Urea Promotes Polyproline II Helix Formation: Implications for Protein Denatured States†

Shelly J. Whittington, Brian W. Chellgren, Veronique M. Hermann, and Trevor P. Creamer*

Center for Structural Biology, Department of Molecular and Cellular Biochemistry, University of Kentucky, 800 Rose Street, Lexington, Kentucky 40536-0298

Received January 21, 2005; Revised Manuscript Received March 1, 2005

ABSTRACT: It is commonly assumed that urea denatures proteins by promoting backbone disorder, resulting in random-coil behavior. Indeed, it has been demonstrated that highly denatured proteins obey random-coil statistics. However, the random-coil model is specified by the global geometric properties of a polymeric chain and does not preclude locally ordered backbone structure. While urea clearly disfavors a compact native structure, it is not clear that the resulting backbone conformations are disordered. Using circular dichroism (CD) spectroscopy, we demonstrate that urea promotes formation of left-handed polyproline II (P_{II}) helical structures in both short peptides and denatured proteins. The observed increase in P_{II} content is sequence-dependent. These data indicate that denatured states possess significant amounts of locally ordered backbone structure. It is time for the formulation of new denatured-state models that take into account the presence of significant local backbone structure. Criteria for such models are outlined.

To understand the process by which a protein folds, it is necessary to acquire knowledge about not just the final folded state of the protein but also its unfolded states. There is a long history of using chemical denaturants to unfold proteins. Characterization of unfolded (or denatured) states began in earnest with the groundbreaking work of Flory (*1*), followed by experimental corroboration by Tanford and colleagues who used guanidine hydrochloride (GdnHCl)[1] as a denaturing agent (*2*). Tanford et al. demonstrated that proteins denatured in high concentrations of GdnHCl exhibit random-coil behavior. However, he warned that denatured proteins do not need to be devoid of structure to obey random-coil statistics (*2*).

Recent work by Plaxco and co-workers (*3*) reconfirmed that chemically denatured proteins obey random-coil statistics. The extent or type of local backbone structure present in denatured states remains unknown. One can imagine four possibilities for the behavior of proteins and peptides in chemical denaturants. At one extreme, denaturants promote complete backbone disorder. All sterically allowable conformations would then be of equal energy. At the other extreme, denaturation only disrupts tertiary packing, whereas the backbone remains highly ordered. Recently Fitzkee and Rose (*4*) deliberately constructed a "physically absurd" model at this extreme and demonstrated that it reproduces random-coil statistics.

One can imagine two possibilities between these two extremes. One is that each residue follows conformational propensities observed in surveys of coil regions in proteins of known structure (*5*−*7*). In coil libraries, each residue would have defined propensities to adopt certain conformations. These propensities would be insensitive to both the local sequence and solution conditions.

The final possibility is in line with the hypothesis of Tiffany and Krimm (*8*, *9*). According to this model, denatured states contain local structure, the specificity of which depends on both sequence and solution conditions. Tiffany and Krimm observed that the circular dichroism (CD) spectra collected for chemically denatured proteins bear a strong resemblance to spectra obtained for proline, lysine, glutamate, and aspartate homopolymers. The latter polymers adopt the left-handed polyproline II (P_{II}) helical structure as their dominant conformation in aqueous solution. This resemblance led Tiffany and Krimm to hypothesize that proteins denatured in urea or GdnHCl possess significant P_{II} helical content (*8*). Much attention has been given to this hypothesis in recent years (*10*−*21*).

Here, we study the effect that urea has upon local backbone structure in a series of peptides and unfolded proteins. Using CD spectroscopy, we demonstrate that urea promotes P_{II} helical structure. The P_{II} helical content increases with increasing concentrations of urea. This is true for both peptides and proteins, supporting the hypothesis of Tiffany and Krimm. Clearly, new/revised models for denatured proteins are required to explain random-coil statistics and the presence of local structure. The lack of specificity of the former needs to be reconciled with the observed specificity in the latter. This is the reconciliation problem outlined by Plaxco and co-workers (*22*). In this work, we outline criteria that new unfolded protein models must obey.

MATERIALS AND METHODS

Peptides (Table 1) were obtained from Peptidogenic Research and Co. (Livermore, CA) and the W. M. Keck

† This work was supported by a grant to T.P.C. from the National Science Foundation (MCB-0110720). The University of Kentucky Center for Computational Sciences provided support for B.W.C.

* To whom correspondence should be addressed. Telephone: (859) 323-6037. Fax: (859) 323-1037. E-mail: trevor.creamer@uky.edu.

[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; IFABP, intestinal fatty-acid-binding protein; P_{II}, polyproline II; R_G, radius of gyration.

10.1021/bi050124u CCC: $30.25
Published on Web 03/29/2005

Table 1: Peptides Employed in This Work

name	sequence[a]
P7	Ac-Pro_7-Gly-Tyr-NH_2
A3	Ac-Pro_3-Ala_3-Pro_3-Gly-Tyr-NH_2
V3	Ac-Pro_3-Val_3-Pro_3-Gly-Tyr-NH_2
KAK	Ac-Lys_2-Ala_7-Lys_2-Gly-Tyr-NH_2
KQK	Ac-Lys_2-Ala_3-Gln-Ala_3-Lys_2-Gly-Tyr-NH_2
KVK	Ac-Lys_2-Ala_3-Val-Ala_3-Lys_2-Gly-Tyr-NH_2

[a] Peptides are blocked to remove electrostatic interactions. The -Gly-Tyr pairs facilitate concentration determination.

Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). They were purified to ≥95% homogeneity by reverse-phase HPLC, and their identities were confirmed using mass spectrometry. Stock solutions were prepared by dissolving peptides in a buffer containing 5 mM potassium phosphate, 5 mM sodium fluoride, and 0.02% sodium azide, with the pH adjusted to 7. The peptide concentration was determined using the method of Brandts and Kaplan (*23*). Absorbance was measured in a 1.0 cm pathlength cuvette in a Beckman-Coulter DU 640B spectrophotometer.

Equine skeletal muscle myoglobin, hen egg-white lysozyme, and bovine β-casein were purchased from Sigma (St. Louis, MO) and used without further purification. Apomyoglobin was generated using the protocol of Teale (*24*). The disulfide bonds in lysozyme were reduced in freshly prepared 5 mM dithiothreitol (DTT). Purified rat intestinal fatty-acid-binding protein (IFABP) was the kind gift of Dr. Ira Ropson of the Pennsylvania State University. We used the Δ27GG variant where 27 residues encompassing a flap-like region over the binding cavity are replaced with two glycine residues (*25*).

Circular dichroism (CD) spectra were measured with a Jasco J-810 spectrapolarimeter employing a 1 mm pathlength quartz cuvette containing solutions of approximately 100 μM peptide or 2−10 μM protein. Spectra were collected at 5 °C with a 0.5 nm resolution and a scan rate of 200 nm min^{-1}. Spectra are the averages of 30 scans. To facilitate analysis of uncertainties, each set of spectra were measured using at least three individually prepared solutions.

RESULTS

Peptides. Proline homopolymers and oligopeptides form P_{II} helices in aqueous solution and possess CD spectra diagnostic for this conformation (*26*−*28*). Such spectra have a strong negative band at 205 nm and a weaker positive band at 228 nm (Figure 1a). Because of the absorbance characteristics of tertiary versus secondary amides, the positions of these bands shift to lower wavelengths for peptides without prolines (*29*). A P_{II} helical peptide devoid of proline would have a positive band around 218−222 nm (*13*). The P_{II} helix is the only secondary structure known to have a positive band in the wavelength range of 218−228 nm (*29*). The intensity of the positive band is taken to be proportional to the P_{II} helical content. The lack of a positive band does not necessarily indicate an absence of P_{II} content (*11*, *13*, *14*). The conformational averaging resulting from the presence of other local structures can lead to negative ellipticity in this region. Positively increasing ellipticity in this wavelength range with changing conditions can indicate an increase in the P_{II} helix content.

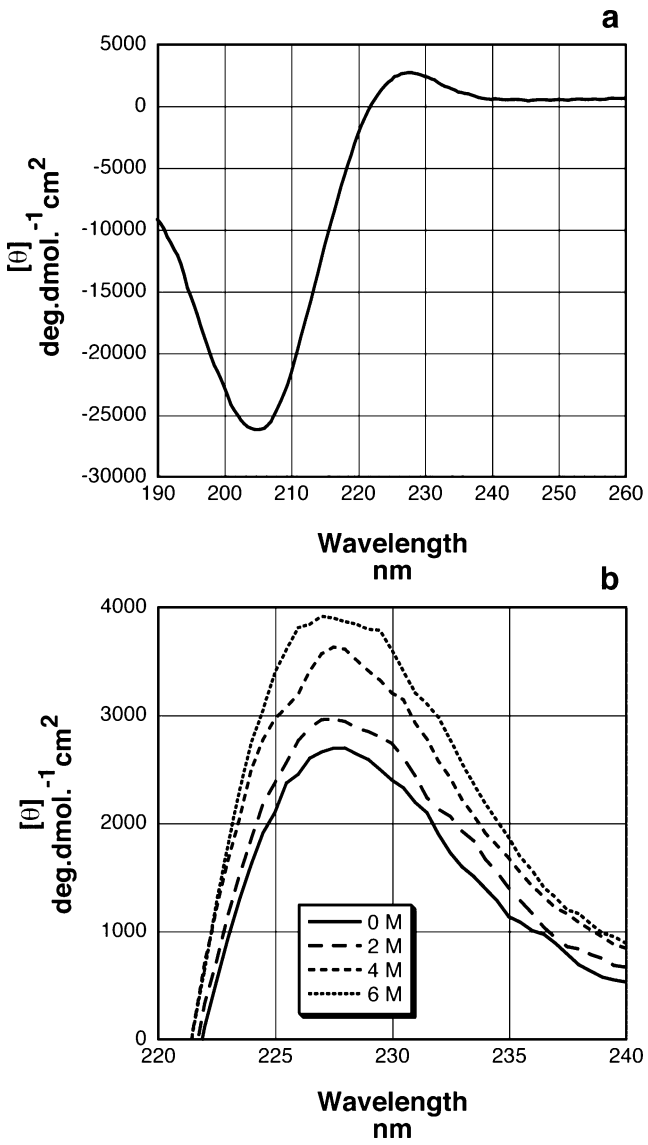

FIGURE 1: (a) CD spectrum of peptide P7 in phosphate buffer at pH 7 and 5 °C. (b) Increase in intensity of P7 positive band as a function of the urea concentration.

It has been shown that the P_{II} content of proline polymers and oligopeptides is significantly increased in the presence of urea or GdnHCl as indicated by an increase in the intensity of the positive band in CD spectra (*9*, *27*). The increasing intensity of the positive band with an increasing urea concentration is shown in Figure 1b for the P7 peptide. Because homopolymers and oligopeptides of proline are sterically restricted to form P_{II} helices (*30*), we interpret this to mean that urea rigidifies the backbone.

