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Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions

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Abstract

The objective of this study was to address the question of whether or not urea and guanidine hydrochloride (GdnHCl) give the same estimates of the stability of a particular protein. We previously suspected that the estimates of protein stability from GdnHCl and urea denaturation data might differ depending on the electrostatic interactions stabilizing the proteins. Therefore, 4 coiled-coil analogs were designed, where the number of intrachain and interchain electrostatic attractions (A) were systematically changed to repulsions (R): 20A, 15A5R, 10A10R, and 20R. The GdnHCl denaturation data showed that the 4 coiled-coil analogs, which had electrostatic interactions ranging from 20 attractions to 20 repulsions, had very similar $[GdnHCl]_{1/2}$ values (average of $\simeq$3.5 M) and, as well, their $\Delta\Delta G_u$ values were very close to 0 (0.2 kcal/mol). In contrast, urea denaturation showed that the $[urea]_{1/2}$ values proportionately decreased with the stepwise change from 20 electrostatic attractions to 20 repulsions (20A, 7.4 M; 15A5R, 5.4 M; 10A10R, 3.2 M; and 20R, 1.4 M), and the $\Delta\Delta G_u$ values correspondingly increased with the increasing differences in electrostatic interactions (20A − 15A5R, 1.5 kcal/mol; 20A − 10A10R, 3.7 kcal/mol; and 20A − 20R, 5.8 kcal/mol). These results indicate that the ionic nature of GdnHCl masks electrostatic interactions in these model proteins, a phenomenon that was absent when the uncharged urea was used. Thus, GdnHCl and urea denaturations may give vastly different estimates of protein stability, depending on how important electrostatic interactions are to the protein.

Keywords: α-helices; coiled-coils; electrostatic interactions; GdnHCl and urea denaturation; hydrophobic interactions; leucine zippers; protein folding; protein stability

Urea and guanidine hydrochloride denaturation curves are generally used to obtain an estimate of the conformational stability of proteins by measuring the differences in conformational stabilities between the native (folded) and the denatured (unfolded) states (Privalov, 1979; Pace, 1986; Shortle, 1989). It had been suggested that unfolding occurs because there are a greater number of identical, noninteracting binding sites on the unfolded conformation than there are on the folded conformation (Tanford, 1970; Creighton, 1993). Urea and GdnHCl molecules are presumed to bind to peptide bonds (Robinson & Jencks, 1965; Roseman & Jencks, 1975) and, as a protein unfolds, more peptide groups are exposed to denaturant molecules (Pace, 1986; Mayo & Baldwin, 1993). For both denaturants, a good correlation has been observed between the number of denaturant molecules bound and a summation of one-half the number of peptide bonds plus the number of aromatic amino acid residues (Lee & Timasheff, 1974; Prakash et al., 1981). Although the above interpretation is generally true, there seems to be a disagreement as to whether the estimates of protein stability are dependent (Pace et al., 1990; Monera et al., 1993, 1994) or independent (Greene & Pace, 1974; Ahmad & Bigelow, 1982; Bolen & Santoro, 1988; Santoro & Bolen, 1988; Bowie & Sauer, 1989; Pace et al., 1990) of the denaturant used.

The differences in the urea and GdnHCl denaturations have been extensively discussed in terms of their differential binding to the protein (Robinson & Jencks, 1965; Tanford, 1970; Roseman & Jencks, 1975; Makhatadze & Privalov, 1992). Surprisingly, very little consideration has been attributed to the role of the differences in the ionic character between urea and GdnHCl

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Abbreviations: GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; TFE, 1,1,1-trifluoroethanol.

in protein denaturation. Twenty years ago, Greene and Pace (1974) reasoned that GdnHCl was 2.8 times more effective than urea in unfolding ribonuclease, but only 1.7 times more effective for lysozyme because the buried polypeptide chain is more polar for ribonuclease than for lysozyme. More recently, Pace et al. (1990) hinted that the much higher ionic strength of the GdnHCl solutions would be expected to suppress effects due to electrostatic interactions among charged groups in proteins. Incidentally, in the course of studying coiled-coil peptides, we observed that 2 coiled-coil analogs that had similar hydrophobic packing but with different electrostatic interactions had identical $[GdnHCl]_{1/2}$ values, but greatly differed in $[urea]_{1/2}$ values (Monera et al., 1993, 1994). Also, when the ratio of GdnHCl to urea in the denaturant mixture was gradually changed, this was accompanied by a stepwise shift in $[denaturant]_{1/2}$ values, the direction of which was dependent on the type of electrostatic interactions in the coiled-coil (Monera et al., 1993). Although we rationalized these observations in terms of the masking of electrostatic interactions in these model proteins by the ionic character of GdnHCl, our previous data could not, and were not intended to, prove this contention. Therefore, the objective of this study was to determine if the estimates of the stability of a protein from GdnHCl and urea denaturation data are affected by the nature of electrostatic interactions present in the protein.

