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Molecular Mechanism for the Denaturation of Proteins by Urea[†]

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ABSTRACT: Understanding protein−solute interactions is one of the sizable challenges of protein chemistry; therefore, numerous experimental studies have attempted to explain the mechanism by which proteins unfold in aqueous urea solutions. On the basis of kinetic evidence at low urea concentrations, ^{1}H NMR spectroscopic analysis, and molecular orbital calculations, we propose a mechanistic model for the denaturation of RNase A in urea. Our results support a direct interaction between urea and protonated histidine as the initial step for protein inactivation followed by hydrogen bond formation with polar residues, and the breaking of hydrophobic collapse as the final steps for protein denaturation. With the proposed model, we can rationalize apparently conflicting results in the literature about the mechanism of protein denaturation with urea.

The denaturation of proteins by urea was first reported around the beginning of the past century (*1*). In the 1930s, urea became the most commonly used denaturant agent in protein folding−unfolding studies, and subsequently, this reactant has been used in numerous studies of protein structure (*2*−*25*). Despite the routine use of urea, our understanding of the molecular mechanism by which this chemical denaturant causes protein unfolding is still rather limited. In the literature, it has been proposed that urea may exert its effect in two ways: by interacting directly with the protein provoking solvation of the polypeptide by urea and water (*7*, *19*) and indirectly by altering the structure of water and causing extensive changes in the behavior of the solvent environment itself that in turn weakens the hydrophobic effect (*8*, *14*). Mirsky and Pauling in 1936 proposed that urea causes protein denaturation by forming hydrogen bonds with protein amino acid side chains (*4*). On the other hand, on the basis of the hydrophobic effect, Tanford proposed that the conditions which led to the unfolding of a globular protein are usually conditions which diminish hydrophobic forces at the protein interior (*15*, *16*). Later, in the 1990s, Makhatadze and Privalov (*17*) on the basis of calorimetric titrations of protein solutions with denaturants showed a direct binding of urea with proteins and provided the thermodynamic characteristics of that reaction. All of these conflicting proposals and interpretations leave several open questions about the mechanism of protein denaturation with chaotropic agents (*4*, *7*, *9*−*12*, *17*, *23*, *25*). The motivation for this work was the development of a molecular model for protein inactivation with urea that could improve our understanding of the protein folding−unfolding phenomena by providing a plausible explanation of this fundamental process in protein structural stability, which has been one of the sizable challenges of modern structural chemistry.

The lack of strong experimental evidence for detailing the molecular mechanism for protein denaturation with urea compelled chemists to use and develop theoretical−-computational approaches to simulate the urea−protein denaturation process (*23*−*33*). With the help of MDS,[1] Bennion and Dagget suggested that urea denatures proteins through both direct and indirect mechanisms (*23*). Also, with MDS the frequency of interactions between urea and the amino acids was quantified (*31*). These authors proposed that urea solvates preferentially aromatic and nonpolar residues so that urea molecules are localized at the protein surface with specific accumulation around the less polar amino acids and the polypeptide backbone (*31*). This high variability of results (experimental and theoretical) in favor of or in opposition to the direct or indirect contact of urea with proteins has prevented an understanding of such fundamental interactions as the folding−unfolding behavior of proteins (*25*−*33*).

In this work, we have developed a model that focuses on understanding the initial steps of the molecular mechanism for protein denaturation by urea, using ribonuclease A as a model enzyme for protein structural studies. Our proposal is based on experimental evidence such as comparisons of enzyme inhibition kinetics at very low urea concentrations with those at high concentrations usually reported for unfolding experiments. Also, we performed ^{1}H NMR on RNase A in the absence and presence of urea to understand the perturbation of the enzyme with the denaturant agent. Furthermore, our experimental results were supported by some quantum chemistry calculations using FMO analysis (*34*−*36*).

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[1]Abbreviations: RNase A, bovine pancreatic ribonuclease A; ^{1}H NMR, proton nuclear magnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MDS, molecular dynamics simulations; FMO, frontier molecular orbital analysis; D, debye.

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MATERIALS AND METHODS

RNase A and cytidine 2′,3′-cyclic monophosphate were purchased from Sigma Chemical Co. (St. Louis, MO). The enzyme was used without any further purification. RNase A concentrations were determined spectrophotometrically using a molar extinction coefficient of 9.800 M^{-1} cm^{-1} at 278 nm and pH 6.5.