The peptides listed in Table 1 were examined to answer the question of what happens to the P_{II} content of nonproline residues in the presence of urea. Two proline-based peptides (peptides A3 and V3) were investigated. Alanine possesses a high propensity to adopt the P_{II} helical structure (*11*, *14*, *27*, *31*, *32*). V3 has been shown to have a low P_{II} content (*14*), although the content can be modulated by small changes in solution conditions (*33*). Because both peptides contain proline residues, each will have a base level of P_{II} content that is due to those residues (*14*, *27*).

The intensities of the positive bands in the CD spectra for peptides P7, A3, and V3 are plotted in Figure 2 as a function

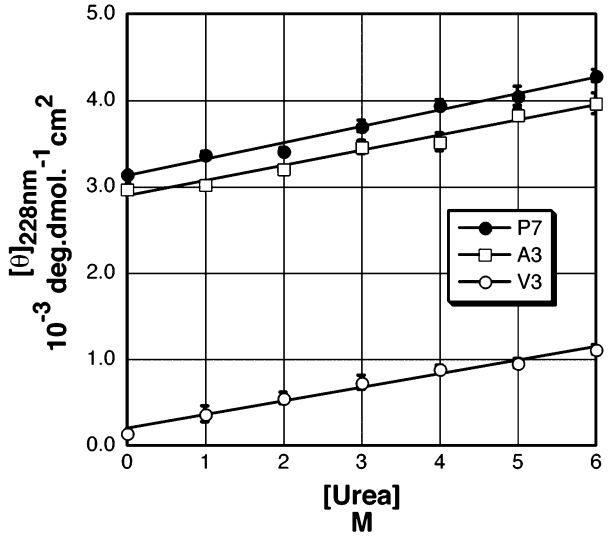

FIGURE 2: Variation in intensity of the positive bands at 228 nm measured in the CD spectra of peptides P7, A3, and V3 with increasing concentrations of urea.

of the urea concentration. Interestingly, the intensities of the positive bands increase linearly with urea concentrations up to 6 M. For P7, the line of best fit has a correlation coefficient of 0.99 and a slope of 190° dmol^{-1} cm^2 M^{-1}. Peptide A3 has a slightly shallower slope of 175° dmol^{-1} cm^2 M^{-1} (correlation coefficient of 0.99). Peptide V3 has a lower slope of 158° dmol^{-1} cm^2 M^{-1} (correlation coefficient of 0.99). The differing slopes suggest that the P_{II}-inducing effect of urea is sequence-dependent.

The alanine-based peptides KAK, KQK, and KVK (Table 1) are devoid of proline and have varying P_{II} helix contents, generally less than proline-based peptides. This is evident from the lack of positive ellipticity in the range of 218−222 nm in CD spectra collected for these peptides in aqueous solution (Figure 3). Valine has a low P_{II} helix-forming propensity; alanine has a high propensity; and glutamine has a higher P_{II} propensity (*14*, *27*, *31*). Upon adding 6 M urea, all three peptides adopt the P_{II} conformation (Figure 3a). The ellipticity at 218 nm is plotted as a function of the urea concentration in Figure 3b. This wavelength corresponds to the position of the positive band that appears at low urea concentrations with these peptides. The increase in P_{II} content for these peptides is nonlinear, showing a rapid increase at lower urea concentrations (up to 3−4 M), followed by more gentle increases, appearing to converge and become asymptotic at high concentrations. We interpret the initial steep increase as being consistent with a transition from a broad ensemble of conformations to an ensemble dominated by the P_{II} conformation. The shallower, more linear increase at higher urea concentrations is probably due to further restriction in the ensemble to the P_{II} conformation.

Equine Apomyoglobin, Reduced Hen Egg-White Lysozyme, and Rat Δ27GG-IFABP. We have chosen to examine a protein from each of three distinct fold classes (*34*): apomyoglobin (predominantly α helical in its native state), reduced lysozyme (mixed α and *β*), and IFABP (mostly *β* sheet). CD spectra for these proteins in concentrations of urea at which they would be considered fully denatured are shown in parts a−c of Figure 4. The absorbance properties of high concentrations of urea prevent collection of spectra at wavelengths below about 215 nm. It is clear that the