Results

Four parallel disulfide-bridged coiled-coils were designed to contain identical hydrophobic packing but different electrostatic interactions (Fig. 1). Potential interchain electrostatic interactions were designed between the charged residues in the g-e′ and e-g′ positions, whereas intrachain electrostatic interactions were possible between charged residues in the b-e, b′-e′, and c′-g′ positions. These coiled-coils were formed by air oxidation of an appropriate combination of 2 35-residue peptides, each containing a cysteine residue at position 33 (Fig. 1). A single leucine at position 19 was substituted with alanine in order to bring the stability down to a workable range.

Figure 2 shows that the 4 disulfide-bridged peptide analogs had very high molar ellipticities in benign medium (Fig. 2A) that did not increase in the presence of a helix-inducing solvent such

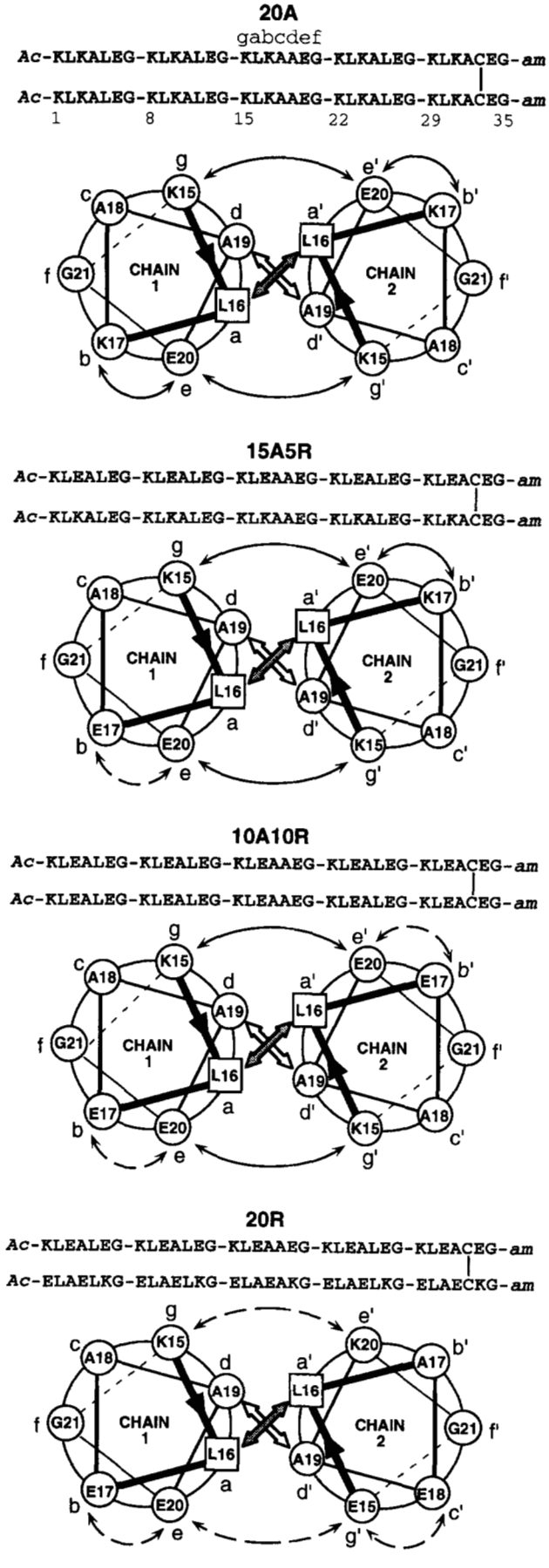

Fig. 1. The amino acid sequence and cross-sectional representations of the 4 coiled-coil analogs. Each strand contains 5 heptad repeats and each residue in a heptad is designated by a-b-c-d-e-f-g, as shown in the 20A analog. Each chain is acetylated in the amino-terminal and amidated in the carboxyl-terminal ends in order to minimize electrostatic interactions at the terminal ends. The cross sections represent the amino acid residues in the middle heptad. Solid arrows represent electrostatic attractions and dashed arrows represent potential electrostatic repulsions. Intrachain electrostatic interactions occur between charged residues (Glu and Lys) at positions b and e in one strand, and e′ and b′ or c′ and g′ in the other strand. Thus, in order to show the number and types of potential electrostatic interactions, the following designations were used for the 4 disulfide-bridged peptides: 20A, which contains potentially 10 interchain and/or 10 intrachain electrostatic attractions; 15A5R, which contains 10 interchain attractions and/or 5 intrachain attractions and 5 intrachain repulsions; 10A10R, which contains 10 interchain attractions and/or 10 intrachain repulsions; and 20R, which contains 10 interchain and/or 10 intrachain electrostatic repulsions.

