the D form at 2 °C. Further, P76A thioredoxin loses this distinctive electrophoretic feature in this gel system, indicating that Pro 76 is the specific proline involved.

The ratio of cis to trans isomers in reduced thioredoxin in the absence of urea cannot be calculated with any certainty from the present data. Very rough estimates of the isomeric ratios may be computed from the $\Delta G°$ values in Table I, which are approximations derived from linear extrapolations of the S-shaped gel bands to zero urea concentrations (Hollecker & Creighton, 1982). From gels with the sample applied as N at 25 °C, $\Delta G°$ values for the cis and trans isomers are 6.5 and 4.5 kcal/mol (Table I). Equivalent values from gels with the sample applied as D are 5.5 and 4 kcal/mol. Assuming that the ratio of cis to trans isomers in unfolded thioredoxin is 30:70 and independent of urea concentration, this gives an approximate range of 8–18% of the protein predicted to be in the trans form in the absence of urea. Dyson et al. (1988) have reported the NH–C$^{\alpha}$H cross peaks of COSY ^{1}H NMR spectra of oxidized and reduced thioredoxin. They have not observed any evidence for a trans configuration at 75–76 which should be detectable in as little as 10% abundance (Jane Dyson and Peter Wright, personal communication). Thus the percentage of trans form in reduced thioredoxin without urea is very small, probably less than a few percent. It is possible that the trans reduced form is significantly populated only in the presence of urea, i.e., that the putative cis–trans equilibrium for reduced thioredoxin is shifted toward trans by the addition of low concentrations of urea.

Our interpretation is compatible with the denaturation studies of Stellwagen, Kelley, and associates on oxidized thioredoxin in guanidine hydrochloride (Kelley & Stellwagen, 1984; Kelley et al., 1986; Shalongo et al., 1987) and in urea (Wilson et al., 1986) and on reduced thioredoxin in guanidine hydrochloride (Kelley et al., 1987). Our scheme shown in Figure 7 is basically equivalent to similar schemes proposed by Kelley et al. (1987) and by Kelley & Richards (1987) except that the folded trans forms (F_T) are not necessarily intermediates, as explained above. In the denaturation studies of Stellwagen, Kelley, and associates, unfolding occurs with a single kinetic phase, while refolding shows three kinetic phases. Hydrodynamic measurements show the transient accumulation of compact forms having a stability less than that of the native protein and give no indication of transient conformations with effective volumes intermediate between those of the native and denatured species. Urea gradient gels identical with those in Figure 2C,D were observed by Wilson et al. (1986). These authors suggest a compact folding intermediate, of lower stability to urea denaturation and containing at least one nonnative peptide isomer. They propose the trans isomer of proline 76 as a likely candidate. Equilibrium measurements of reduced thioredoxin denaturation by guanidine hydrochloride (Kelley et al., 1987) and of oxidized thioredoxin by urea (Wilson et al., 1986) show simple two-state behavior.

The structural basis for the difference in stability between the putative cis and trans isomers of Ile 75–Pro 76 in either oxidized or reduced thioredoxin can only be a speculation at this point. The trans configuration destabilizes the folded state and/or stabilizes the urea-denatured state, as compared to cis. In the crystal structure[2] of oxidized thioredoxin, which contains the cis configuration, Ile 75 and Pro 76 side chains are almost all buried, and both pack against one side of the disulfide group. Isomerization to trans should rotate the side chain of Ile 75 into the solvent. It seems likely that this would primarily affect the folded state, destabilizing it by moving hydrophobic groups into water. But this does not offer an answer to the closely related question of why oxidized thioredoxin is so much more stable than reduced. (Rephrased in terms of our model,

this question becomes why is the cis oxidized form so much more stable than either trans oxidized, cis reduced, or trans reduced.) This is not likely to be due primarily to unfavorable entropic effects of the S–S bond on the denatured state, because the difference in stability between trans oxidized and trans reduced is relatively small. It therefore seems more likely that the unusual stability of cis oxidized thioredoxin arises from the stabilization of the folded state, possibly by the formation of a hydrophobic cluster around the S–S group.

The high degree of conservation of proline at position 76 suggests that it may be involved in the enzymatic function of thioredoxin. It has been proposed that the cis configuration at Pro 76 is required for binding of ligands such as thioredoxin reductase, since several of its side-chain atoms are part of a hydrophobic surface patch close to the S–S bridge (Eklund et al., 1984). In addition, the backbone atoms and the Cδ and Cβ atoms of Pro 76 are packed around the sulfur of Cys 35 on the buried side of the S–S. An intriguing possibility is that linkage of the disulfide/sulfhydryl equilibrium to Pro 76 cis–trans isomerization may be part of the enzymatic mechanism, which involves an oxidation/reduction proton transfer at the active site S–S bridge. In this regard it is interesting to note that *cis*-proline–*trans*-proline isomerization is acid catalyzed (Dunker, 1982) and that the highly conserved acidic side chain of Asp 26 is only slightly exposed to solvent and is located at the bottom of a deep cleft on the buried (Cys 35) side of the S–S bridge near Pro 76.[2]

C32S, C32,35S, and 33R34 Thioredoxins. Urea gradient gels of the other three thioredoxin variants studied may similarly be interpreted. When Cys 32 is replaced by serine, both F_T and F_C species are present and slowly interconverting at 25 and 2 °C (Figure 5A–C). This is the same as observed for reduced wild type (Figure 2E–H). However, there is an additional interesting feature. When C32S is first unfolded and then refolded at 2 °C, three distinct electrophoretic species are observed (Figure 5D). Two species unfold at similar, but not identical, urea concentrations. Their lower stability suggests that they are in the F_T form. The third has a higher stability to urea denaturation, as expected for the F_C form.

