Dissecting Contributions to the Denaturant Sensitivities of Proteins†

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ABSTRACT: Understanding the molecular basis for protein denaturation by urea and guanidinium chloride (GdmCl) should accommodate the observation that, on a molar basis, GdmCl is generally 2−2.5-fold more effective as a protein denaturant than urea. Previous studies [Smith, J. S., and Scholtz, J. M. (1996) *Biochemistry 35*, 7292−7297] have suggested that the effects of GdmCl on the stability of alanine-based helical peptides can be separated into denaturant and salt effects, since adding equimolar NaCl to urea enhanced urea-induced unfolding to an extent that was close to that of Gdm. We reinvestigated this observation using an alanine-based helical peptide (alahel) that lacks side chain electrostatic contributions to stability, and compared the relative denaturant sensitivities of this peptide with that of tryptophan zipper peptides (trpzip) whose native conformations are stabilized largely by cross-strand indole ring interactions. In contrast to the observations of Smith and Scholtz, GdmCl was only slightly more powerful as a denaturant of alahel than urea in salt-free buffer (the denaturant *m* value m_{GdmCl}/m_{urea} ratio = 1.4), and the denaturation of alahel by urea exhibited only a small dependence on NaCl or KCl. The trpzip peptides were much more sensitive to GdmCl than to urea (m_{GdmCl}/m_{urea} = 3.5−4). These observations indicate that the m_{GdmCl}/m_{urea} ratio of 2−2.5 for proteins results from a combination of effects on the multiple contributions to protein stability, for which GdmCl may be only slightly more effective than urea (e.g., hydrogen bonds) or considerably more effective than urea (e.g., indole−indole interactions).

Of the two most commonly used protein denaturants, guanidinium chloride (GdmCl)[1] is generally found to be 2−2.5 times more effective as a denaturant than urea (*1*, *2*). Recent studies have tended to support the idea that these denaturants promote protein unfolding by favorable interaction with groups exposed upon unfolding (*3*−*6*) [rather than via generalized effects of denaturants on water structure (*7*−*9*)]. The extent to which different protein groups contribute to the denaturant sensitivities of proteins, and the origin of the enhanced denaturant power of GdmCl over urea, is not fully understood. Here we address each of these points by measuring the absolute and relative denaturation power of GdmCl and urea on two model peptides. One of these, a polyalanine-based helical peptide (alahel) (*10*, *11*), is stabilized by (helical) hydrogen bonding. The other, from the family of tryptophan zipper peptides (*12*) (trpzip), is stabilized by cross strand indole ring interactions. These peptides serve to separate, to a first approximation, the denaturant sensitivities of two contributions to the conformational stability of proteins, i.e., hydrogen bonds and interactions involving the side chain indole group of tryptophan.

Previous studies have addressed the question of interaction-specific contributions to protein denaturant sensitivities, focusing largely on hydrogen bonding and hydrophobic interactions (*6*, *13*−*17*). However, numerous studies of denaturant effects on free energies of transfer into water of compounds chosen to represent the different types of groups found in proteins have yielded contradictory conclusions. Nozaki and Tanford found GdmCl to be 2−3 times more effective than urea in solubilizing small molecules representing the peptide backbone or amino acid side chains of proteins (*14*). They also found that GdmCl was particularly effective (compared to urea) in promoting transfer of the peptide bond (diglycine or triglycine) into water. Nandi and Robinson found that urea and GdmCl were approximately equally effective in solubilizing *N*-acetyl(glycine)$_n$ ethyl esters when *n* equaled 2. As *n* was increased above 2, a nonlinear relationship between the transfer free energy and *n* for GdmCl resulted in an increasingly greater $\Delta G_{GdmCl}/\Delta G_{urea}$ ratio (*15* and references therein). Smith and Scholtz suggested, from the denaturation of a polyalanine-based helical peptide, that urea and GdmCl are equally effective in unfolding peptide helices if one can account for the salt effects of the ionic denaturant GdmCl (i.e., urea with equimolar NaCl was equally powerful as a denaturant as GdmCl) (*17*). Since helix formation in these peptides is dominated by hydrogen bonding (*18*), this observation implies a large intrinsic salt effect on the stability of (at least helical) hydrogen bonds. However, complications resulting from side chain electrostatic contributions to the stability of the helical peptide used by Smith and Scholtz (*17*) may have

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[1] Abbreviations: BSA, bovine serum albumin; GdmCl, guanidinium chloride; LEM, linear extrapolation method.