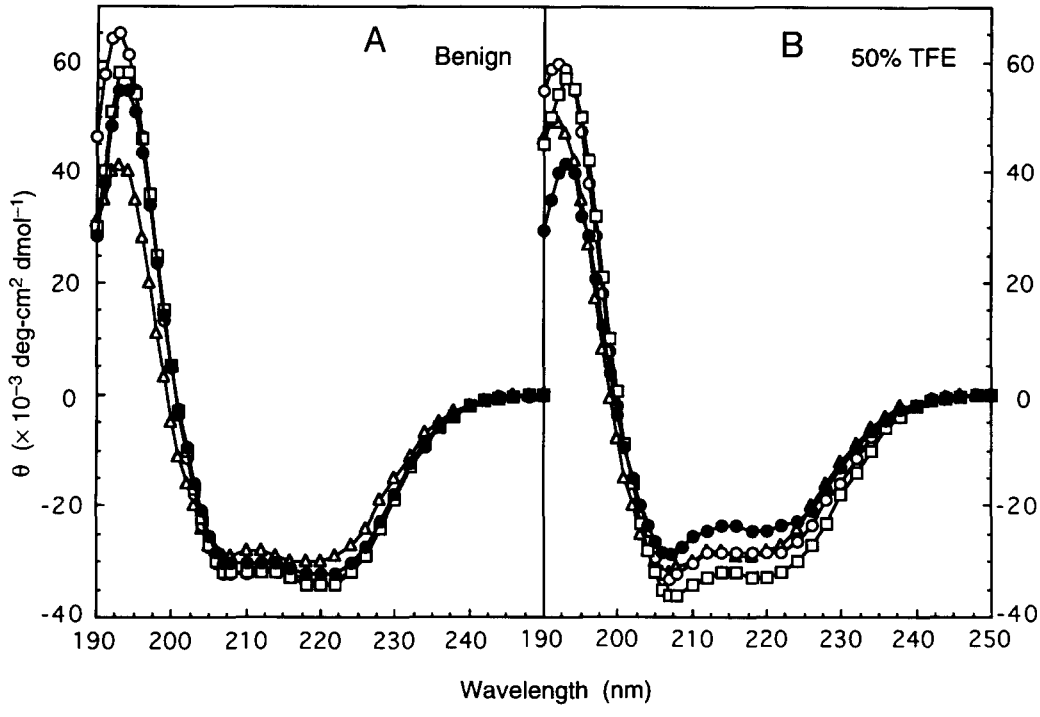

Fig. 2. CD spectra of the 4 coiled-coil analogs under (**A**) benign conditions and (**B**) in the presence of 50% TFE. ○, 20A; □, 15A5R; △, 10A10R; ●, 20R.

as TFE (Fig. 2B). Their CD profiles in benign medium showed that the 2 minima at 220 and 208 nm had a molar ellipticity ratio of ~1, which had been previously attributed to coiled-coil structures (Lau et al., 1984; Zhou et al., 1992a, 1992b; Zhu et al., 1992; Monera et al., 1993, 1994). In the presence of TFE, which disrupts hydrophobic interactions, the CD profiles became more characteristic of single-stranded α-helices. In addition, size-exclusion chromatography (data not shown) indicated that the 3 peptides had similar retention times with each other, which corresponded to those of 2-stranded disulfide-bridged coiled-coils. Peptide 20R appeared to undergo nonspecific aggregation of coiled-coils under benign conditions, probably due to the packing of the oppositely charged sides of the 2 stranded coiled-coils (Fig. 1). However, this observation does not alter the outcome of this study because 20R remained the least stable and its $[\text{urea}]_{1/2}$ value was consistent with the other 3 analogs (Table 1).