Steady-State Kinetic Analysis. RNase A enzyme activity was determined according to the method of delCardayré and Raines (*37*) with some modifications by assaying the enzyme at 20 °C and using a different buffer composition [100 mM MOPS (pH 6.5)]. The difference in molar absorptivity ($\Delta\varepsilon$) between the substrate cytidine 2′,3′-cyclic monophosphate and its hydrolysis product, the nucleotide 3′-phosphate, was obtained by UV spectroscopy and was 1450 M^{-1} cm^{-1} at 287 nm. Kinetic parameters were determined by a linear least-squares regression analysis of the initial velocity from individual progress curves. Data are the average of at least six independent experiments.

Inhibition of RNase A Catalysis. Inhibition of ribonucleolytic activity was assessed by using cytidine 2′,3′-cyclic monophosphate as a substrate in the presence of urea (100 and 300 mM).

NMR Studies. Spectra were recorded on a Bruker DRX 400 instrument with a transmitter frequency of 400.13 MHz. The spectra are the result of 256 scans, 1 s (delay time) at 293.4 K, and were processed with Bruker Topspin version 2.1. The concentration of RNase A in all solutions used for NMR spectroscopy was 40 mg/mL prepared as follows. (a) The RNase A was dissolved in D_2O (pH 6.9). (b) the RNase A was dissolved in acetic/acetate buffer (pH 5.0) in D_2O. (c) The RNase A was dissolved in acetic/acetate buffer (pH 5.0) in D_2O, and 1.0 M urea.

Theoretical Methods. All the calculations and optimizations for the urea and the 20 natural amino acids were performed using Gaussian 98 at the RHF/6-31G(d) level (*38*).

RESULTS AND DISCUSSION

Kinetic Studies of RNase A at Low Urea Concentrations. Ribonuclease A is a thoroughly studied enzyme, and the mechanism of folding and unfolding as well as its kinetic properties has been described in detail (*18*, *39*−*42*). To evaluate the effect of urea on proteins, we studied the effect of urea on RNase A activity at low concentrations. As seen in Figure 1, urea inhibition of RNase A follows a simple competitive model suggesting the possibility that this compound forms adducts with residues at the active center of the enzyme. The urea concentrations utilized were significantly below the pretransition region concentration for unfolding suggested by Wu and Wang, which also showed competitive inhibition for RNase A in aqueous urea solutions (*39*).

Taken together, these results indicate that urea inhibits RNase A competitively over a concentration range from 100 mM to 4.0 M and suggest that urea with its high dipolar moment is a competitive inhibitor and that a very high concentration (> 4.0 M) of it could denature the enzyme, beginning the interaction with the protein at the active center. Our results are in concordance with the results published by Nelson and co-workers (*40*), who observed that compounds with high dipolar moments (phosphate and sulfate) can bind charged residues within the catalytic center of RNase A, provoking competitive inhibition of the enzyme and thus preventing urea denaturation. Both sulfate and phosphate anions exhibited maximum protection against enzyme denaturation with urea at pH 5.5. This last result suggests that protonation of groups on the catalytic center rather than on the anionic molecule brings about an increased level of binding as the pH is lowered. Urea with its high dipolar moment (3.86 D) could attack histidines at the active site in the same way as iodoacetate, another compound with a high dipolar moment (5.12 D). This monohaloacetate produces a more efficient carboxymethylation of histidines in protein when it is used at pH 5.5, indicating that it has a preference for protonated histidine at the active center of proteins (*41*). Moreover, iodoacetate does not produce carboxymethylation of histidines in the active center of RNase in the presence of urea at pH 5.5, which could indicate that the interaction of urea with protonated histidine prevents iodoacetate attacks (*42*). Here, we report that urea at low concentrations exhibits the same behavior as other molecules with a high dipolar moment that attack protonated histidines at the active site which resulted in enzyme inhibition under our experimental conditions. On the other hand, our experimental results are in disagreement with MDS results which indicated that urea preferentially binds apolar amino acids (*26*, *31*).