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given rise to an overestimation of both the GdmCl potency for helix denaturation in a salt free solution and intrinsic salt effects on hydrogen bond contributions to stability. We have used a related polyalanine-based helical peptide that lacks side chain electrostatic contributions to stability, and find small intrinsic salt effects on helical stability, and a relatively small increased effectiveness of GdmCl over urea in denaturing peptide helices. On the other hand, GdmCl is much stronger as a denaturant of trpzip peptides than urea. These observations indicate that the overall effectiveness of denaturants is a summation of different effects on the different types of noncovalent interactions that contribute to the overall stability of proteins.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Characterization. The following peptides were synthesized by G. Bloomberg of the Bristol Centre for Molecular Recognition: a polyalanine-based helical peptide (*10*, *11*) [denoted here as alahel; this is peptide E7 in the series of peptides characterized by Scholtz et al. (*10*)], Ac-AAQAAAEQAAAAQAAY-NH_2; trpzip1 (*12*), SWTWEGNKWTWK-NH_2; trpzip2 (*12*), SWTWENGKWTWK-NH_2; and KWTWK-NH_2 (*19*). Using methods previously described for peptide purification and characterization by our group (*20*, *21*), each peptide was purified by HPLC and confirmed to be at least 96% pure by analytical HPLC and to have the predicted *m*/*e* ratio by mass spectrometry.

Spectroscopic Measurements. All spectroscopic measurements were taken in a buffer composed of 10 mM potassium phosphate (pH 7.0). Peptide concentrations of stock solutions were measured using either the Tyr absorbance of alahel (ϵ_{275} = 1450 M^{-1} cm^{-1}) or the Trp absorbance of the trpzip peptides (ϵ_{280} = 5600 M^{-1} cm^{-1} per mole of Trp). Circular dichroism spectra were obtained in cuvettes with a path length of 2 mm using a Jobin-Yvon CD6 spectropolarimeter with the temperature of the cuvette holder maintained using a Haake circulating water bath. Accurate temperatures were obtained by direct measurement within samples using a Hanna H198801 thermocouple thermometer. All denaturant stock solutions were made by dissolving the required amount of solute into a minimal volume of phosphate buffer, and the pH was readjusted to pH 7.0 with orthophosphoric acid or potassium hydroxide before the volume was carefully adjusted with phosphate buffer to give the correct molarity of the solute.

Analysis of Spectroscopic Data. (a) Alahel. Data were collected in molecular ellipticity mode, and were converted to mean residue ellipticity θ after subtracting relevant blank spectra. The fraction of helix (f_H) was determined from the θ_{222} value using (*22*)

$$f_H = \frac{\theta_{222} - \theta_r}{0.8125(-44000 + 250T) - \theta_r} \quad (1)$$

where T is the temperature in degrees Celsius, θ_r is the temperature-dependent value of θ_{222} for the random coil form of the peptide, and the constant 0.8125 (i.e., $1 - {}^3/_{16}$) corrects for the three non-hydrogen-bonded amide carbonyls in the C-terminally carboxamidated peptide. We estimated that θ_r has a value of $2200 - 53T$ from the data plotted in Figure 2 of ref *22*. We analyzed the denaturant dependence of helix content using the Zimm−Bragg theory (*23*), in which the value of f_H is fitted to eq 2, to determine a value for s, the helix propagation parameter. The analysis essentially followed that of Baldwin and colleagues (*6*) in which the helix nucleation constant, σ, was fixed at 0.003; n is the number of amide bonds in the peptide. We used the linear extrapolation method (LEM) (*24*, *25*) to analyze the denaturation concentration dependence of s according to eq 3

$$f_H = \frac{\sigma s}{(s-1)^3}\left(\frac{ns^{n+2} - (n+2)s^{n+1} + (n+2)s - n}{n\{1 + [\sigma s/(s-1)^2][s^{n+1} + n - (n+1)s]\}}\right) \quad (2)$$

$$\ln s = \ln s_0 - \frac{m[\text{denaturant}]}{RT} \quad (3)$$

where s_0 is s in the absence of denaturant, R is the gas constant (1.987 cal mol^{-1} K^{-1}), and m is the per-residue Gibbs energy of helix propagation as a function of the molar denaturant concentration.