The denaturation profiles in Figure 3A clearly showed that the 4 coiled-coil analogs, which had electrostatic interactions that ranged from 20 attractions to 20 repulsions, had nearly identical

Table 1. *GdnHCl and urea denaturation data of the four coiled-coil analogs*

Coiled-coil	Denaturant	$[D]_{1/2}$[a]	$[G]_{1/2}/[U]_{1/2}$[b]	$\Delta\Delta G_u$[c]	Slope, m[d]	m_G/m_U[e]	t_m (°C)
20A	GdnHCl	3.5	0.47	0	1.87	2.49	n.d.[f]
	Urea	7.4		0	0.75		
15A5R	GdnHCl	3.4	0.63	0.2	1.71	2.22	75
	Urea	5.4		1.5	0.77		
10A10R	GdnHCl	3.6	1.12	0.2	1.49	1.49	62
	Urea	3.2		3.7	1.00		
20R	GdnHCl	3.6	2.57	0.2	1.44	1.21	54
	Urea	1.4		5.8	1.19		

[a] $[D]_{1/2}$ represents the $[\text{GdnHCl}]_{1/2}$ or $[\text{urea}]_{1/2}$ values.
[b] The ratio of the $[\text{denaturant}]_{1/2}$ values in GdnHCl to those in urea.
[c] The difference in free energy of unfolding in kcal/mol, as calculated from the equation of Sali et al. (1991) between peptide 20A and each of the other 3 peptides.
[d] Slopes of the free energy plots in Figure 3B and D, in kcal mol^{-1} M^{-1}.
[e] The ratio of the slope values in GdnHCl denaturation to those in urea.
[f] Not determined because this peptide was less than half unfolded at 75 °C.

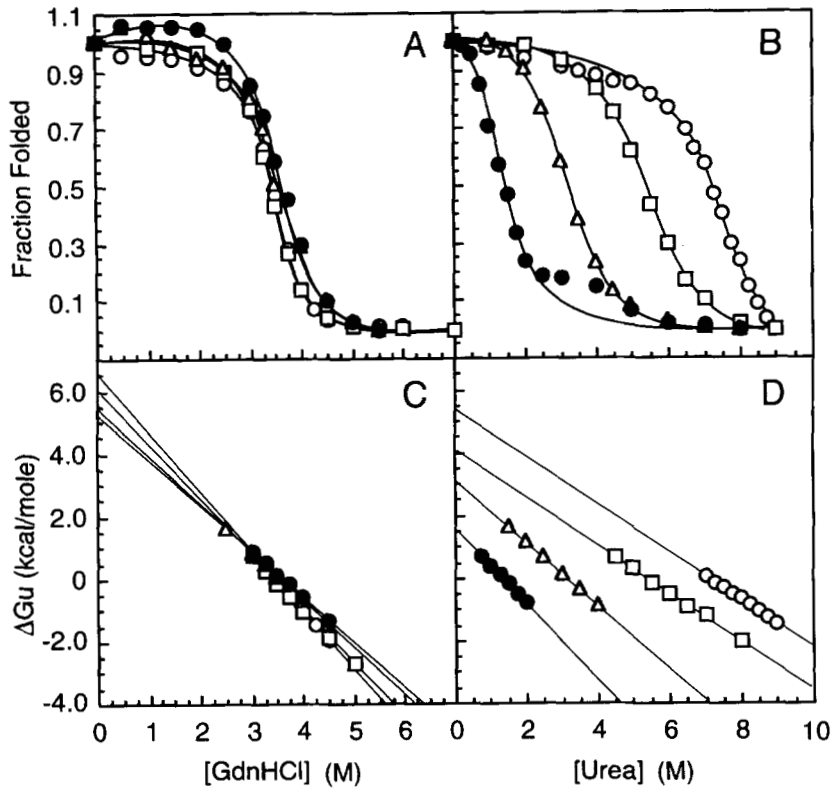

Fig. 3. **(A)** GdnHCl and **(B)** urea denaturation profiles of the 4 coiled-coils. **C,D:** Plots of the free energy of unfolding in the presence of denaturant versus the denaturant concentrations. ○, 20A; □, 15A5R; △, 10A10R; ●, 20R.

$[GdnHCl]_{1/2}$ values at around 3.5 M (Table 1). In contrast, Figure 3B and Table 1 showed a proportional decrease in $[urea]_{1/2}$ values when the electrostatic interactions were changed stepwise from 20 attractions to 20 repulsions. Consequently, the ratio of the $[denaturant]_{1/2}$ values of GdnHCl relative to urea ($[G]_{1/2}/[U]_{1/2}$) was lowest when all electrostatic interactions were attractions (0.47 for 20A; Table 1) and increased proportionately when the electrostatic attractions were changed stepwise to repulsions (0.63 for 15A5R, 1.12 for 10A10R, and 2.57 for 20R).