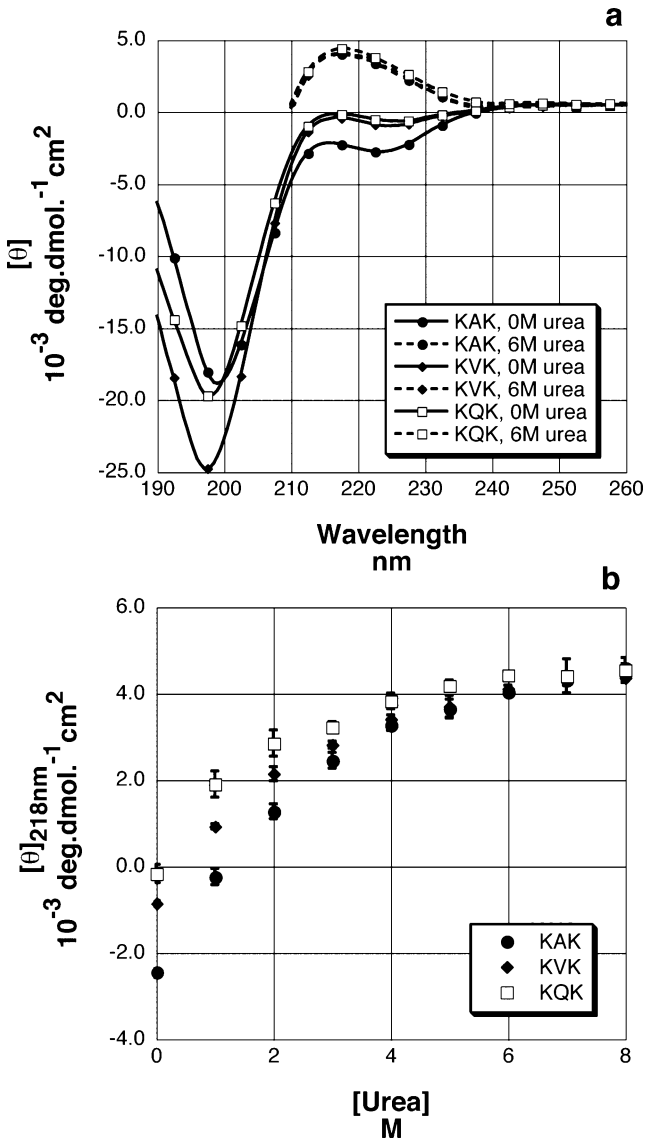

FIGURE 3: (a) CD spectra of the alanine-based KAK, KVK, and KQK peptides collected at 5 °C in the absence and presence of 6 M urea. Note that the KAK and KVK 6 M urea spectra lie on top of one another. Spectra in the presence of 6 M urea are truncated at 210 nm because of the absorbance properties of urea at lower wavelengths. (b) Increase in intensity of the ellipticity at 218 nm as a function of the urea concentration for KAK, KVK, and KQK.

ensemble of backbone conformations is changing for each protein as the urea concentration is increased. Furthermore, the spectra differ from one protein to the next, indicating that there is sequence-specificity in the ensemble of denatured states. For all three proteins, the ellipticity around 218−222 nm increases with an increasing denaturant. Apomyoglobin and IFABP possess a local maximum in this vicinity, indicating an increase in the P_{II} helix content. Although lysozyme does not possess an obvious local maximum, we interpret the increasing ellipticity in this wavelength range as indicative of an increasing P_{II} helix content.

β-Casein. *β*-Casein is an intrinsically disordered protein (*35*, *36*). This allows for examination of the effects of urea over the full range of concentrations rather than just high concentrations. CD spectra for *β*-casein in urea concentrations ranging from 0 to 8 M are shown in Figure 4d. As with the other proteins examined, there is a clear increase in ellipticity around 218−225 nm. A local maximum appears

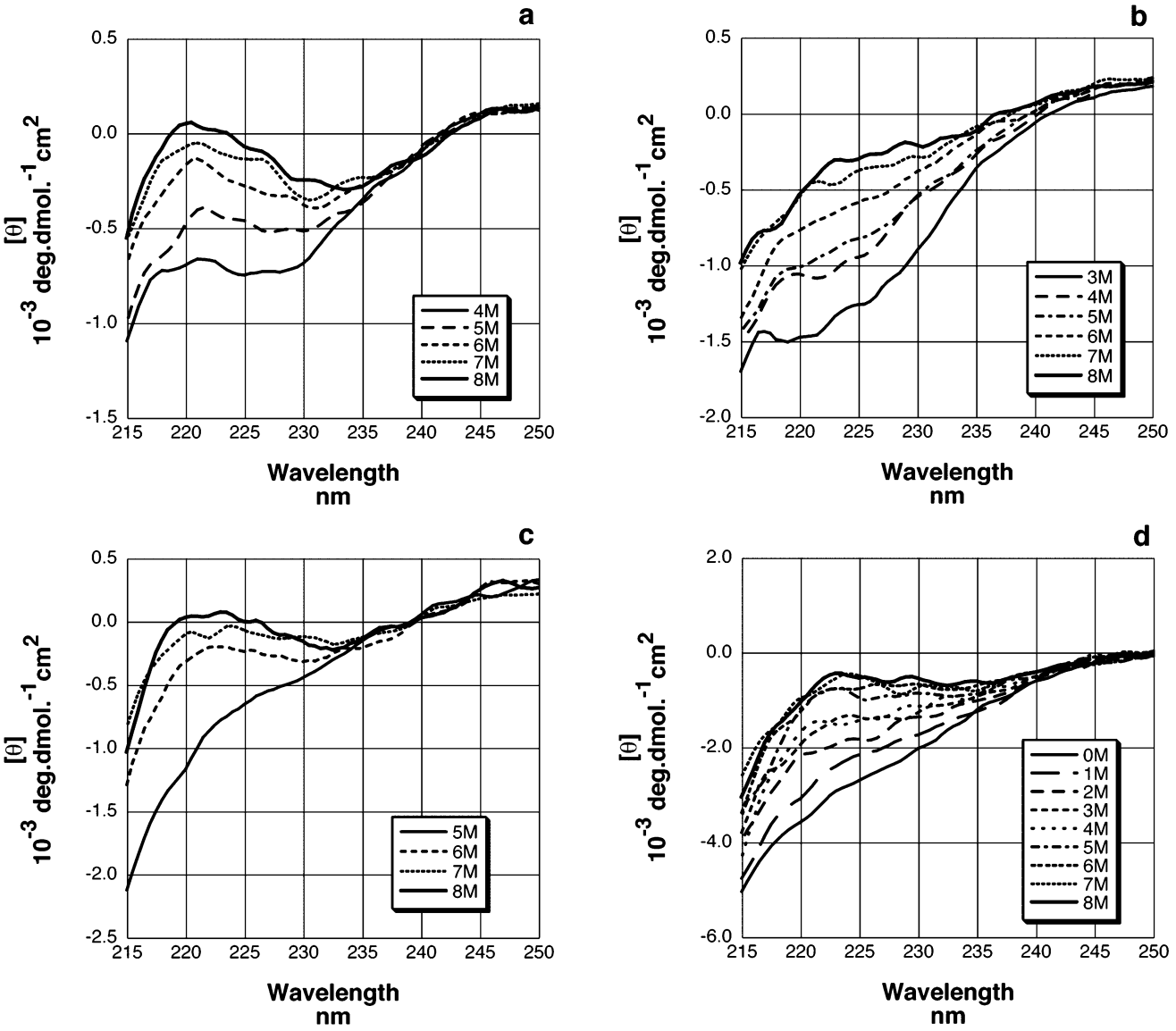

FIGURE 4: CD spectra collected at 5 °C for (a) equine apomyoglobin, (b) hen egg-white lysozyme, (c) rat 27GG-IFABP, and (d) β-casein in increasing concentrations of urea.

