Biochemistry. In the article "Guanidine hydrochloride stabilization of a partially unfolded intermediate during the reversible denaturation of protein disulfide isomerase" by Nihmat A. Morjana, Barry J. McKeone, and Hiram F. Gilbert, which appeared in number 6, March 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 2107–2111), the authors request the following correction be noted. On p. 2107, right column, lines 22–29 should read as follows: The two forms of PDI appear to represent dimeric and tetrameric species in which a metastable tetramer without intermolecular disulfides is present (M. Kruzel and H.F.G., unpublished observations).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 tetramer to the dimer; under the conditions of our experiments, the PDI is dimeric.

This correction was necessitated by subsequent experiments that detected an error in the original calibration of the gel-filtration column (M. Kruzel and H.F.G., unpublished observations). Since the thermodynamic parameters are independent of protein concentration, this does not alter the conclusions regarding the stabilization of the folding intermediate by guanidine hydrochloride.

Biochemistry. In the article "Alanine scanning site-directed mutagenesis of the zinc fingers of transcription factor ADR1: Residues that contact DNA and that transactivate" by Sushil K. Thukral, Michael L. Morrison, and Elton T. Young, which appeared in number 20, October 15, 1991, of Proc. Natl. Acad. Sci. USA (88, 9188–9192), an error was made in sequencing or otherwise characterizing one of the ADR1 mutant plasmids. This mutant, in which glutamate 117 had been mutated to alanine (E117A), has a 10- to 20-fold reduced DNA binding activity whereas it was reported in Table 1, line 15, column 6, that its DNA binding activity was equivalent to that of wild-type ADR1 (1.0). We request that the value 0.05 be inserted at this position in Table 1. Thus, our interpretation that glutamate 117 in finger one is essential for a step in transcription that occurs after DNA binding is unwarranted. We regret this error and any inconvenience that it may have caused the scientific community.

Cell Biology. In the article "Calmodulin-binding domain of recombinant erythrocyte β-adducin" by Dominick A. Scarazzuzono and Jon S. Morrow, which appeared in number 8, April 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 3398–3402), it is requested that the following corrections be noted. (i) On p. 3399, line 15 of the legend to Fig. 2 should read "was a variable amount of proteolytic products between 41 . . . .". (ii) On p. 3400, the calmodulin-binding sequence shown in Fig. 3 should read "k42qqke . . . ."

Medical Sciences. In the article "Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element" by Nitzan Resnick, Tucker Collins, William Atkinson, David T. Bonthron, C. Forbes Dewey, Jr., and Michael A. Gimbrone, Jr., which appeared in number 10, May 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 4591–4595), the authors request that the following correction be noted. Table 2 on p. 4594 should read as follows.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Location, nt</th>
<th>Sequence</th>
<th>Ref(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-B</td>
<td>Human</td>
<td>−125</td>
<td>TCTCAGAGAACC</td>
<td>20, 21</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Feline</td>
<td>−125</td>
<td>TCTCAGAGAACC</td>
<td>23</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Murine</td>
<td>−125</td>
<td>TCTCAGAGAACC</td>
<td>24</td>
</tr>
<tr>
<td>tPA</td>
<td>Human</td>
<td>−945</td>
<td>GTCTCCTGCTC</td>
<td>26</td>
</tr>
<tr>
<td>tPA</td>
<td>Rodent</td>
<td>−252</td>
<td>CCTTCAGAACC</td>
<td>26</td>
</tr>
<tr>
<td>tPA</td>
<td>Murine</td>
<td>−252</td>
<td>CCTTCAGAACC</td>
<td>26</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Human</td>
<td>−1219</td>
<td>CCTGCGTGCTC</td>
<td>27</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Murine</td>
<td>−401</td>
<td>AGCTGCGTGCTC</td>
<td>28</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Murine</td>
<td>−1314</td>
<td>GTGAAGAGAACC</td>
<td>28</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Human</td>
<td>−644</td>
<td>GGTGCTAGAACC</td>
<td>29</td>
</tr>
</tbody>
</table>

Location of the SSRE core binding sequence is indicated relative to the initiation of transcription. Note that the core binding sequence (GAGACC) of the SSRE identified in this study and its complementary sequence (GCTTCC) are conserved among the PDGF-B promoters of several species and in unrelated genes that are also responsive to shear stress in vascular endothelium.
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 5PW (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 Amino 8000 (Urbana, IL) and Amino–Bowman (Urbana, IL) spectrophurometers 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-camphorsulfonic 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-
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 $\theta_{222}$ 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):

$$\Delta G = \Delta G_0 - m[D],$$

where $\Delta G_0$ 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 $\Delta G$ against [D]. At any denaturant concentration, the observed signal intensity (fluorescence or CD), $S_{\text{obs}}$, is given by

$$S_{\text{obs}} = S_N f_N + S_U f_U,$$

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 $\Delta G = -RT \ln(f_U/f_N)$,

