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Guanidine hydrochloride stabilization of a partially unfolded intermediate during the reversible denaturation of protein disulfide isomerase

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ABSTRACT **The reversible denaturation of protein disulfide isomerase proceeds through intermediates that are stabilized by interaction with guanidine hydrochloride. At pH 7.5, the equilibrium denaturation by urea is completely reversible and the transition can be reasonably well-described by a two-state model involving only native and denatured forms. In comparison, the equilibrium denaturation by guanidine hydrochloride occurs in two distinct steps. In the presence of a low constant amount of guanidine hydrochloride (0.5–1.4 M), urea denaturation also becomes biphasic, suggesting the accumulation of an intermediate species that is stabilized by specific interaction with guanidine hydrochloride but not by high concentrations of other salts or other denaturants.**

Protein disulfide isomerase (PDI; EC 5.3.4.1) is a multifunctional protein (M_r = 57,000) that is located in the lumen of the endoplasmic reticulum where it is thought to catalyze thiol–disulfide exchange reactions that are essential for the post-translational formation of disulfide bonds in newly synthesized proteins (1–6). The primary sequence of PDI shows two internally homologous domains (7) that contain the two active site regions of each monomer. One domain is located near the N terminus and the other is near the C terminus. The two domains are ≈30% identical to *Escherichia coli* thioredoxin, a redox-active dithiol/disulfide-containing protein. Each thioredoxin-like domain contains a dithiol/disulfide center (WCGHCK) that comprises the two independent active sites (8).

PDI accelerates the renaturation of disulfide-containing proteins; therefore, the enzyme could find application in the renaturation of disulfide-containing proteins produced as insoluble misfolded inclusion bodies in bacterial expression systems (9). Since many refolding strategies employ denaturants such as urea or guanidine hydrochloride (Gdn·HCl), we were initially interested in evaluating the stability of PDI toward these denaturants. During the course of these studies, we noticed an unusual situation in which the transition between native and unfolded states appeared to be a simple two-state process in urea but involved a stable partially unfolded intermediate state in Gdn·HCl. For many proteins, denaturation is a cooperative two-state process (10, 11); however, deviation from a simple two-state transition is observed when stable intermediates occur on the folding/unfolding pathway (12). By fluorescence and CD spectroscopy, we have detected a partially folded intermediate during the reversible denaturation of PDI that is specifically stabilized by relatively low concentrations of Gdn·HCl.

MATERIALS AND METHODS

Materials. Glutathione, insulin (bovine pancreas), and glutathione reductase (yeast type III) were purchased from Sigma. Dithiothreitol (DTT) was purchased from Boehringer Mannheim. Gdn·HCl was sequanal grade from Pierce. Urea (ultra pure) was from ICN. Urea solutions were prepared immediately before use. Glass-distilled deionized water was used for all experiments.

PDI was prepared from fresh bovine liver by the method of Lambert and Freedman (13). The purity of the enzyme was >95% as judged by polyacrylamide gel electrophoresis. The enzyme (1.5–2 mg/ml) was stored at −20°C in 20 mM sodium phosphate (pH 6.3). HPLC on a DEAE 5WP (Waters) anion-exchange column (eluted with a linear gradient of 0–0.5 M NaCl over 30 min) or gel filtration on a Bio-Sil SEC250 (Bio-Rad) column revealed two major PDI species in a 1:0.7 ratio. Both peaks had PDI activity, both proteins migrated as a single 57-kDa band during SDS/PAGE under reducing and nonreducing conditions, and the N-terminal 10 residues of both species were identical to the sequence of PDI. Two forms of PDI that are resolved by gel-filtration HPLC have been reported previously and attributed to proteolysis near the C terminus (14); however, the suggested C terminus of one of the two peaks could not be found in the deduced cDNA sequence of PDI. The two forms of PDI appear to represent monomeric and dimeric species in which a metastable dimer without intermolecular disulfides is induced by freezing in phosphate buffer (M. Kruzel and H.F.G., unpublished observations). Overnight incubation of the preparation at pH 7.5 and 22°C results in essentially complete (>90%) conversion of the dimer to the monomer; under the conditions of our experiments, the PDI is monomeric. In addition, Gdn·HCl denaturation profiles for the two forms of PDI isolated from HPLC are identical to each other and identical to those of the mixture.