The free energy of unfolding in the absence of denaturant ($\Delta G_u^{H_2O}$) may not be the most reliable basis for comparing the stability between analogs because small errors in the slope values are amplified on extrapolation, leading to corresponding errors in $\Delta G_u^{H_2O}$ values (Green et al., 1992). To minimize the effect due to slope differences, Sali et al. (1991) proposed that more accurate comparisons between analogs can be made based on the difference in free energy of unfolding ($\Delta\Delta G_u$) at their respective $[denaturant]_{1/2}$ values (see Materials and methods). Calculated on this basis, the $\Delta\Delta G_u$ values clearly showed an identical trend as those of the $[denaturant]_{1/2}$ values, that is, GdnHCl denaturation data showed that the $\Delta\Delta G_u$ between the coiled-coil with the largest number of electrostatic attractions (20A) and that with the largest number of electrostatic repulsions (20R) was negligible (20A − 20R = 0.2 kcal/mol; Table 1). In contrast, the $\Delta\Delta G_u$ values from urea denaturation proportionately increased with increasing differences in the number and types of electrostatic interactions (20A − 15A5R = 1.5 kcal/mol; 20A − 10A10R = 3.7 kcal/mol; and 20A − 20R = 5.8 kcal/mol).

The CD spectroscopic scans at different temperatures (Fig. 4) showed that each coiled-coil analog exhibited a well-defined isodichroic point at around 202 nm. This suggests that the denaturation can be described on the basis of a 2-state structural transition, presumably from 2-stranded coiled-coil to random coil (Pace, 1975; Matthews et al., 1987). The temperature denaturation profiles, based on change in ellipticity at 220 nm ($[\theta]_{220}$), also showed progressive decrease in stabilities with the stepwise change from electrostatic attractions to repulsions (Fig. 4, insets). The melting temperatures (t_m) (Table 1) correlated very well with both the $[urea]_{1/2}$ and with the $\Delta\Delta G_u$ values for these peptide analogs (Fig. 5A,B, respectively). These results were consistent with the urea denaturation data and reflect the contributions of electrostatic interactions on the stability of these coiled-coils.

Discussion

The search for a better understanding of the process of protein folding, and the stability of the resulting structure, has been long and difficult. This is not only because of the tremendous complexity of the structure of native proteins, but also due to our limited understanding of the solvent denaturation process (Pace, 1986; Dill, 1990; Pace et al., 1990; Engel et al., 1991; Makhatadze & Privalov, 1992; Creighton, 1993; Thompson et al., 1993). The problem in using native proteins as models in comparing urea and GdnHCl denaturations lies in the difficulty in varying a particular type of interaction and in isolating its effects from the multitude of interactions that determine the overall stability of the protein. In this study, we addressed these problems by using the 2-stranded coiled-coil as a model protein because this motif contains only 1 type of secondary structure, the α-helix. The coiled-coil serves as an ideal model where