^{1}H NMR Studies of Urea−*RNase A Interactions*. To corroborate that urea has a direct or short-range interaction with RNase A, we conducted high-resolution ^{1}H NMR studies to check for a possible interaction of urea with the protonated histidines of the active site of RNase A, which was suggested by our inhibitory kinetics experiments. Markley (*43*) performed ^{1}H NMR of RNase A assigning histidine C(2)-H and C(4)-H, which correspond to His 12 and His 119, respectively, at the active center of the enzyme. Following the Markley assignation of protons, we did not detect the peaks that would correspond to protons at C(4) and C(2) at the histidines implicated in enzyme catalysis when the spectrum was recorded at pH 6.9 (Figure 2a). At pH 5.0, in the absence of substrate, the equilibrium is displaced to the protonated form of histidines at the active center, due to the acidic environment below the pK_a of both weak acidic histidines (5.4 and 6.4, respectively) at the active site of RNase A. When we decreased the pH of the enzyme solution to 5.0, we observed two signals with chemical shifts at 7.04 and 8.35 ppm (Figure 2b). The first corresponds to the proton of C(4), and the second corresponds to C(2)-H, both from H12 and H119 rings, respectively, according to the NMR assignment when both histidines are protonated (*43*). Signals at 7.04 and 8.35 ppm were perturbed when the sample was treated with 1.0 M urea (Figure 2c). The perturbation of these signals was not observed with 0.5 M urea (results not shown). The addition of urea produced no significant pH change in the RNase solution (ΔpH 0.06), eliminating the possibility that the changes in chemical shift at δ 7.04 and 8.35 are a consequence of pH variation by urea addition. Perturbation of the H12 proton signal at δ 7.04 at C(4), together with the competitive inhibition kinetic results, is indicative that urea could exert a favorable electrostatic interaction with positively charged histidines (H12 in the case of RNase A). In the spectrum in Figure 2b, two more sharp peaks at 7.43 (H1′) and 8.06 ppm (H1) are observed, suggesting that hydrogen atoms on C(4) and C(2) are perceiving an unshielding effect due to a protonated imidazole ring of the histidine and that both histidines are protonated at pH 5.0. Furthermore, we also observed well-defined signals at δ 8.60 and 8.62 that correspond to the other protons of the histidines present in the RNase A structure, and these signals are indicative of protonation of the imidazole rings of all RNase A histidines. In addition, urea perturbs the amide signals (between 7.50 and 9.50 ppm), as observed in Figure 2c, and the region that corresponds to tyrosine aromatic protons between 6.40 and 6.80 ppm (Figure 2c).

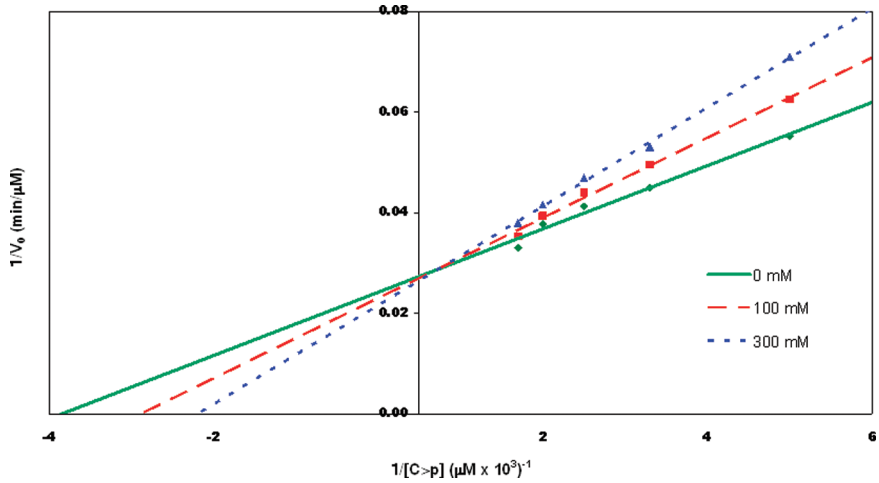

FIGURE 1: Lineweaver−Burk plot for the inhibition of RNase A by urea. The reciprocal of the initial velocity of the hydrolysis of cytidine 2′,3′-cyclic monophosphate (C > p) is represented as a function of the reciprocal of its millimolar concentration. The increase in absorbency at 287 nm was determined in the presence of 0.5 μM RNase in the absence and presence of urea.