Methods. PDI activity, measured by the glutathione-dependent reduction of insulin, was determined as described by Morjana and Gilbert (15). Fluorescence measurements were performed on SLM Aminco 8000 (Urbana, IL) and Aminco–Bowman (Urbana, IL) spectrofluorometers with the cell compartments maintained at 23°C. The fluorescence emission spectrum (excitation at 280 nm) of PDI is red-shifted from 340 to 352 nm upon denaturation with either urea or Gdn·HCl. The maximum difference between the fluorescence of native and denatured PDI was obtained at an emission wavelength of 370 nm (excitation at 280 nm). CD spectra were recorded at 23°C with a Jasco (Easton, MD) J-500 A spectropolarimeter calibrated with a 0.1% *d*-10-camphosulfonic acid solution.

Denaturation/Renaturation Experiments. Denaturation was induced by incubation of PDI (2.1–7.4 μM) with various concentrations of Gdn·HCl or urea for 24 h at room temperature in 0.2 M potassium phosphate, pH 7.5/5 mM EDTA. For experiments with reduced PDI, 2 mM DTT was included. Renaturation was performed using PDI that had been dena-

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Abbreviations: PDI, protein disulfide isomerase; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride.

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tured by a 24-h incubation with 6 M Gdn·HCl or 8 M urea. The denatured PDI was diluted 1:20 into the appropriate concentration of denaturant, and the mixtures were incubated at room temperature for another 24 h.

Data Analysis. The variation in fluorescence intensity or θ_{222} with urea concentration was analyzed by a simple two-state model. At a given concentration of denaturant [D], the free energy for conversion of the native (N) to the unfolded (U) state, at any given denaturant concentration [D] was assumed to vary according to the empirical relationship (16):

$$\Delta G = \Delta G_0 - m[\mathrm{D}], \qquad [1]$$

where ΔG_0 is the free energy for converting the native to the unfolded state extrapolated to zero denaturant and m is an empirical constant corresponding to the slope of a plot of ΔG against [D]. At any denaturant concentration, the observed signal intensity (fluorescence or CD), S_{obs}, is given by

$$S_{obs} = S_N f_N + S_U f_U, \qquad [2]$$

where S_N and S_U represent the signal intensities of the native and unfolded protein, and f_N and f_U represent the fraction of the protein present in the native and unfolded states at any concentration of denaturant [D] (17). Since $f_N + f_U = 1$ and $\Delta G = -RT \ln(f_U/f_N)$,

$$S_{obs} = \frac{S_N + S_U e^{-(\Delta G_0 - m[\mathrm{D}])/RT}}{1 + e^{-(\Delta G_0 - m[\mathrm{D}])/RT}}, \qquad [3]$$

where R is the gas constant and T is the absolute temperature. The denaturation profiles of PDI in urea monitored by fluorescence or CD were fit directly to Eq. 3 by a nonlinear least squares routine using the Marquart algorithm (18). Additional terms were also included to account for the small linear effects of the denaturant on the intrinsic signal intensity of the native and unfolded protein (17, 19) but these had no significant effect on the values of ΔG_0 or m.

Denaturation of PDI by Gdn·HCl or by urea in the presence of Gdn·HCl was analyzed by a three-state model in which the accumulation of an intermediate state (I) is significant (Eq. 4).

$$N \overset{K_{N\to I}}{\rightleftharpoons} I \overset{K_{I\to U}}{\rightleftharpoons} U \qquad [4]$$

The signal intensity (fluorescence or CD) observed at any denaturant concentration is given by