at about 223 nm at higher urea concentrations, indicating an increasing P_{II} helix content. β-Casein has a high proline content (16% of the residue content), which would shift the observed local maximum to a higher wavelength than those observed for the other proteins.

DISCUSSION

CD spectroscopy is incapable of determining the presence of random coil. This absorbance technique measures the average local backbone conformational ensembles of peptides and proteins. The random coil is defined by global geometric properties of a chain such as the radius of gyration (R_G). CD spectroscopy and other ensemble-based spectroscopies that directly probe the backbone cannot measure such properties. This makes CD spectroscopy an ideal method for examining the local backbone behavior of denatured proteins. Given that an unfolded or denatured polypeptide chain exists in an ensemble of conformations, a technique that probes the ensemble is more useful than one that provides higher resolution information on a single structure.

All six peptides (Table 1) possess significant P_{II} helix signals in CD spectra collected in the presence of urea. For peptides P7, A3, and V3, this is not surprising. Tiffany and Krimm (*9*) demonstrated over 30 years ago that the P_{II} content of homopolymers of proline could be increased by the addition of urea or GdnHCl. P7, A3, and V3 are all proline-based; therefore, their behavior (Figure 2) could be due to the effects of urea upon the prolines. The alanine-based peptides KAK, KQK, and KVK are however devoid of proline and yet clearly adopt the P_{II} conformation in urea (Figure 3). Similar behavior has been observed recently by Kallenbach and co-workers for a peptide of sequence Ac−$O_2A_7O_2$−NH_2 (O = ornithine) in GdnHCl (*37*). The P_{II} helix-inducing effect of urea is therefore not limited to proline. The differing slopes obtained from Figure 2 and the behavior observed for the three nonproline-based peptides in Figure 3 indicate that the P_{II}-inducing effects of urea are sequence-dependent.

The magnitudes of the ellipticities at 218 nm for KAK, KVK, and KQK (Figure 3) appear to contradict existing data that indicate valine has the lowest P_{II} helix-forming propensity (*16*, *27*, *31*). Eker et al. (*38*) have shown that a trivaline peptide prefers more extended β-strand-like conformations to the P_{II} helical conformation, providing additional evidence

that valine has a low P_{II} helix-forming propensity. The alanine-based KAK, KVK, and KQK peptides employed in this work can readily adopt other secondary structures. The high α-helix-forming propensity of alanine (*39*) will result in these peptides having a significant tendency to adopt that conformation. This is reflected in the negative bands observed at 222 nm in Figure 3a. Valine possesses a low α-helix propensity (*39*), reducing the amount of that conformation in the KVK peptide when compared to KAK. Because the alanines in KVK are occupying the α-helical conformation to a lesser extent, we believe they default to a higher P_{II} content, leading to a higher overall P_{II} content for KVK when compared to KAK. Note that the valine in KVK is not necessarily adopting the P_{II} conformation to a significant extent. These data reinforce the notion that the observed P_{II} contents, both in the presence and absence of urea, are sequence-dependent.

Spectra collected for the four proteins in increasing urea concentrations demonstrate that the ensemble of backbone conformations, as measured by CD spectroscopy, change with the urea concentration (Figure 4). Increasing ellipticities and the appearance of local maxima in the wavelength range of the positive band diagnostic of P_{II} helix content indicate that these denatured proteins gain P_{II} helix content as the urea concentration is increased. Each protein has its own distinct set of CD spectra in urea, indicating that they have different ensembles of backbone conformations. The effects of urea are clearly sequence-dependent.

The CD data collected for the four proteins agree with previous studies. Recently, Wright and co-workers used residual dipolar couplings to characterize unfolded states of apomyoglobin (*40*). They found that for apomyoglobin in 8 M urea the backbone favored extended β and P_{II} conformations. These findings are in part echoed in our CD data, which indicate an increase in the P_{II} content at high urea contents (Figure 4a). The denatured lysozyme spectra (Figure 4b) are similar to those obtained recently by Vernaglia et al. (*41*) who used GdnHCl as a denaturant. These authors demonstrated that denatured lysozyme could form amyloid fibrils. We see no evidence of such a process in our experiments, most likely a result of differences in solution conditions. The Δ27GG-IFABP spectrum in 8 M urea is similar to that obtained by Burns et al. (*42*) for full-length IFABP in 8.2 M urea.

β-Casein is an intrinsically disordered protein possessing P_{II} helix (*35*, *36*). On the basis of the presence of P_{II} structure in the absence of urea, plus the high proline content of β-casein (16%), it would be reasonable to expect a significant increase in the P_{II} content upon addition of urea. In support of this assertion, there is an increase in ellipticity at wavelengths corresponding to the P_{II} diagnostic positive band with an increasing urea concentration (Figure 4b). Interestingly, Qi et al. (*35*) find that a peptide corresponding to residues 1−25 of β-casein gains P_{II} structure upon addition of sodium dodecyl sulfate (SDS). In contrast, Kallenbach and co-workers (*37*) find that SDS promotes α-helical structure in an alanine-based peptide known to form a P_{II} helix. There are clearly important sequence and cosolvent effects that require further examination.