(b) Trpzip. The CD data for trpzip peptides were collected in the molecular ellipticity mode, and peptide-free blank spectra were subtracted. The molecular ellipticity at 227 nm (θ_{227}) was used as a measure of the folded state. We used the data of Cochran et al. (see Figure 1 of ref *12*) and estimated f_B for trpzip1 to be 0.60 at 42 °C, where f_B is the fraction of the folded (β-hairpin) peptide. Using this estimate, we calculated f_B of trpzip2 at 42 °C to be 0.85, in excellent agreement with the data of Cochran et al. (*12*). A value for θ_u, the molecular ellipticity at 227 nm for the unfolded peptide, of 29 400 deg cm^2 mol^{-1} was determined from the spectrum of the KWTWK-NH_2 peptide, measured at a molar concentration twice that of trpzip, so that the overall concentration of tryptophan was equivalent. Values for f_B at each addition of denaturant were determined using eq 4 in which θ_{max} = 921 000 deg cm^2 mol^{-1} and θ_u = 29 400 deg cm^2 mol^{-1}.

$$f_B = \frac{\theta_{227} - \theta_u}{\theta_{max} - \theta_u} \quad (4)$$

RESULTS

Alahel. Figure 1 shows a series illustrating the effects of urea and GdmCl on the CD spectra of both alahel and trpzip1. The CD spectrum of alahel is characterized by a positive band near 190 nm and a double minimum near 208 and 222 nm. At 2 °C in 10 mM phosphate buffer, the ellipticity at 222 nm corresponds to a fractional helical content of ~0.46.

Using the Zimm−Bragg analysis, the variation of the helix propagation parameter (expressed as ln s), as a function of denaturant content, was determined for sets of data collected at 2, 8, 15, and 22 °C for both urea and GdmCl (see Figures 2 and 3 for representative data). The m values, determined by linear regression, are listed in Table 1, and the temperature dependence of m for denaturant-induced unfolding of alahel is shown in Figure 4.

In light of previous evidence of an intrinsic salt effect on helix stability (*17*), we further determined m values for alahel at 2 °C in the presence of NaCl or KCl, with and without