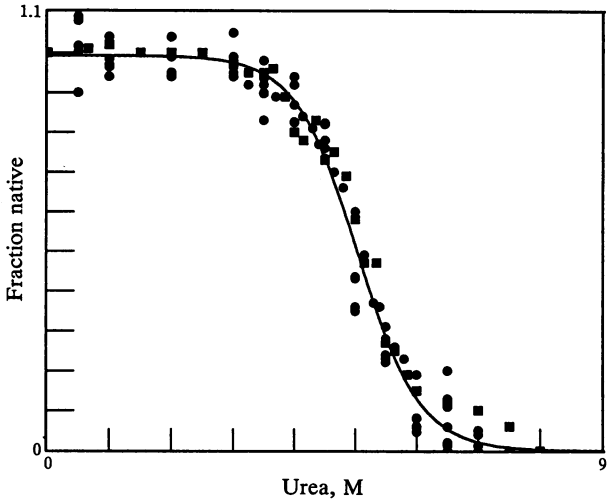

FIG. 1. Denaturation–renaturation transitions of PDI induced by urea under equilibrium conditions at 23°C in 0.2 M potassium phosphate, pH 7.5/5 mM EDTA/2 mM DTT. The final concentration of protein was 2 μM. Measurements were carried out after 24 h of incubation at room temperature with various concentrations of urea. ●, Unfolding data measured by fluorescence; ■, refolding data measured by CD at 222 nm. The solid curve is drawn according to Eq. 3 by using the values shown in Table 1.

$$S_{obs} = \frac{S_N + S_I \exp\{-(\Delta G_{N\to I} - m_{N\to I}[\mathrm{D}])/RT\} + S_U \exp\{-(\Delta G_{N\to I} - m_{N\to I}[\mathrm{D}])/RT\}\exp\{-(\Delta G_{I\to U} - m_{I\to U}[\mathrm{D}])/RT\}}{1 + \exp\{-(\Delta G_{N\to I} - m_{N\to I}[\mathrm{D}])/RT\} + \exp\{-(\Delta G_{N\to I} - m_{N\to I}[\mathrm{D}])/RT\}\exp\{-(\Delta G_{I\to U} - m_{I\to U}[D]/RT\}}, \qquad [5]$$

where S_N, S_I, and S_U represent, respectively, the intrinsic signal intensities of the native, intermediate, and unfolded states. $\Delta G_{N\to I}$ and $\Delta G_{I\to U}$ are the free energies for the N → I and I → U conversions, respectively, extrapolated to [D] = 0, and $m_{N\to I}$ and $m_{I\to U}$ are the m values for the same conversions. Data were fit directly to Eq. 5 by nonlinear least squares. The data were also analyzed by a model that allows for a linear change in the signal due to the fully folded and unfolded states with Gdn·HCl concentration. The $\Delta G_{N\to I}$ and $m_{N\to I}$ values were not significantly affected by this procedure; however, the $\Delta G_{I\to U}$ and $m_{I\to U}$ were altered by up to 50% since the linear regions after the second transition are short and not well-defined. The values reported are the results of analyses in which the change after the second transition was assumed to be independent of the Gdn·HCl concentration.

RESULTS

Urea Denaturation of PDI. With urea denaturation, PDI exhibits a single reversible unfolding transition when monitored by fluorescence or by CD (Fig. 1). The concentration of urea required to half-denature the enzyme is 4.8 M. The free energy of unfolding extrapolated to zero urea (ΔG_0) and the m value are shown in Table 1. In 8 M urea, the residue ellipticity is -2500 ± 400 deg·cm^2·dmol^{-1} compared to $-10{,}700 \pm 1300$ deg·cm^2·dmol^{-1} for the native enzyme. The

Table 1. Equilibrium denaturation of PDI by urea and Gdn·HCl as followed by fluorescence or CD

	S_I	$m_{N\to I}$, kcal/liter	$\Delta G_{N\to I}$, kcal/mol	$m_{I\to U}$, kcal/liter	$\Delta G_{I\to U}$, kcal/mol
Urea (fluorescence and CD)	—	1.2 ± 0.04	5.8 ± 0.3	—	—
Gdn·HCl					
Fluorescence	0.39 ± 0.04	2.7 ± 0.2	5.4 ± 0.3	1.6 ± 0.2	7.6 ± 0.9
CD	0.36 ± 0.03	3.1 ± 0.3	5.2 ± 0.5	1.1 ± 0.16	4.0 ± 0.7

S_I is the fraction of the total change in signal intensity remaining in the intermediate. The subscripts for m and ΔG refer to the m and ΔG values for the conversion of the native protein (N) to the intermediate (I) and the intermediate to the unfolded protein (U) as given in Eq. 5.