The mechanism by which urea promotes P_{II} helical structure is not clear. Nozaki and Tanford (*43*) and Robinson and Jencks (*44*) proposed that urea interacts favorably with the polypeptide backbone. Such an interaction would influence the conformational behavior of the backbone, perhaps favoring the P_{II} helical conformation. This in turn would be modulated by the nature of the side chains, leading to the observed sequence dependence.

Plaxco and co-workers (*3*) recently performed an analysis of radii of gyration (R_G) obtained from scattering experiments on denatured proteins. They concluded that these follow the statistical behavior expected for a random coil with excluded volume. According to the work of Flory (*1*), the R_G of such chains should depend on chain length, not sequence, and only weakly upon solution conditions. Plaxco and co-workers (*3*) note that average R_G values determined in scattering experiments do not change with increases in the denaturant concentration once past the midpoint of the unfolding transition. These data provide significant evidence for the random-coil behavior of highly denatured proteins.

The fact that denatured proteins are random coils is seemingly at odds with our observations. We have found significant local backbone structure that is sensitive to both the urea concentration and sequence. Increasing the urea concentration leads to an increase in P_{II} helix content in both peptides and proteins. One might expect an increase in extended P_{II} helix content would lead to an increase in R_G. Segel et al. (*45*) have examined the behavior of cytochrome *c* denatured with GdnHCl. Using small-angle X-ray scattering, they demonstrated that R_G was insensitive to the GdnHCl concentration in the range of ∼3.5−5 M. However, analysis of associated Kratky plots indicated that the average size of the denatured protein increased with an increasing denaturant concentration. Furthermore, Fitzkee and Rose (*4*) demonstrated that R_G values are insensitive to local backbone structure. Random-coil behavior is an important spatial constraint given that denatured proteins conform to such statistics. However, random-coil behavior does not impose limits upon the types of local conformations available to denatured proteins.

We now return to the four proposed models for backbone conformational behavior in denatured proteins. These are (1) completely disordered backbone conformations (i.e., all sterically allowed conformations are isoenergetic), (2) backbone conformations that obey the distributions observed in coil libraries, (3) a Tiffany and Krimm-like model consisting of a dynamic ensemble of conformations where local stretches of sequence fluctuate in and out of secondary structures, and finally (4) a highly structured model in which much of the secondary structure remains intact but the tertiary structure is abolished. As pointed out by Fitzkee and Rose (*4*), the last of these models is physically absurd, even though it reproduces random-coil statistics. As such, this model can be discarded without further discussion.

On the basis of our data and that of others, we can dismiss the first of these models. Clear signals indicating significant P_{II} helical content have been observed, negating the idea that the backbone is completely disordered in the presence of high concentrations of urea. Indeed, we find just the opposite result, with the backbone becoming more ordered at higher concentrations. Specifically, we find that urea induces local P_{II} helical structure.

The coil library model can also be dismissed as a model for denatured states. Although such libraries demonstrate varying P_{II} propensities for residues (*46*), the levels are not

overly significant. More conclusively, propensities derived from coil libraries are by definition independent of both sequence and solution conditions. However, our data indicate that both the sequence and increasing urea concentration affect the ensemble of backbone conformations.

By default, this analysis leaves the Tiffany and Krimm-like model. As first suggested by these workers (*8*), proteins unfolded in the presence of high concentrations of chemical denaturant possess significant P_{II} helix content. We are not implying that high concentrations of urea induce a single long P_{II} helix, but rather that the propensity for segments of the polypeptide chain to adopt this conformation is higher under such conditions. We are also not stating that certain stretches of sequence will always be in the P_{II} helical conformation when the protein is chemically denatured. Rather, the backbone will fluctuate between various allowed conformations with the distribution of states being determined by both sequence and solution conditions. Protein denaturation using urea and GdnHCl (*9*, *27*) can then be considered a transition from a globally structured state to a dynamic ensemble of locally structured states.

New models of protein denatured states are required. Such models need to satisfy the following criteria:

(1) Protein chains modeled under high denaturant concentrations must obey random-coil statistics because it has been demonstrated unequivocally that highly denatured proteins conform to such behavior (*3*).

(2) Denatured-state models must allow formation of local backbone structure. The data presented here indicate significant levels of P_{II} helix in the presence of high concentrations of urea, but the presence of other structures is not precluded. Consequently, new models must include propensities to adopt all allowable local structures.

(3) Propensities to adopt local backbone structures need be sensitive to both solution conditions and local sequence. Our data indicate that backbone ensembles possess both urea sensitivity and sequence dependence. Other workers have suggested that increasing the temperature increases the β-strand content of unfolded proteins (*11*, *47*).

(4) Finally, new denatured-state models must allow for the presence of residual nonlocal structure in denatured proteins. There is increasing evidence that some proteins retain interactions between nonlocal sections of sequence even in the presence of high levels of the chemical denaturant (*48*−*51*).

The development of new denatured-state models will require considerable additional information, not the least of which are propensities for residues to adopt various conformations under a variety of solution conditions. The sequence dependencies of these propensities also need to be determined. A number of groups are in the process of obtaining propensity data and analyzing their determinants (for examples, see refs *10*−*21*). It seems likely that useful new models for protein denatured states will soon emerge. This will lead to better descriptions of where proteins start the folding process and ultimately to a full understanding of the folding process.

ACKNOWLEDGMENT

The authors thank George Makhatadze, Rohit Pappu, Gary Pielak, and George Rose for helpful discussions and Steven Smith for assistance. We are grateful to Dr. Ira Ropson for the gift of purified Δ27GG-IFABP.