When Cys 32 and Cys 35 are both replaced by serine, one band is observed at 25 °C for N and D (Figure 5E,F). At 2 °C, there are two bands, corresponding to F_T and F_C (Figure 5G,H). The increased intensity of the lower stability band for D at 2 °C (Figure 5H) supports this interpretation. The appearance of one thick band on the 25 °C gels reflects a rapid interconversion of F_T and F_C, while at 2 °C these interconvert slowly on the electrophoresis time scale.

The behavior of the oxidized variant having an arginine insertion is similar (Figure 6A–D). Oxidized 33R34 shows only the $F_C \Leftrightarrow U_C$ transition at 25 °C for both N and D. Broadened bands of high stability are observed at 25 °C (Figure 6A,B). A lower stability band corresponding to F_T is resolved at 2 °C for both N and D (Figure 6C,D). The increased intensity of the lower stability band for the D sample supports its assignment to F_T (Figure 6D). Reduced 33R34 is far less stable than its oxidized counterpart. Further, multiple bands are observed under all conditions (Figure 6E–H). Attempts are being made to separate and characterize the multiple species of this variant and of C32S. Observation of multiple-folded electrophoretic species for this and other thioredoxin variants suggests that there could be complications in the interpretation of urea denaturation data obtained with techniques that measure average spectroscopic properties.

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Structural Domains and Conformational Changes in Nuclear Chromatin: A Quantitative Thermodynamic Approach by Differential Scanning Calorimetry†

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ABSTRACT: A good deal of information on the thermodynamic properties of chromatin was derived in the last few years from optical melting experiments. The structural domains of the polynucleosomal chain, the linker, and the core particle denature as independent units. The differential scanning calorimetry profile of isolated chromatin is made up of three endotherms, at ~74, 90, and 107 °C, having an almost Gaussian shape. Previous work on this matter, however, was mainly concerned with the dependence of the transition enthalpy on external parameters, such as the ionic strength, or with the melting of nuclei from different sources. In this paper we report the structural assignment of the transitions of rat liver nuclei, observed at 58, 66, 75, 92, and 107 °C. They are representative of the quiescent state of the cell. The strategy adopted in this work builds on the method developed for the investigation of complex biological macromolecules. The heat absorption profile of the nucleus was related to the denaturation of isolated nuclear components; electron microscopy and electrophoretic techniques were used for their morphological and molecular characterization. The digestion of chromatin by endogenous nuclease mimics perfectly the decondensation of the higher order structure and represented the source of several misinterpretations. This point was carefully examined in order to define unambiguously the thermal profile of native nuclei. The low-temperature transitions, centered around 58 and 66 °C, arise from the melting of scaffolding structures and of the proteins associated with heterogeneous nuclear RNA. The conformational change of the linker domain occurs at 75 °C. Finally, the endotherms at 92 and 107 °C reflect the denaturation of the core particle placed within an expanded loop and the 30-nm fiber, respectively. The stability of chromatin higher order structure appears to be enthalpy dependent, because a large decrease in the denaturation heat is associated with unfolding. Starting from these assignments, we gained an insight into the structural changes underlying the progress of the cell along the cycle. The 107 °C endotherm is dominant in both G_0 and S phase, but actively dividing hepatocytes are characterized by a reproducible broadening of the thermal profile at 100 °C, related to the regional unwinding of the 30-nm fiber occurring during transcription and DNA replication.

A few years ago (Nicolini et al., 1983) we embarked upon an investigation on the denaturation enthalpy of both calf thymus chromatin and rat liver nuclei. Our experiments revealed several intriguing features of the melting behavior of these materials. In spite of an extremely heterogeneous composition, the denaturation process resulted in strikingly simple DSC[1] profiles. Three major endotherms, having an almost Gaussian shape, were observed at 74, 90, and 107 °C for both chromatin and nuclei. In the latter case, an additional broad peak at ~58 °C was present. Moreover for isolated chromatin, the heat absorbed at 90 and 107 °C was found to increase strongly with the concentration of monovalent salt between 10 and 50 mM, where the transition from the 10-nm fiber to the helical structure occurs (Thoma et al., 1979). We drew the conclusion that DSC methods could be successfully applied in order to investigate the condensation process of

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[1] Abbreviations: DSC, differential scanning calorimetry; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BN, nuclei prepared in batch; FN, nuclei isolated in flow; NM, nuclear matrix; Tris, tris(hydroxymethyl)aminomethane; hnRNA, heterogeneous nuclear RNA; SDS, sodium dodecyl sulfate; IF, intermediate filaments; kbp, kilobase pair(s); kDa, kilodalton(s); RNase, ribonuclease.

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