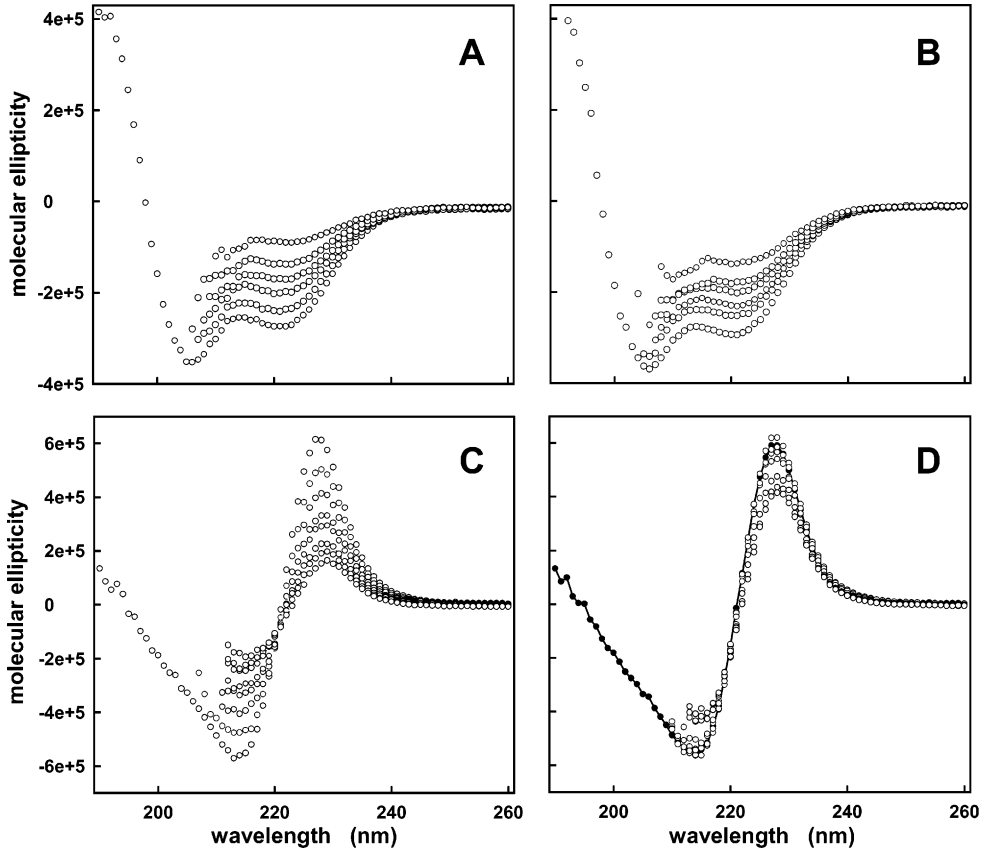

FIGURE 1: Denaturant dependence of the circular dichroism spectra of alahel at 2 °C (A and B) and trpzip1 at 42 °C (C and D) in GdmCl (A and C) and urea (B and D), in 10 mM potassium phosphate buffer (pH 7.0). The denaturant concentrations in panels A and B are (from bottom to top) 0, 0.5, 1, 1.5, 2, and 3 M. GdmCl concentrations in panel C are (from top to bottom at 227 nm) 0−3 M in 0.5 M steps. Urea concentrations in panel D are 0−5.8 M in 1 M steps. Data for the 0 M urea sample in panel D are highlighted (●) to illustrate the small increase in ellipticity obtained for trpzip1 in concentrations of urea of 0.5−1 M (see the text). The peptide concentration was 40 μM in all cases.

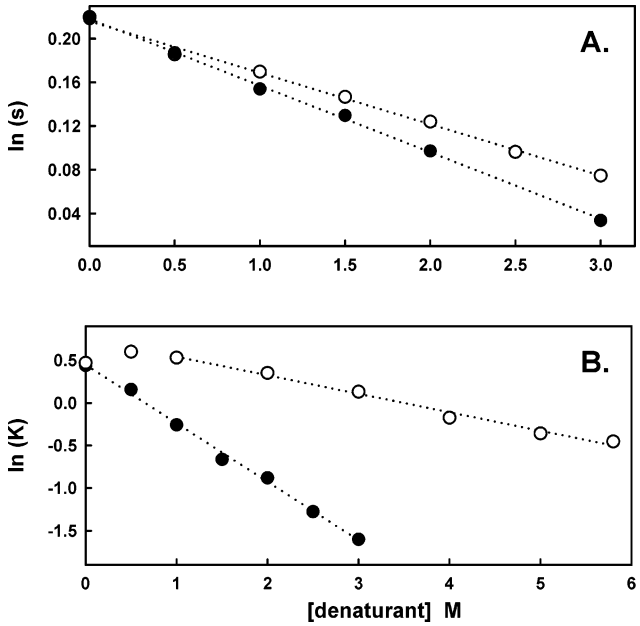

FIGURE 2: Denaturant dependence of ln(*s*) for alahel at 2 °C (A) and of ln(*K*) for trpzip1 at 42 °C (where *K* is the equilibrium constant K_{FU}) (B). In each panel, filled circles are GdmCl data and empty circles are urea data. Dotted lines are linear regression data. In panel B, the regression line was fitted only to the urea data between 1 and 5.8 M (see the text).

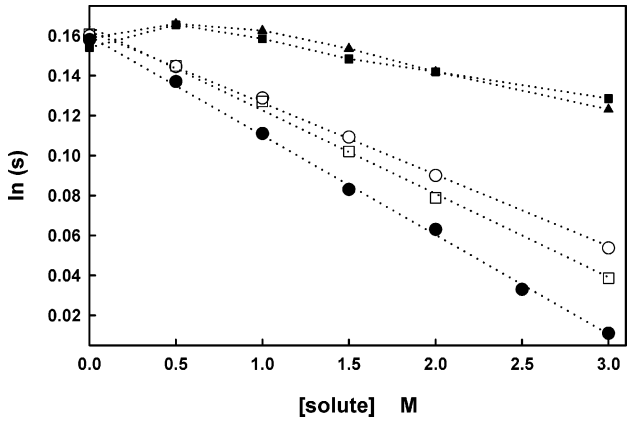

FIGURE 3: Solute dependence of ln(*s*) for alahel at 15 °C. Solutes are urea (○), GdmCl (●), urea with equimolar KCl (□), NaCl (▲), and KCl (■). Dotted lines for the ○, ●, and □ symbols are linear regression fits. Dotted lines for the ■ and ▲ symbols are drawn to guide the eye.

equimolar urea. In contrast to the study of Smith and Scholtz (*17*), we found that these salts had weak effects on the *m* value for urea-induced denaturation of alahel, and on the stability of alahel in a denaturant-free solution. In the case of alahel, the denaturation power of urea is not greatly enhanced by salt, and our *m* value for Gdm-induced unfolding of alahel is considerably lower than that described for the peptide studied in ref *17*. As described in the Discussion, we believe that the $i-i+4$ charge interactions in the peptide studied in ref *17* contribute to helical stability, and that the large enhancement of urea-induced unfolding

Table 1: *m* Values for Solute Effects on Alahel and Trpzip1

Alahel		
	m (cal mol^{-1} M^{-1})[a]	
	2 °C	15 °C[b]
GdmCl	34	28
urea	26	20
urea and KCl (equimolar)	28	23
KCl[c]	5	6
NaCl[c]	6	7

Trpzip1 (42 °C)	
	m (cal mol^{-1} M^{-1})[d]
GdmCl	410
GdmCl (without the contribution from the E_5K_8 salt bridge)	358
urea	126

[a] *m* values for alahel are per residue. [b] GdmCl and urea *m* values for other temperatures are given in Figure 4. [c] *m* values are "apparent" values since salt effects on helix stability are biphasic (see the text and Figure 3). [d] *m* values for trpzip1 are per mole of the 12-residue peptide.