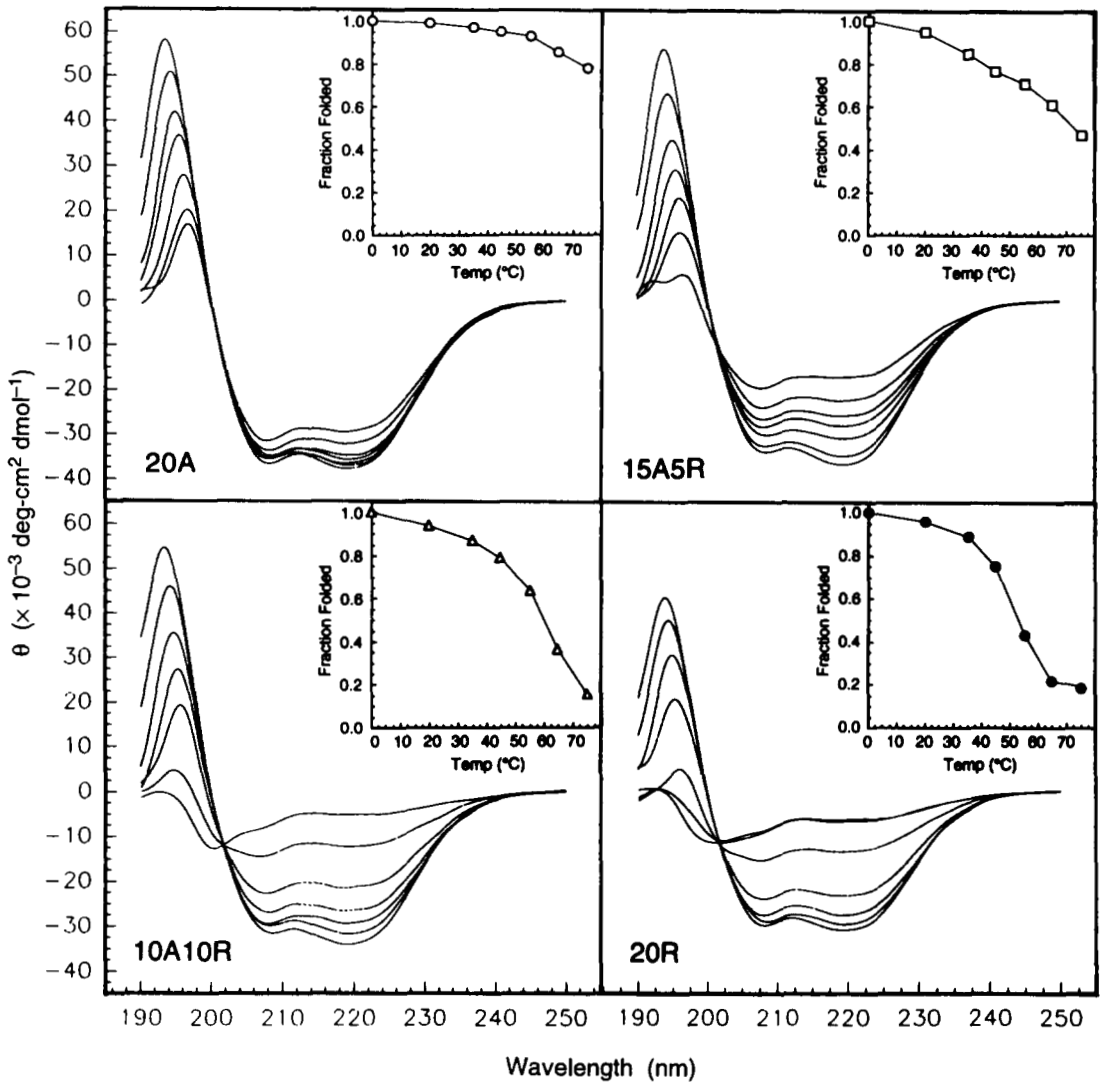

Fig. 4. CD profiles of the 4 coiled-coil analogs at different temperatures. **(A)** 20A, **(B)** 15A5R, **(C)** 10A10R, and **(D)** 20R. Going from bottom to top, the CD scans were at 5 °C, 20 °C, 35 °C, 45 °C, 55 °C, 65 °C, and 75 °C. The fractions of proteins folded at various temperatures are shown in the insets. ○, 20A; □, 15A5R; △, 10A10R; ●, 20R.

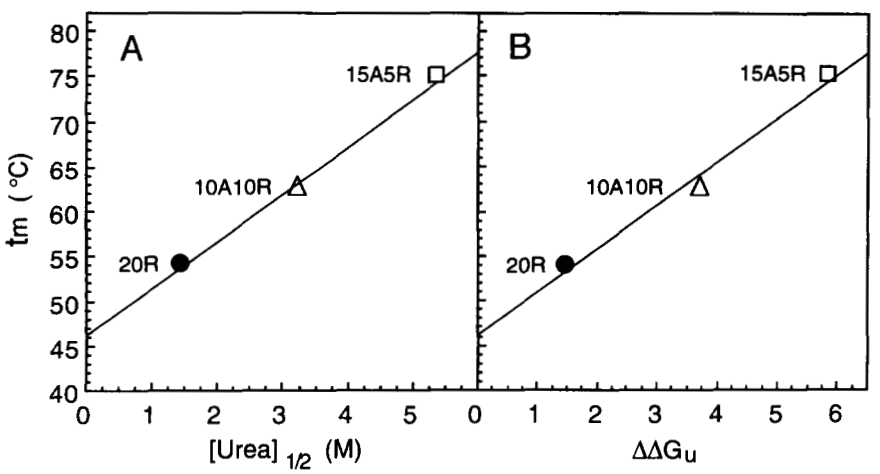

Fig. 5. Relationship between melting temperatures (t_m) and **(A)** $[urea]_{1/2}$ and **(B)** $\Delta\Delta G_u$ of 3 peptide analogs. Peptide 20A was not included because it was too stable under the temperature range employed in this study. □, 15A5R; △, 10A10R; ●, 20R.

charged amino acid residues can be systematically positioned and varied along each α-helical chain in order to study the effect of electrostatic interactions on protein stability from GdnHCl and urea denaturation data.