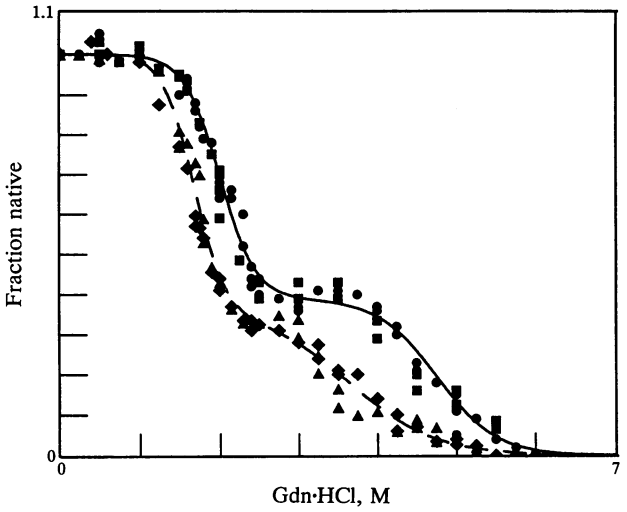

FIG. 2. Denaturation–renaturation transitions of PDI induced by Gdn·HCl under equilibrium conditions at 23°C in 0.2 mM potassium phosphate, pH 7.5/5 mM EDTA/2 mM DTT. The final concentration of protein was 2 μM. Measurements were carried out after 24 h of incubation at room temperature with various concentrations of Gdn·HCl by using fluorescence (solid line) and CD at 222 nm (dashed line). Unfolding (●) and refolding (■) data obtained using fluorescence and unfolding (▲) and refolding (◆) data obtained using CD are as indicated. Data were fit to the three-state model of Eq. 5 and are plotted as the fraction of PDI present in the native state. The curves are drawn using the values in Table 1.

presence of the reducing agent DTT (2 mM) has no significant effect on the denaturation/renaturation process (data not shown), suggesting that none of the disulfide bonds of PDI contribute significantly to the stability of the protein. The enzymatic activity is completely recovered when PDI, denatured in 8 M urea, is dialyzed against the same buffer or diluted to a urea concentration of 0.6 M.

Gdn·HCl Denaturation of PDI. In contrast to denaturation in urea, the denaturation and renaturation of PDI in Gdn·HCl exhibits multiple phases when monitored by fluorescence or by CD (Fig. 2). The Gdn·HCl denaturation data were fit to a three-state model (Eq. 5), and the values of the free energies of unfolding extrapolated to zero denaturant ($\Delta G_{N\rightarrow I}$ and $\Delta G_{I\rightarrow U}$) and the corresponding m values for the two steps are given in Table 1. The intermediate state is characterized by a θ_{222} of -4800 ± 500 deg·cm^2·dmol^{-1} compared to $-10{,}700 \pm 1300$ deg·cm^2·dmol^{-1} for native PDI and a fluorescence intensity that is ≈60% of the way between native and denatured enzyme. The $\Delta G_{N\rightarrow I}$ and $m_{N\rightarrow I}$ values are similar, if not identical for the first transition (N → I) whether observed by CD or fluorescence; however, the transition between the intermediate and unfolded states is significantly different when observed by fluorescence or by CD (Table 1). The renaturation curves and denaturation curves are indistinguishable, and PDI denatured by 6 M Gdn·HCl and subjected to dialysis or dilution to 0.5 M Gdn·HCl regains >90% of its original activity. As with urea denaturation, the reduction of the disulfides of PDI by DTT has no significant effect on the denaturation behavior in Gdn·HCl. Maintaining the ionic strength constant at 6 M by the addition of NaCl does not significantly alter the denaturation profile.