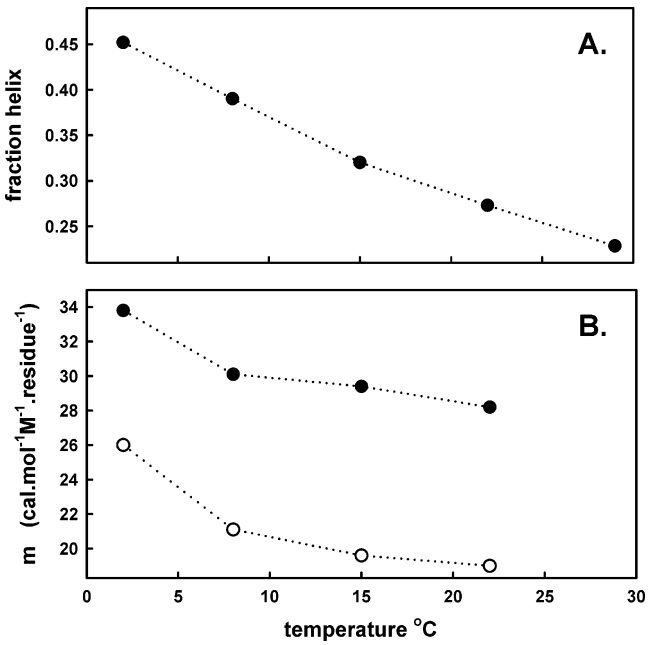

FIGURE 4: (A) Temperature dependence of the helix content of alahel in 10 mM potassium phosphate buffer (pH 7.0). (B) Temperature dependence of *m* for GdmCl (●) and urea (○) denaturation of alahel in potassium phosphate buffer.

by salt and the high *m* value for GdmCl (and curved plots of ln *s* vs [GdmCl]) are not representative of intrinsic solute effects on a peptide helix lacking side chain electrostatic contributions to stability. A separate series of measurements was made at 15 °C, to establish whether these conclusions apply at temperatures closer to those used in studies of denaturant-induced protein unfolding (Figure 3 and Table 1). The *m* values for GdmCl and urea-induced unfolding of alahel at 15 °C are smaller than at 2 °C (see also Figure 4), but the ratio of *m* values ($m_{GdmCl}/m_{urea} = 1.4$) is similar. Likewise, equimolar KCl enhances urea-induced unfolding by only a small amount, and intrinsic salt effects are weak. The small *enhancement* of helical stability at NaCl and KCl concentrations up to ~1 M (Figure 3) is due to screening of destabilizing helix dipole charges as described previously (*26*, *27*).

Since the *m* values for effects of denaturant on helix stability were significantly smaller at 15 °C than at 2 °C (Table 1), we determined the temperature dependence of *m*. The rather small temperature dependence of helix content in alahel (Figure 4) allowed reliable determination of *m* values for urea- and GdmCl-induced unfolding at temperatures up to (at least) 22 °C, where the fractional helical content f_H is 0.24. The *m* value for urea was around 20 ± 1 cal mol^{-1} M^{-1} residue^{-1} between 8 and 22 °C but increased to 26 cal mol^{-1} M^{-1} residue^{-1} at 2 °C. Likewise, the *m* value for GdmCl denaturation was around 28 ± 1 cal mol^{-1} M^{-1} residue^{-1} between 8 and 22 °C but increased to 34 cal mol^{-1} M^{-1} residue^{-1} at 2 °C. At all temperatures, the ratio of *m* values (m_{GdmCl}/m_{urea}) was 1.4 ± 0.1.