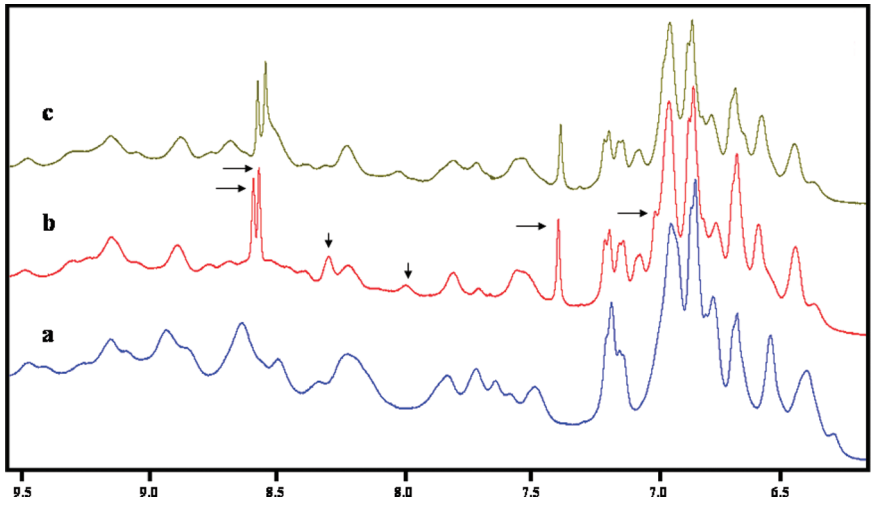

FIGURE 2: RNase A ^{1}H NMR spectra at 400 MHz in D_2O in the range of 6.0−9.5 ppm. (a) RNase A in D_2O (pH 6.9). (b) RNase A in acetic/acetate buffer (pH 5.0) in D_2O. (c) RNase A in acetic/acetate buffer (pH 5.0) in D_2O in the presence of 1.0 M urea. The concentration of RNase A was 40 mg/mL in all cases.

The protonation of all histidines of RNase A could explain the increased sensitivity of papain and RNase A to urea at low pH observed by Wu and Wang (*39*). At low pH, urea could interact easily with charged amino acids, forming much more stable adducts with these amino acids and causing inactivation of the enzyme followed by denaturation at the high concentration that they used in their experiments. Moreover, the ^{1}H NMR spectrum (Figure 3) recorded between 0.0 and 5.0 ppm, which corresponds to protons of aliphatic groups in the protein spectrum, did not exhibit appreciable chemical displacement or signal perturbations at pH 5.0 in the presence of 1.0 M urea. This result indicates that at low urea concentrations (through 1.0 M) no significant interaction takes place between urea and nonpolar amino acids, lending support to our suggestion that competitive inhibition by urea results from an initial interaction with positively charged amino acids from the active center of the enzyme. In addition, our results could help explain the stability to denaturation by urea provided by treatment of RNase A with anion and polyanion compounds at low pH found by Nelson and co-workers (*40*). The enzyme inhibition of RNase A found by these authors could prevent the interaction of urea with the catalytic charged residues at the active site, causing the interference with the first and fundamental steps of RNase A inactivation and denaturation.

Molecular Orbital Calculations. From the point of view of FMO analysis, the donor−acceptor reactivity between two molecules is determined by the highest occupied molecular orbital (HOMO) of the electron donor and the lowest unoccupied molecular orbital (LUMO) of the electron acceptor (*34*−*36*). This theory is a convenient way to study molecular interactions using the electronic properties of the isolated species (*34*−*36*). From this perspective, a first approach to understanding the interaction between urea and amino acids is a comparison of the energies of the HOMO of the urea, localized in the oxygen atom, and the LUMO of each of the natural amino acids. As schematically presented in Figure 4, this interaction is more favorable with the positively charged amino acids whose LUMO values are 1 order of magnitude lower than those of the apolar ones (Table 1). On the basis of FMO analysis, we propose that urea and the cationic residues of RNase can be stabilized by three-center, four-electron (N−H−O) interactions. These three-center, four-electron interactions partially compensate for the electronic deficit of the nitrogen atoms, in the cationic residues, which share