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

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 $\Delta G_0$ 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).

$$N \rightleftharpoons I \rightleftharpoons U$$

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

$$S_{\text{obs}} = \frac{S_N f_N + S_I e^{-(\Delta G_{N\rightarrow I} - m_{N\rightarrow I}[D])/RT} + S_U e^{-(\Delta G_{N\rightarrow U} - m_{N\rightarrow U}[D])/RT} \exp\left(-\frac{\Delta G_{I\rightarrow U} - m_{I\rightarrow U}[D]}{RT}\right)}{1 + e^{-(\Delta G_{N\rightarrow I} - m_{N\rightarrow I}[D])/RT} + e^{-(\Delta G_{N\rightarrow U} - m_{N\rightarrow U}[D])/RT} \exp\left(-\frac{\Delta G_{I\rightarrow U} - m_{I\rightarrow U}[D]}{RT}\right)}.$$

where $S_N$, $S_I$, and $S_U$ represent, respectively, the intrinsic signal intensities of the native, intermediate, and unfolded states. $\Delta G_{N\rightarrow I}$ and $\Delta G_{I\rightarrow U}$ are the free energies for the N $\rightarrow$ I and I $\rightarrow$ U conversions, respectively, extrapolated to [D] = 0, and $m_{N\rightarrow I}$ and $m_{I\rightarrow U}$ 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 $\Delta G_{N\rightarrow I}$ and $m_{N\rightarrow I}$ values were not significantly affected by this procedure; however, the $\Delta G_{I\rightarrow U}$ and $m_{I\rightarrow U}$ 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

![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.](image_url)

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

<table>
<thead>
<tr>
<th>Urea (fluorescence and CD)</th>
<th>$m_{N\rightarrow I}$, kcal/liter</th>
<th>$\Delta G_{N\rightarrow I}$, kcal/mol</th>
<th>$m_{I\rightarrow U}$, kcal/liter</th>
<th>$\Delta G_{I\rightarrow U}$, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (fluorescence and CD)</td>
<td>1.2 ± 0.04</td>
<td>5.8 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>Gdn-HCl</td>
<td>2.7 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>1.6 ± 0.2</td>
<td>6.1 ± 0.16</td>
<td>4.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>4.0 ± 0.7</td>
<td>7.6 ± 0.9</td>
<td>4.0 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

Where $S_I$ is the fraction of the total change in signal intensity remaining in the intermediate. The subscripts for $m$ and $\Delta G$ refer to the $m$ and $\Delta G$ 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.
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 monomeric.

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 non-denaturing 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 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

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.

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.
Biochemistry: Morjana et al.

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

<table>
<thead>
<tr>
<th>Gdn-HCl, M</th>
<th>$S^0$</th>
<th>$m_{N-I}$, kcal/liter</th>
<th>$\Delta G_{N-I}$, kcal/mol</th>
<th>$m_{I-U}$, kcal/liter</th>
<th>$\Delta G_{I-U}$, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>1.2 ± 0.1</td>
<td>5.8 ± 0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>0.64 ± 0.10</td>
<td>1.9 ± 0.7</td>
<td>5.7 ± 2</td>
<td>0.7 ± 0.1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>0.9</td>
<td>0.56 ± 0.04</td>
<td>1.2 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>1.35</td>
<td>0.64 ± 0.05</td>
<td>1.0 ± 0.2</td>
<td>2.1 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>5.2 ± 0.8</td>
</tr>
</tbody>
</table>

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 $\rightarrow$ 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 $N \rightleftharpoons I$ and $I \rightleftharpoons U$ 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 $\Delta G$ 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 $\Delta G_{N-U}$ of 5.8 ± 0.3 kcal/mol rather than 9 kcal/mol and the observation that the residual ellipticity 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 $\Delta G_{N-I}$ is independent of the denaturant, then $\Delta G_{I-U}$ in urea would be expected to have a value of 3–3.5 kcal/mol and an $m_{I-U}$ 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 $S^0_I = 0.4$, $m_{N-I} = 1.0$ kcal/liter, $\Delta G_{N-I} = 5.2$ kcal/mol, $m_{I-U} = 0.7$ kcal/liter, and $\Delta G_{I-U} = 3.5$ 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.

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 $\approx$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 (pl $= 4.2$) (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-a-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 (I). 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

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 ($\Delta G$ 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.
Gdn-HCl could result from unfolding of one these domains, consistent with the retention of \( \approx 40\% \) of the secondary structure in the intermediate.

We thank the Atherosclerosis and Lipoprotein Group of the Department of Medicine, Baylor College of Medicine, for the use of fluorescence and CD instrumentation. This instrumentation was provided by a capital equipment grant from the National Science Foundation (PCM-8413751). This research was supported by grants from the National Institutes of Health (GM-40379) and the Texas Advanced Technology Program.