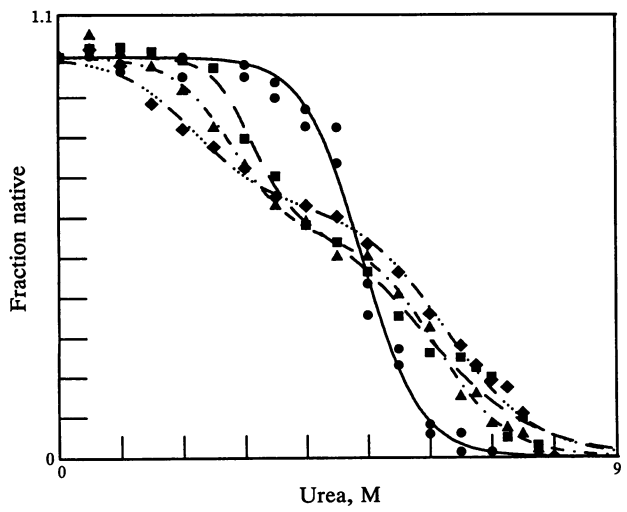

FIG. 3. Urea-induced denaturation of PDI at 23°C in the presence of Gdn·HCl. Fluorescence of PDI as a function of urea concentration was measured in 0.2 M sodium phosphate, pH 7.5/5 mM EDTA/2 mM DTT in the presence of Gdn·HCl at 0 M (●), 0.5 M (■), 0.9 M (▲), and 1.35 M (◆). The curves are drawn according to Eq. 5 with the values shown in Table 2.

Effect of Gdn·HCl on PDI Denaturation. Moderate concentrations of Gdn·HCl (0.5–1.35 M) alter the urea-induced denaturation so that a biphasic denaturation curve results (Fig. 3). Fitting of the data to a three-state model (Eq. 5) yields ΔG_0 (extrapolated to zero urea) and m values for the different concentrations of Gdn·HCl (Table 2).

DISCUSSION

The equilibrium denaturation of PDI by urea is completely reversible, and changes in the fluorescence and CD spectra may be described reasonably well by a simple two-state denaturation/renaturation model. However, Gdn·HCl-induced denaturation of the same protein shows the presence of a stable folding intermediate that is significantly populated at equilibrium. This intermediate retains a significant amount of secondary structure, amounting to ≈40% that of the native protein. The folding intermediate observed in Gdn·HCl is not due to differential denaturation of monomeric and dimeric PDI since the denaturation profile is independent of the PDI concentration over a 3.5-fold range and gel-filtration HPLC indicates that PDI is monomomeric.

The observation of a stable intermediate in the denaturation of PDI by Gdn·HCl but not urea could be accounted in several ways. The simplest would involve incomplete denaturation by urea so that only the first transition to produce the metastable intermediate is observed. There may be some residual secondary structure in 8 M urea (see below); the residue ellipticity at 222 nm in 8 M urea shows that the denaturation transition is ≈85% as complete as in 6 M Gdn·HCl.

Alternatively, Gdn·HCl could stabilize an intermediate, increasing its equilibrium concentration. If nondenaturing concentrations of Gdn·HCl stabilize a folding intermediate, relatively low concentrations of Gdn·HCl might also lead to accumulation of this intermediate during urea-induced denaturation. Such behavior is observed experimentally. When Gdn·HCl (0.5–1.4 M) is present during the urea-dependent denaturation of PDI, the denaturation profile becomes distinctly biphasic (Fig. 3), reminiscent of that observed with Gdn·HCl-induced denaturation. The ΔG_0 of the N ⇌ I transition, extrapolated to zero urea, is a linear function of the fixed Gdn·HCl concentration (Table 2), and extrapolation of this plot to zero Gdn·HCl provides an independent estimate of the free energy of the N ⇌ I transition of 6.2 ± 0.6 kcal/mol (1 cal = 4.184 J) in the absence of any denaturant, a value similar to that observed in urea alone. In addition, the m value (2.9 ± 0.7 kcal/liter) determined from the dependence of the urea denaturation on the fixed Gdn·HCl concentration is also similar to that for the N ⇌ I transition observed during Gdn·HCl denaturation. Thus, the effects of low concentrations of Gdn·HCl appear to be similar for both