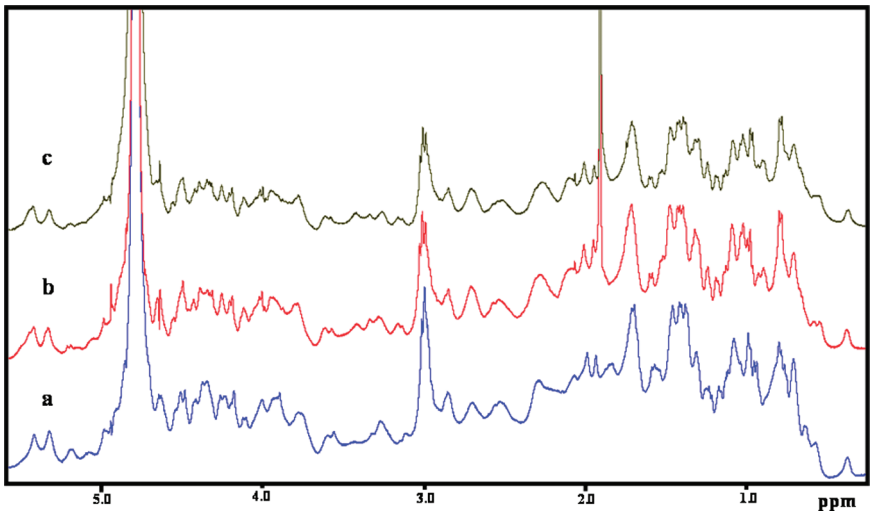

FIGURE 3: RNase A ^{1}H NMR spectra at 400 MHz in D_2O in the range of 0.0−5.0 ppm. (a) RNase A in D_2O (pH 6.9). (b) RNase A in acetic/acetate buffer (pH 5.0) in D_2O. (c) RNase A in acetic/acetate buffer (pH 5.0) in D_2O in the presence of 1.0 M urea. The concentration of RNase A was 40 mg/mL in all cases.

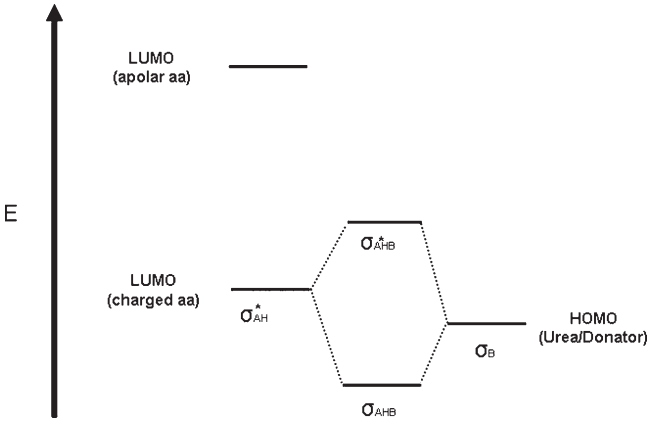

FIGURE 4: Schematic representation of frontier molecular orbital energy. Qualitative comparison of the HOMO of urea against the LUMO of cationic amino acids and nonpolar amino acids.

Table 1: Calculated Frontier Molecular Orbital Energies[a]

	urea	amino acids (protonated)	amino acids (apolar)
E_{HOMO} (eV)	-7.36		
E_{LUMO} (eV)		-5.31	-0.48

[a] Comparisons of the average energy of positively charged amino acids and the average energy of apolar amino acids against urea energy, at the RHF/6-31G(d) level.

their lone pairs with the proton. These interactions between the HOMO of urea and the LUMO of cationic residues, which are favorable for the formation of the acid−base adduct, can help to explain the competitive inhibition of RNase A at low urea concentrations. Furthermore, this interaction can explain the observed perturbation at 7.04 and 8.35 ppm signals in the ^{1}H NMR spectrum in the presence of urea (Figure 2c). In addition, the FMO analysis is indicative of a very weak interaction between urea and hydrophobic residues, explaining the lack of signal perturbation in the hydrophobic region of the ^{1}H NMR spectrum in the presence of 1.0 M urea (Table 1). For a more detailed picture of the interaction between urea and charged amino acids, we need to take into account the hydrophobic environment and the detailed dynamics of these molecules in the protein core.