The results of this study clearly show that GdnHCl cannot distinguish the contributions of electrostatic interactions to the stability of the coiled-coil, but the effects of these electrostatic interactions are effectively monitored in urea. These results strongly support our earlier contention that the difference in the estimates of their stabilities was due to the ionic property of GdnHCl and its absence in urea (Monera et al., 1993, 1994). It is well understood that GdnHCl is a salt and, therefore, expected to ionize in aqueous solution. At low concentrations, Gdn^+ and Cl^- ions are presumed to mask the positively and negatively charged amino acid side chains, thereby reducing or even totally eliminating any stabilizing or destabilizing electrostatic interactions. The initial slight increase in stability observed in the GdnHCl denaturation of 20R may be explained in terms of the masking of electrostatic repulsions at low GdnHCl concentrations. Note that 20A and 15A5R, which have more electrostatic attractions than repulsions, did not exhibit this initial increase in stability. The recent report that RNase T1 was slightly stabilized by low concentrations GdnHCl, whereas RNAse A was not, was rationalized in terms of competitive binding of the Na^+ and Gdn^+ ions into the cation binding site in RNase T1 (Pace et al., 1990; Mayr & Schmid, 1993). However, it may also be possible to explain their results in terms of the masking effect of GdnHCl on electrostatic repulsions that might be present in the protein.

At high concentrations, GdnHCl becomes a denaturant, regardless of the types of electrostatic interactions present in the protein. The binding of the Gdn^+ ions to the proteins (Tanford, 1970; Greene & Pace, 1974; Pace, 1986) is presumed to predominate and to push the equilibrium toward the unfolded state. In addition, the high ionic strength of the GdnHCl solution is expected to mask the electrostatic interactions in these analogs (Pace, 1990; Monera et al., 1993, 1994). Thus, the coiled-coil analogs that differed only in terms of their electrostatic interactions had the same apparent stability. Taken together, these results suggest that the estimates of protein stability from GdnHCl denaturation studies would likely be a relative measure of the contributions of hydrophobic interactions.

On the other hand, the denaturing action of urea was presumed to be mainly based on its ability to bind to the protein (Tanford, 1970; Prakash et al., 1981; Pace, 1986). Because the urea molecule is uncharged, it is not expected to have any significant effect on the intermolecular and intramolecular electrostatic interactions in the protein. Thus, the $[urea]_{1/2}$ or free energy of unfolding from urea denaturations indicates the net stability of the protein, that is, the sum of the stabilizing effect of hydrophobic interactions and the stabilizing effect of electrostatic attractions (or the destabilizing effect of electrostatic repulsions). The results from temperature denaturation studies were also consistent with the urea denaturation data (Fig. 5) and support the role of electrostatic interactions in stabilizing (or destabilizing) the coiled-coil structures.

Previously, we attempted to mimic the electrostatic masking effect of GdnHCl denaturation by conducting urea denaturation in the presence of KCl (Monera et al., 1993). The results, however, showed a proportional increase in the $[urea]_{1/2}$ values with increasing KCl concentration, regardless of the type of electrostatic interactions. This was interpreted to mean that KCl was not as effective as GdnHCl in masking electrostatic interactions, as previously found in a separate study (Zhou et al., 1994). In addition, when GdnHCl denaturation of similar coiled-coils was conducted in the presence of KCl, there was no significant change in the $[GdnHCl]_{1/2}$ values, suggesting that no additional electrostatic masking effect or increase in hydrophobic interactions was brought about by KCl (Monera et al., 1993).

It had been suggested that the slope (m) of the plots between ΔG_u and [denaturant] is a measure of the ability of the denaturant to unfold a protein (Greene & Pace, 1974), and this has been supported by the recent observation that the m value is proportional to the number of denaturant binding sites exposed on unfolding (Mayo & Baldwin, 1993). Greene and Pace (1974) also reported that, based on the denaturation of small proteins, the ratio of the m values between GdnHCl and urea ($m_{(GdnHCl)}/m_{(urea)}$) were in the range of 2.4–2.9 for polar groups and can vary from 1.6 to 2.3 for apolar side chains. Consistent with the above suggestion, the ratios of the m values of these analogs were highest (2.49 for 20A; Table 1) when the coiled-coil was stabilized by both hydrophobic interactions and 20 potential electrostatic attractions. When the number of electrostatic attractions were gradually decreased and, as a result, gradually increasing the contribution of hydrophobic packing relative to the contributions of electrostatic attractions, the ratio of the slopes proportionately decreased (1.21 for 20R; Table 1). These results can be rationalized in terms of the resultant effects of denaturation and electrostatic interactions. When electrostatic attractions predominate over repulsions, such as in 20A, the denaturing effect of urea may be partially opposed by stabilizing effects of electrostatic attractions, resulting in a shallower slope. Conversely, when electrostatic repulsions predominate over attractions, such as in 20R, the denaturing effect of urea acts in concert with the destabilizing effect of electrostatic repulsions, resulting in a steeper slope (Table 1).