Table 2. Equilibrium denaturation of PDI in urea containing a fixed concentration of Gdn·HCl

Gdn·HCl, M	S_I	$m_{N\rightarrow I}$, kcal/liter	$\Delta G_{N\rightarrow I}$, kcal/mol	$m_{I\rightarrow U}$, kcal/liter	$\Delta G_{I\rightarrow U}$, kcal/mol
0	—	1.2 ± 0.1	5.8 ± 0.3	—	—
0.5	0.64 ± 0.10	1.9 ± 0.7	5.7 ± 2	0.7 ± 0.1	4 ± 1
0.9	0.56 ± 0.04	1.2 ± 0.2	3.5 ± 0.5	0.9 ± 0.1	6.4 ± 0.9
1.35	0.64 ± 0.05	1.0 ± 0.2	2.1 ± 0.5	0.8 ± 0.1	5.2 ± 0.8

Details are as in Table 1.

Gdn·HCl and urea-dependent denaturation, consistent with the stabilization of a folding intermediate by Gdn·HCl.

The second transition (I → U) is more difficult to quantitate because it occurs at higher denaturant concentrations and produces a somewhat smaller signal change; however, it appears that an increasing Gdn·HCl concentration (in urea-dependent denaturation) increases the free energy difference between the intermediate and unfolded states (Table 2). With urea denaturation in the presence of Gdn·HCl, the sum of the free energy changes for the N ⇌ I and I ⇌ U transitions is nearly constant (9 ± 1.4 kcal/mol), particularly at the intermediate concentrations of Gdn·HCl where the accuracy of measurement of the individual ΔG values is greatest. This suggests that the estimated free energy difference between native and unfolded states is reasonably independent of the concentration of Gdn·HCl and that the accumulation of the intermediate results from a stabilization by Gdn·HCl (Fig. 4). The sum of the m values (2.2 ± 0.4 kcal/liter) appears to decrease slightly with increasing Gdn·HCl concentration; however, given the errors in the two values of m that make up this sum, it is difficult to determine whether this variation is significant.

The fact that denaturation by urea in the absence of Gdn·HCl is characterized by a $\Delta G_{N\rightarrow U}$ of 5.8 ± 0.3 kcal/mol rather than 9 kcal/mol and the observation that the residue elipticity in 8 M urea is significantly higher than in 6 M Gdn·HCl implies that denaturation in urea may also involve an intermediate that is simply less stable in the absence of Gdn·HCl and difficult to detect experimentally. If the model of Fig. 4 is correct and the $\Delta G_{N\rightarrow U}$ is independent of the denaturant, then $\Delta G_{I\rightarrow U}$ in urea would be expected to have a value of 3–3.5 kcal/mol and an $m_{I\rightarrow U}$ value of 0.8–1.2 kcal/liter. In fact, a curve drawn through the data of Fig. 1 using a three-state model in which S_I = 0.4, $m_{N\rightarrow I}$ = 1.0 kcal/liter, $\Delta G_{N\rightarrow I}$ = 5.2 kcal/mol, $m_{I\rightarrow U}$ = 0.7 kcal/liter, and $\Delta G_{I\rightarrow U}$ = 3.5 kcal/mol is indistinguishable from the curve shown that was drawn using a two-state model and the values in Table 1. Thus, the inferred stability of the intermediate in the absence of Gdn·HCl is consistent with the inability to observe it experimentally.