REFERENCES

1. Flory, P. J. (1969) *Statistical Mechanics of Chain Molecules*, John Wiley and Sons, New York.
2. Tanford, C. (1968) Protein denaturation, *Adv. Protein Chem. 23*, 121−282.
3. Kohn, J. E., Millett, I. S., Jacob, J., Zagrovic, B., Dillon, T. M., Cingel, N., Dothager, R. S., Seifert, S., Thiyagarajan, P., Sosnick, T. R., Hasan, M. Z., Pande, V. S., Ruczinski, I., Doniach, S., and Plaxco, K. W. (2004) Random-coil behavior and the dimensions of chemically unfolded proteins, *Proc. Natl. Acad. Sci. U.S.A. 101*, 12491−12496.
4. Fitzkee, N. C., and Rose, G. D. (2004) Reassessing random-coil statistics in unfolded proteins, *Proc. Natl. Acad. Sci. U.S.A. 101*, 12497−12502.
5. Serrano, L. (1995) Comparison between the ϕ distribution of the amino acids in the protein database and NMR data indicates that amino acids have various ϕ propensities in the random coil conformation, *J. Mol. Biol. 254*, 322−333.
6. Smith, L. J., Bolin, K. A., Schwalbe, H., MacArthur, M. W., Thornton, J. M., and Dobson, C. M. (1996) Analysis of main chain torsion angles in proteins: Prediction of NMR coupling constants for native and random coil conformations, *J. Mol. Biol. 255*, 494−506.
7. Swindells, M. B., MacArthur, M. W., and Thornton, J. M. (1995) Intrinsic propensities of amino acids, derived from the coil regions of known structures, *Nat. Struct. Biol. 2*, 596−603.
8. Tiffany, M. L., and Krimm, S. (1968) New chain conformations of poly(glutamic acid) and polylysine, *Biopolymers 6*, 1379−1382.
9. Tiffany, M. L., and Krimm, S. (1973) Extended conformations of polypeptides and proteins in urea and guanidine hydrochloride, *Biopolymers 12*, 575−587.
10. Shi, Z., Woody, R. W., and Kallenbach, N. R. (2002) Is polyproline II a major backbone conformation in unfolded proteins? *Adv. Protein Chem. 62*, 163−240.
11. Shi, Z., Olson, C. A., Rose, G. D., Baldwin, R. L., and Kallenbach, N. R. (2002) Polyproline II structure in a sequence of seven alanine residues, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9190−9195.
12. Ferreon, J. C., and Hilser, V. J. (2003) The effect of the polyproline II (PPII) conformation on the denatured state entropy, *Protein Sci. 12*, 447−457.
13. Rucker, A. L., and Creamer, T. P. (2002) Polyproline II helical structure in protein unfolded states: Lysine peptides revisited, *Protein Sci. 11*, 980−985.
14. Chellgren, B. W., and Creamer, T. P. (2004) Short sequences of non-proline residues can adopt the polyproline II helical conformation, *Biochemistry 43*, 5864−5869.
15. Eker, F., Cao, X., Nafie, L., and Schweitzer-Stenner, R. (2002) Tripeptides adopt stable structures in water. A combined polarized visible Raman, FTIR, and VCD spectroscopy study, *J. Am. Chem. Soc. 124*, 14330−14341.
16. Eker, F., Griebenow, K., Cao, X., Nafie, L. A., and Schweitzer-Stenner, R. (2004) Preferred peptide backbone conformations in the unfolded state revealed by the structure analysis of alanine-based (AXA) tripeptides in aqueous solution, *Proc. Natl. Acad. Sci. U.S.A. 101*, 10054−10059.
17. Drozdov, A. N., Grossfield, A., and Pappu, R. V. (2004) Role of solvent in determining conformational preferences of alanine dipeptide in water, *J. Am. Chem. Soc. 126*, 2574−2581.
18. Pappu, R. V., and Rose, G. D. (2002) A simple model for polyproline II structure in unfolded states of alanine-based peptides, *Protein Sci. 11*, 2437−2455.
19. Garcia, A. E. (2004) Characterization of non-α helical conformations in Ala peptides, *Polymer 45*, 669−676.
20. Kentsis, A., Mezei, M., Gindin, T., and Osman, R. (2004) Unfolded state of polyalanine is a segmented polyproline II helix, *Proteins 55*, 493−501.
21. Mezei, M., Fleming, P. J., Srinivasan, R., and Rose, G. D. (2004) Polyproline II helix is the preferred conformation for unfolded polyalanine in water, *Proteins 55*, 502−507.
22. Millett, I. S., Doniach, S., and Plaxco, K. W. (2002) Toward a taxonomy of the denatured state: Small angle scattering studies of unfolded proteins, *Adv. Protein Chem. 62*, 241−262.
23. Brandts, J. F., and Kaplan, L. J. (1973) Derivative spectroscopy applied to tyrosyl chromophores. Studies on ribonuclease, lima