Trpzip1. The CD spectrum of the trpzip peptides (Figure 1) is characterized by strong minima (215 nm) and maxima (227 nm) due to exciton coupling between pairs of Trp indole groups from opposing strands of the *β*-hairpin (*12*). The relationship between ellipticity and the fraction of folded peptide, f_B, is less clear for trpzip peptides [compared to the well-characterized relationship between θ_{222} and f_H (eq 1)], and there is no formalism for characterizing partial unfolding equivalent to the Zimm−Bragg analysis of helical states. We used the following approach for determining *m* values for denaturant-induced unfolding of trpzip1. First, we used the estimate of the f_B for trpzip1 in pH 7.0 buffer determined by Cochran et al. of 0.60 at 42 °C. This is consistent with the value of 0.85 for f_B for trpzip2 under equivalent conditions, since extrapolating our measured θ_{227} values for each trpzip peptide back to an f_B of 1.0 gave a common value for $\theta_{227(max)}$ of 9.21 × 10^5 deg cm^2 mol^{-1}. The nature of the CD signal in trpzip peptides presumably depends on the precise orientation between the indole rings, and the possibility of solute-induced alterations of the CD spectrum due to small perturbations of ring interactions *within the folded state* cannot be discounted. We assessed this by determining the effects of denaturant on the spectrum of trpzip2 (*12*). At 20 °C, this peptide is resistant to urea, and to GdmCl, up to at least 1 M GdmCl (not shown). Apart from small red shifts in the position of the maximum (227 to 229 nm), neither urea nor GdmCl at 1 M significantly affected the magnitude of the spectral maximum. We used the CD spectrum of the KWTWK-NH_2 peptide to determine a value of θ_u, the molecular ellipticity at 227 nm for the unfolded peptide. Finally, we assumed that peptide unfolding is two-state so that f_B corresponds to the equilibrium between folded (F) and unfolded (U) forms. The *m* values for denaturant-induced unfolding were thus determined using the LEM in which *s* and s_0 in eq 3 are replaced with *K* and K_0, respectively, where *K* is the equilibrium constant K_{FU}. Note that addition of urea to trpzip1 at concentrations up to ~1 M resulted in small increases in the ellipticity at 227 nm (Figure 1D), consistent with a small stabilization of the folded state or a small perturbation of the folded state structure. We used data between 1 and 5.8 M urea to calculate *m* values for urea-induced unfolding since the data are linear in this range (see Figure 2B).

The calculated *m* value for urea-induced unfolding of trpzip1 at 42 °C was 126 cal mol^{-1} M^{-1}; for GdmCl denaturation, the corresponding value of *m* was 410 cal mol^{-1} M^{-1}. Note that these values are per mole of a 12-residue peptide (rather than per residue in the case of alahel), since

it is not obvious how contributions to these *m* values should be divided among the various interactions that contribute to the stability of trpzip1 (see the Discussion). It is possible, however, to account for the contribution from the salt bridge interaction between Glu5 and Lys8 (*12*). Lowering the pH from 7.0 to 2.4 (to protonate the side chain carboxylate of Glu5) resulted in a reduction of f_B from 0.60 to 0.54 (not shown) at 42 °C. Since this salt bridge interaction should be completely attenuated (at pH 7.0) by the increased ionic strength during the course of the addition of GdmCl to a concentration of 3 M, the contribution of the salt bridge interaction was subtracted from the GdmCl *m* value (but not the urea *m* value). The *m* value for GdmCl-induced denaturation of trpzip1, removing the contribution from the salt bridge, is 358 cal mol^{-1} M^{-1} (see Table 1).

DISCUSSION

The availability of novel small folded peptides is significantly advancing the characterization of specific noncovalent interactions and their contributions to protein stability. Here we have shown that these peptides may provide detailed information about the contributions of specific noncovalent interactions to the denaturant sensitivities of proteins. This effectively bridges the gap between small molecule studies and proteins, where the complexity of the multiple interactions stabilizing the native state precludes straightforward analysis of denaturant mechanisms. One question concerns the large (2−2.5-fold) enhanced activity of GdmCl over urea in denaturing proteins. The data presented here demonstrate that this ratio is not representative of the respective abilities of the denaturants to unfold α-helix, where GdmCl is only ∼1.4 times as effective as urea. On the other hand, GdmCl is nearly 4 times as effective in unfolding the trpzip1 peptide in which Trp−indole interactions dominate contributions to stability. These observations indicate that the 2−2.5-fold enhanced effectiveness of GdmCl over urea for protein denaturation results from the summation of different effects on multiple interactions that stabilize the native state, for which GdmCl is either only slightly more effective than urea (α-helix) or considerably more effective that urea (Trp−indole interactions). We discuss below whether the very strong enhanced ability of GdmCl to unfold the trpzip peptide is representative of hydrophobic interactions in general or is specific to Trp alone, or to Trp and the other aromatic amino acids.