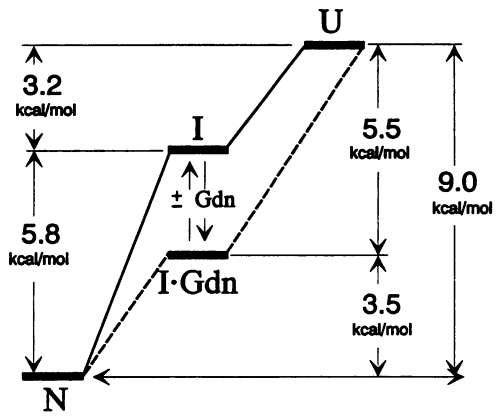

FIG. 4. Effect of Gdn·HCl on the stability of the folding intermediate observed in the denaturation of PDI. The native (N), intermediate (I), and unfolded (U) states are represented by horizontal bars. The relative stabilities (ΔG_0 in kcal/mol) are shown on the diagram. The position of the intermediate in the absence of Gdn·HCl is represented as 5.8 kcal/mol less stable than the native state; however, no detectable intermediate is actually observed in urea (see text for details). The stability of the intermediate in the presence of Gdn·HCl (I·Gdn) is shown for a Gdn·HCl concentration of 0.9 M.

The thermodynamic parameters for the first denaturation transition in Gdn·HCl are similar when observed by fluorescence or CD. However, the secondary structure of this intermediate appears to be less stable than the structure monitored by fluorescence; i.e., the CD signal disappears significantly before the fluorescence change is complete. Because the intermediate is denatured only at high concentrations of Gdn·HCl, it is difficult to determine whether this may be an artifact of baseline drift. However, it would appear that the intermediate may lose much of its secondary structure before complete exposure of the tryptophan to solvent.

Stabilizing interactions between Gdn·HCl and the native state have been noted previously. Pace *et al.* (19) found that Gdn·HCl increases the stability of the native state of ribonuclease T1 by ≈2 kcal/mol and Havel *et al.* (20) have noted a Gdn·HCl-induced dimerization of bovine growth factor that occurs at much lower concentrations of Gdn·HCl than urea. The increased stability of the intermediate folding state that is observed for PDI denaturation in the presence of Gdn·HCl could result from specific stabilizing interactions between the intermediate and Gdn·HCl through binding, from an effect of Gdn·HCl on electrostatic shielding through an ionic strength effect, or from an effect of Gdn·HCl on the structure of water (21). The effect is most likely not the result of electrostatic shielding since the inclusion of NaCl to maintain a constant ionic strength of 6 M has no effect on Gdn·HCl denaturation. The lack of an effect of NaCl would also imply that the stabilization of the intermediate is not due to the anion and that the stabilizing effect exhibits some specificity for the guanidinium cation. Goto *et al.* (21) have found that anions stabilize molten globule states of cytochrome *c* and apomyoglobin at low pH where the protein is positively charged and the intermediate state is more positively charged than the native state. PDI is a very acidic protein (pI = 4.2) (13), and at pH 7.5 the protein will be negatively charged. However, to specifically stabilize the intermediate relative to the native state, the number of cation binding sites would have to increase upon formation of the intermediate. PDI denaturation does not fit with the classic description of a "molten globule" state (12, 22). In contrast to the intermediate state observed in PDI denaturation, the "molten globule" state of apo-α-lactalbumin, which is stable at low ionic strength, low pH, and at intermediate concentrations of denaturant, exhibits a far-UV CD spectrum that is similar to that of the native protein (22).

Monomeric PDI has two active site regions, one near the N terminus and another near the C terminus; both are homologous to each other and to the redox active protein thioredoxin (1). Using a pattern recognition approach that evaluates the potential structural resemblance of a domain of given primary sequence to the thioredoxin structural motif, Ellis *et al.* (23) have proposed that the C-terminal domain of PDI is more closely related to the thioredoxin structure than the N-terminal domain. Thus, the two melting transitions might represent differences in the stability of these two structural domains. The intermediate that is stabilized by

Gdn·HCl could result from unfolding of one these domains, consistent with the retention of ≈40% of the secondary structure in the intermediate.

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