bean inhibitors, insulin, and pancreatic trypsin inhibitor, *Biochemistry 12*, 2011−2024.
24. Teale, F. W. J. (1959) Cleavage of the haem-protein link by acid methylethyl ketone, *Biochim. Biophys. Acta 35*, 543.
25. Ogbay, B., Dekoster, G. T., and Cistola, D. P. (2004) The NMR structure of a stable and compact all-β-sheet variant of intestinal fatty acid-binding protein, *Protein Sci. 13*, 1227−1237.
26. Tiffany, M. L., and Krimm, S. (1968) Circular dichroism of poly-L-proline in an unordered conformation, *Biopolymers 6*, 1767−1770.
27. Kelly, M., Chellgren, B. W., Rucker, A. L., Troutman, J. M., Fried, M. G., Miller, A.-F., and Creamer, T. P. (2001) Host−guest study of left-handed polyproline II helix formation, *Biochemistry 40*, 14376−14383.
28. Helbecque, N., and Loucheux-Lefebvre, M. H. (1978) Synthesis and circular dichroism studies of two polypeptides H-[Gly-(Pro)3]-*n*-OH and H-[Gly-(Pro)4]*n*-OH, *Int. J. Pept. Protein Res. 11*, 353−362.
29. Woody, R. W. (1992) Circular dichroism and conformation of unordered polypeptides, *Adv. Biophys. Chem. 2*, 37−79.
30. Creamer, T. P. (1998) Left-handed polyproline II helix formation is (very) locally driven, *Proteins 33*, 218−226.
31. Rucker, A. L., Pager, C. T., Campbell, M. N., Qualls, J. E., and Creamer, T. P. (2003) Host−guest scale of left-handed polyproline II helix formation, *Proteins 53*, 68−75.
32. Schweitzer-Stenner, R., Eker, F., Griebenow, K., Cao, X., and Nafie, L. A. (2004) The conformation of tetraalanine in water determined by polarized Raman, FT-IR, and VCD spectroscopy, *J. Am. Chem. Soc. 126*, 2768−2776.
33. Chellgren, B. W., and Creamer, T. P. (2004) Effects of H_2O and D_2O on polyproline II helical structure, *J. Am. Chem. Soc. 126*, 14734−14735.
34. Levitt, M., and Chothia, C. (1976) Structural patterns in globular proteins, *Nature 261*, 552−558.
35. Qi, P. X., Wickham, E. D., and Farrell, H. M., Jr. (2004) Thermal and alkaline denaturation of bovine β-casein, *Protein J. 23*, 389−402.
36. Syme, C. D., Blanch, E. W., Holt, C., Jakes, R., Goedert, M., Hecht, L., and Barron, L. D. (2002) A Raman optical activity study of the rheomorphism in caseins, synucleins, and tau, *Eur. J. Biochem. 269*, 148−156.
37. Liu, Z., Chen, K., Ng, A., Shi, Z., Woody, R. W., and Kallenbach, N. R. (2004) Solvent dependence of PII conformation in model alanine peptides, *J. Am. Chem. Soc. 126*, 15141−15150.
38. Eker, F., Griebenow, K., and Schweitzer-Stenner, R. (2003) Stable conformations of tripeptides in aqueous solution studied by UV circular dichroism spectroscopy, *J. Am. Chem. Soc. 125*, 8178−8185.
39. Chakrabartty, A., Kortemme, T., and Baldwin, R. L. (1994) Helix propensities of the amino acids measured in alanine-based peptides without helix-stabilizing side-chain interactions, *Protein Sci. 3*, 843−852.
40. Mohana-Borges, R., Goto, N. K., Kroon, G. J., Dyson, H. J., and Wright, P. E. (2004) Structural characterization of unfolded states of apomyoglobin using residual dipolar couplings, *J. Mol. Biol. 340*, 1131−1142.
41. Vernaglia, B. A., Huang, J., and Clark, E. D. (2004) Guanidine hydrochloride can induce amyloid fibril formation from hen egg-white lysozyme, *Biomacromolecules 5*, 1362−1370.
42. Burns, L. L., Dalessio, P. M., and Ropson, I. J. (1998) Folding mechanism of three structurally similar β-sheet proteins, *Proteins 33*, 107−118.
43. Nozaki, Y., and Tanford, C. (1963) The solubility of amino acids and related compounds in aqueous urea solutions, *J. Biol. Chem. 238*, 4074−4081.
44. Robinson, D. R., and Jencks, W. L. (1965) The effect of compounds of the urea-guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds, *J. Am. Chem. Soc. 87*, 2462−2470.
45. Segel, D. J., Fink, A. L., Hodgson, K. O., and Doniach, S. (1998) Protein denaturation: A small-angle X-ray scattering study of the ensemble of unfolded states of cytochrome *c*, *Biochemistry 37*, 12443−12451.
46. Avbelj, F., and Baldwin, R. L. (2003) Role of backbone solvation and electrostatics in generating preferred peptide backbone conformations: Distributions of ϕ, *Proc. Natl. Acad. Sci. U.S.A. 100*, 5742−5747.
47. Yang, W. Y., Larios, E., and Gruebele, M. (2003) On the extended β-conformation propensity of polypeptides at high temperature, *J. Am. Chem. Soc. 125*, 16220−16227.
48. Neri, D., Billeter, M., Wider, G., and Wuthrich, K. (1992) NMR determination of residual structure in a urea-denatured protein, the 434-repressor, *Science 257*, 1559−1563.
49. Saab-Rincon, G., Froebe, C. L., and Matthews, C. R. (1993) Urea-induced unfolding of the α subunit of tryptophan synthase: One-dimensional proton NMR evidence for residual structure near histidine-92 at high denaturant concentration, *Biochemistry 32*, 13981−13990.
50. Shortle, D., and Ackerman, M. S. (2001) Persistence of native-like topology in a denatured protein in 8 M urea, *Science 293*, 487−489.
51. Klein-Seetharaman, J., Oikawa, M., Grimshaw, S. B., Wirmer, J., Duchardt, E., Ueda, T., Imoto, T., Smith, L. J., Dobson, C. M., and Schwalbe, H. (2002) Long-range interactions within a nonnative protein, *Science 295*, 1719−1722.

BI050124U