The *m* value for urea denaturation of alahel at 2 °C (26 cal mol^{-1} M^{-1}) is similar to values previously obtained for related polyalanine-based helical peptides [23 cal mol^{-1} M^{-1} (*6*) and 24 cal mol^{-1} M^{-1} (*17*)]. However, our data for salt effects, for salt and urea as cosolutes, and for GdmCl differ markedly from those obtained by Smith and Scholtz, who found very large *m* values for GdmCl (up to 50 cal mol^{-1} M^{-1}), and a value for NaCl (27.4 cal mol^{-1} M^{-1}) that is greater than that obtained for urea (*17*). We suspect that these values contain large contributions from electrolyte effects on helix-stabilizing side chain charge interactions in the peptides studied in ref *17*. When those studies were carried out, sequence-dependent electrostatic contributions to helix stability were less well characterized, compared to the high degree of detail established by systematic studies of Serrano (*27*). Using the helix prediction algorithm AGADIR (*27*), the 14-mer peptide of the series studied by Smith and Scholtz is predicted to have an f_H of 0.44 in 0.01 M NaCl at pH 7.0 compared to an f_H of 0.31 in 1 M NaCl. For comparison, the AGADIR prediction of alahel gives the following: f_H = 0.46 in 0.01 M NaCl at pH 7.0 and f_H = 0.48 in 1 M NaCl. This considerable salt dependence of helical stability in the peptides previously studied accounts for the very high GdmCl and NaCl *m* values, and is probably responsible for the curved plots of ln *s* versus solute concentration obtained in some cases by these authors.

We consistently found that the *m* values for both urea and GdmCl were around 20% higher at 2 °C than the values obtained between 8 and 22 °C. A possible explanation may be the enhanced structuring of water at temperatures approaching its freezing point, and the suppression of water structuring by the denaturants, which increases denaturant effectiveness at low temperatures. In support of this interpretation, Smith and Scholtz determined an *m* value for helix denaturation by urea in 3 M NaCl (in which the freezing point of water is highly suppressed) at 0 °C of ∼19 cal mol^{-1} M^{-1} (*17*), similar to the limiting value obtained in this study. Regardless of whether this is the correct explanation, we suggest that it may be preferable to determine *m* values in studies of isolated helical peptides at temperatures around 10−15 °C rather than near 0 °C. We also suggest that the limiting values obtained in the range of 8−22 °C for urea (m = 20 cal mol^{-1} M^{-1}) and GdmCl (m = 28 cal mol^{-1} M^{-1}) may be suitable values for considering helix contributions to denaturant activities on proteins, which are generally determined at temperatures well above 0 °C.

Why is GdmCl ∼1.4-fold more effective than urea in unfolding an isolated peptide helix? The dominant contribution to the stability of isolated helical peptides lacking side chain electrostatic contributions to stability is the enthalpic contribution of intramolecular hydrogen bond formation in water (*18*, *28*). Consistent with the absence of significant burial of hydrophobic surface upon helix formation in a (largely) polyalanine peptide, and the near-zero heat capacity change upon unfolding (*18*), the hydrophobic effect makes a minimal contribution to helix stability in isolated polyalanine-based peptides. In line with recent evidence (*3*), these denaturants unfold helices by competing with intramolecular hydrogen bonds. It is not obvious why GdmCl should be more effective than urea in doing this, but the relative activities might relate to the partitioning of these weakly hydrated solutes (*29*, *30*) between the bulk solution and the polypeptide surface. For example, Courtenay et al. (*31*) measured preferential interaction coefficients using vapor pressure osmometry and calculated partition coefficients that characterize accumulation of solutes (like urea and Gdm$^+$) near protein surfaces. The ratio of partition coefficients for accumulation of urea ($K^{nat}_{urea} \sim 1.1$) and Gdm$^+$ ($K^{nat}_{Gdm^+} \sim 1.6$) at the surface of BSA of ∼1.5 is similar to the ratio of denaturant effectiveness (∼1.4) measured here.