Model for Early Steps of Urea Denaturation of Proteins. Because of the high solubility of urea in aqueous solution and its small size, it could penetrate to the interior of the protein interacting to various degrees with amino acid residues and generating a cooperative effect for protein denaturation. As a part of this cooperative effect, the unfolded protein is also stabilized in the aqueous solution, preventing protein refolding. Obviously, the quantity of urea within the protein and the degree of unfolding that it generates will depend on urea concentration. With all of this in mind, we propose a direct mechanism for the initial steps in protein denaturation by urea that is in opposition to the direct or short-range current model based on the disturbance of hydrophobic force as the first step for protein unfolding. In the low-dielectric constant core of the protein, the strong donor capability and high dipolar moment of urea will potentiate its reactivity to protonated residues, causing immediate interaction with charged amino acids situated at the active site (as is the case for RNase A). With a urea concentration between 0.1 and 1.0 M, according to our inhibition kinetics results, ^{1}H NMR spectroscopy data, and the FMO analysis, the probable first step in this process of unfolding is the interaction of urea with cationic amino acids, primarily with histidine. Immediately after or in parallel, urea will establish hydrogen bonds with polar noncharged residues (Q and N), negatively charged amino acids (D and E), the amidic linkage of the peptide backbone, and probably, to a lesser degree, the hydroxyl protons from tyrosine perhaps in a sequential or hierarchical process, dictated by the reactivity of all these molecular species with urea at the protein surface and within the protein structure. These interactions cause instability in the secondary structure (H, K, E, and Q are typically α helix formers, and D and N are typically β strand formers) of the protein and weakening of its tertiary structure. This disruption of three-dimensional structure results in the exposure of aliphatic and aromatic residues, and the partially unfolded polypeptide is most likely stabilized as an amphipathic compound in aqueous media with segments of the partially unfolded polypeptide chain forming clusters of hydrophobic residues and the amidic backbone interacting with the aqueous urea solution. In a treatment of the hydrodynamic properties of proteins, Scheraga and Mandelkern (*44*) proposed that the denaturation process appears to involve an increase in the effective volume of

the protein due to swelling instead of uncoiling of the polypeptide chain with the increasing urea concentration, a result that can be interpreted as a direct association of cosolvent molecules (urea) with the protein structure. This denatured protein with exposed hydrophobic residues is probably stabilized by enclosure of its hydrophobic sectors in a cagelike structure, minimizing the contact with the aqueous phase and the entropy loss of the system. These proposals are partially supported by our experimental and theoretical studies and by X-ray studies of urea, which has been reported to form cagelike structures with hydrophobic compounds whose dimensions allow the arrangement of aliphatic chains of several sizes (*45*−*47*). Our experimental results using low urea concentrations strongly suggest a direct interaction with the formation of an acid−base adduct of urea with cationic residues from the protein structure as the initial step of protein denaturation and contradict the model of indirect interaction as the first step for protein unfolding. Furthermore, our proposal can be supported by the calorimetric results of Makhatadze and Privalov (*17*). It is suggested that urea interacts directly and mainly with polar groups of proteins. Their conclusion based on the correlation analysis of the number of binding sites and structural characteristics of proteins suggests that the binding site for urea is likely to be formed by several hydrogen-bonding groups; also, they assumed that the urea has a low probability of interaction with exposed nonpolar groups of proteins. We do not discard the possibility that structural modification of semicrystalline water by high urea concentrations (between 4.0 and 9.0 M) has implications for protein fold stability, but we suggest that this alteration of the solvent environment could be among the last steps of protein denaturation by urea.

The prevalent model for urea denaturation of proteins has been based on the increase in solubility of nonpolar amino acid residues when they are transferred from water to urea denaturant solutions and in this way explains the disruption of hydrophobic collapse as the fundamental step for protein denaturation (*15*, *16*). The possibility that a urea/water solution can form cagelike structures that trap nonpolar compounds suggests a different explanation for the increased solubility reported by Whitney and Tanford (*10*) and Nozaki and Tanford (*11*) for nonpolar amino acids in urea solutions. An alternative interpretation of their results is that the aliphatic and aromatic residues were somehow isolated from the bulk of the hydrophilic solution by enclosure in these structures, which contrasts with the interpretation of these authors that this behavior is explained by the increasing solubility of nonpolar solutes in a urea/water solution and that this indicates disruption of hydrophobic collapse as the primary step of protein denaturation. Our experimental results, supported by theoretical calculations, indicate that at low urea concentrations the first attack is on the positively charged amino acids.

CONCLUSION

According to our experimental and theoretical studies, we conclude that urea−protein interaction occurs in a hierarchical way, disturbing first charged histidine and then amidic linkage, while the hydrophobic region remains unaffected at low urea concentrations. The molecular mechanism for protein denaturation with urea, discussed in this paper, could improve our understanding of protein folding−unfolding events by providing an explanation for the steps of this fundamental phenomenon in protein structural stability.

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