In conclusion, the results of this study are significant in at least 2 ways. First, as far as we are aware of, this is the first systematic demonstration of the ionic effects of GdnHCl in the context of its ability to mask electrostatic interactions during protein denaturation. Though GdnHCl is a salt and urea is uncharged, their modes of denaturation have often been erroneously discussed in the same context or are considered analogous. The differential effect of GdnHCl and urea on the electrostatic interactions may enable us to monitor selectively the contribution of a particular type of interaction, that is, hydrophobic or electrostatic interactions. If the effects of the changes in electrostatic interactions are to be assessed, or if the overall stability of the protein is of interest, urea or temperature denaturation becomes the obvious choice. However, GdnHCl is more appropriate in determining the specific contributions of hydrophobic or nonionic interactions on protein stability. Second, and more importantly, the results clearly show that for a particular protein the estimates of the free energy of unfolding from GdnHCl and urea denaturations can potentially be very different, depending on the nature of electrostatic interactions stabilizing the protein structure. This possibility had been hinted or inferred before (Pace et al., 1990; Mayr & Schmid, 1993; Monera et al., 1993, 1994) but is unequivocally proven in this study by designing a homologous series of coiled-coils that differed only in the number and types of electrostatic interactions. Thus, the use of the 2 denaturants should not be treated as analogous to each other or as a matter

of convenience but, instead, should be based on a rational consideration of the electrostatic nature of the protein.

Materials and methods

Peptide synthesis and purification

The peptide analogs were synthesized by standard solid-phase methodology and purified by reversed-phase HPLC. The disulfide-bridged coiled-coils were formed by air oxidation of an appropriate pair of 35-residue starting peptides. Peptide authenticities were verified by amino acid analysis and mass spectrometry. Details of the synthesis and purification procedures were similar to those described previously (Monera et al., 1993).

Characterization of the coiled-coils

Each of the parallel, disulfide-bridged coiled-coil analogs was characterized by CD spectroscopy under benign conditions (50 mM phosphate, 0.1 M KCl, pH 7) and in the presence of 50% TFE in the same buffer. In addition, the analogs were also subjected to size-exclusion HPLC in 50 mM phosphate buffer, pH 7, containing 0.1 M KCl, as described previously (Monera et al., 1993).

GdnHCl and urea denaturation studies

Stock solutions of GdnHCl (8 M) and urea (10 M) were prepared in 50 mM phosphate at pH 7.0. Then, aliquots of these denaturants were used to prepare a series of solutions containing different concentrations of GdnHCl and urea. The mean residue ellipticities of the different solutions were measured at 20 °C by CD at 220 nm. Assuming a 2-state model, the molar fraction of folded peptide (f) was calculated from the equation $f = ([\theta] - [\theta]_u)/([\theta]_n - [\theta]_u)$, where $[\theta]$ is the observed mean residue ellipticity at any particular denaturant concentration and $[\theta]_n$ and $[\theta]_u$ are the mean residue ellipticities of the native (folded) and denatured (unfolded) states, respectively. The free energy of unfolding at each denaturant concentration (ΔG_u) was calculated from the equation $\Delta G_u = -RT \ln(f/1 - f)$ and then plotted against [denaturant]. Estimates of the free energy of unfolding in the absence of denaturant ($\Delta G_u^{H_2O}$) can then be obtained by linear extrapolation method (Pace, 1986).

Because the main interest in this study was to determine the difference in free energy of unfolding between analogs ($\Delta\Delta G_u$), as opposed to their absolute ($\Delta G_u^{H_2O}$) values, $\Delta\Delta G_u$ was calculated using the equation suggested by Sali et al. (1991): $\Delta\Delta G_u = \{(m_1 + m_2)/2\}\{([\text{denaturant}]_{1/2})_2 - ([\text{denaturant}]_{1/2})_1\}$, where m is the slope term from the equation $\Delta G_u = \Delta G_u^{H_2O} - m$ [denaturant] (Pace, 1986).

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