In contrast to the small enhanced denaturant activity of GdmCl over urea measured for helix unfolding, GdmCl was 3.5-fold more effective than urea as a denaturant of trpzip1. Although dominated by the cross-strand indole ring pairings, the interactions contributing to the folded state of trpzip peptides are heterogeneous. We apply the following argument to estimate the contribution of the Trp indole interactions to denaturant sensitivities in trpzip1. Swapping the central order of Asn and Gly in trpzip2 to Gly and Asn in trpzip1

considerably destabilizes the folded state, so we assume that the β-turn makes only a small contribution to the folded state stability in trpzip1, and to the denaturant dependence. For example, the position of Asn at the $i + 1$ position of the β-turn in trpzip1 limits potential contributions from hydrogen bonds involving the side chain amide to turn stability (*32*), as is apparent in the NMR structures of trpzip1 and tripzip2 [*12*; Protein Data Bank (PDB) entries 1LE0 and 1LE1, respectively]. We therefore neglect any significant contribution from the central four β-turn residues to denaturant sensitivity. Subtracting out the contribution of the salt bridge interaction leaves 358 cal mol^{-1} M^{-1} for GdmCl denaturation (see the Results). The trpzip peptides contain four cross-strand peptide bonds. Unfortunately, the relative contribution of hydrogen bonding and Trp indole interactions can only be estimated. However, if the per-residue values for urea (20 cal mol^{-1} M^{-1}) and GdmCl (28 cal mol^{-1} M^{-1}) determined on alahel are applied to the four residues in trpzip1 that have both amide carbonyl and NH groups involved in cross-strand hydrogen bonds [Thr3, Glu5, Lys8, and Thr10 (*12*)], then the per-indole *m* values for denaturant attenuation of indole−indole interactions are around 12 cal mol^{-1} M^{-1} for urea [(126 − 4 × 20)/4] and 61 cal mol^{-1} M^{-1} [(358 − 4 × 28)/4] for GdmCl. If, as suggested by Searle et al. (*33*), cross-strand hydrogen bonding makes only a small contribution to the stability of small β-hairpins, then the per-indole *m* values for denaturant attenuation of indole−indole interactions for both urea and GdmCl will be somewhat larger than these estimates. However, the conclusion that GdmCl is considerably more effective (by 3−5-fold) than urea in attenuating indole−indole interactions in trpzip1 is clear.

Is there something special about the interaction of GdmCl with the Trp indole group that makes it particularly effective in competing with indole−indole interactions? We recently showed that the Gdm^+ cation is only weakly hydrated (*29*, *34*), forming hydrogen bonds in the molecular plane, but interacting weakly with waters above and below the molecular plane. We suggested that the Gdm^+ ion is able to form "stacking" interactions with the Trp indole (and probably other aromatic amino acid side chains) in which weakly hydrating waters are displaced from the interacting surfaces into bulk water with a favorable entropic contribution to the free energy of exposure (*29*, *34*). This scenario is similar to that suggested by Kuharski and Rossky (*35*) and Muller (*36*) for the dominant effect of denaturants in promoting the exposure of hydrophobic amino acid side chains by displacing hydration shell waters, and is supported by observations of aromatic stacking with the arginine guanidine group (structurally homologous with Gdm^+) in protein crystal structures (*37*). In addition, the guanidine group is a positively charged, delocalized π-system (*38*). The dipole−dipole nature of the indole interaction, apparent in the staggered offset orientations of the interacting indole rings in the trpzip NMR structures (*12*; PDB entries 1LE0 and 1LE1), and the strong interaction between the Trp indole ring and alkali metals (*39*), supports an electrostatic contribution to the indole−Gdm^+ interaction. While urea is likely to be effective in displacing waters from the surface of nonpolar amino acid side chains (*35*, *36*), it lacks these specific features that may make Gdm^+ a particularly good denaturant of structure stabilized by interactions involving aromatic groups. These considerations indicate that the interactions of the denaturants with the trpzip peptides are unlikely to be representative of their interactions with the aliphatic side chains of amino acids that contribute to stabilizing hydrophobic interactions in proteins. However, the availability of small peptides in which hydrophobic interactions provide the dominant contribution to stability (*32*, *40*, *41*) will be useful in extending the approach described here to measuring the relative effectiveness of urea and GdmCl in attenuating hydrophobic interactions.

Concluding Comments. The relative abilities of urea and GdmCl to attenuate specific contributions (hydrogen bonding and indole ring interactions) to protein stability differ markedly, consistent with the expectation that the general 2−2.5-fold enhanced effectiveness of GdmCl over urea as a protein denaturant results from different effects on the heterogeneous contributions to protein stability. GdmCl is only slightly more effective than urea in attenuating hydrogen bond interactions but is considerably more effective against indole−indole interactions (and probably all hydrophobic interactions involving aromatic amino acid side chains). What remains to be determined is the relative effectiveness of these denaturants on the more generalized hydrophobic interactions involving the aliphatic amino acid side chains. The strategy of isolating these interactions within small model peptides in which dominant contributions to stability may be "designed in" should allow the relative effectiveness of different denaturants to be determined for these contributions to protein stability.

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