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The Hydrophobic Effect: A New Insight from Cold Denaturation and a Two-State Water Structure

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ABSTRACT: Herein we provide a new insight into the hydrophobic effect in protein folding. Our proposition explains the molecular basis of cold denaturation, and of intermediate states in heat and their absence in cold denaturation. The exposure of non-polar surface reduces the entropy and enthalpy of the system, at low and at high temperatures. At low temperatures the favorable reduction in enthalpy overcomes the unfavorable reduction in entropy, leading to cold denaturation. At high temperatures, folding/unfolding is a two-step process: in the first, the entropy gain leads to hydrophobic collapse, in the second, the reduction in enthalpy due to protein-protein interactions leads to the native state. The different entropy and enthalpy contributions to the Gibbs energy change at each step at high, and at low, temperatures can be conveniently explained by a two-state model of the water structure. The model provides a clear view of the dominant factors in protein folding and stability. Consequently, it appears to provide a microscopic view of the hydrophobic effect and is consistently linked to macroscopic thermodynamic parameters.

KEY WORDS: cold denaturation, hydrophobic effect, entropy, water structure, molten globule, α-lactalbumin; molecular simulations.

I. INTRODUCTION: THE APPARENT PARADOX

Owing to the critical importance of cold denaturation in the understanding of protein folding and stability, it has attracted considerable attention over the years. In his classic review of cold denaturation, Privalov has noted that the denaturation of proteins after heating makes intuitive sense.[1] Raising the temperature leads to heat absorption, with an increase in enthalpy and entropy. Hence, unfolding at high temperatures seems natu-

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ral. Native folded proteins are more structured, have higher stabilities, and lower entropies than their heat-denatured forms. Thus, one would have expected that denatured proteins would be inherently unstructured, flipping between a large number of extended conformational states. As native proteins exist at lower temperatures than their heat denatured ones, or than their intermediate states, it appears logical to assume that as the temperature further decreases, internal motion would be further reduced, leading to further optimization of protein packing and stability. Therefore, it has been a surprising experimental observation that this is not the case. Most proteins denature after cooling, with a characteristic cold denaturation transition state temperature (T'_G). Furthermore, paradoxically, the unfolding at low temperatures is accompanied by a *decrease* in entropy. This apparent paradoxical phenomenon has been difficult to reconcile. Hence, a key point in the understanding of cold denaturation is an explanation of the decrease in entropy.

To understand this intriguing phenomenon, it is convenient to consider a fluctuating two-state model of water structure.[2] Exposed non-polar surface area promotes the ordering of the water molecules. At low temperatures, "normal" hexagonal low density, enthalpically favored ice structure (termed "Ice-Ih") prevails. Hence, here exposure of non-polar surface area further optimizes the H-bond network. Consequently, the entropy of the system is reduced. On the other hand, the enthalpy is also reduced. The favorable gain in enthalpy is apparently more significant than the unfavorable entropy loss, driving protein denaturation at low temperatures. At higher temperatures, liquid ("Ice-II" type) water increasingly predominates. Liquid forms are characterized by their higher density and increasing molecular fluctuations. Exposure of non-polar surface area to liquid forms also has the effect of ordering the water molecules. However, unlike the situation where hexagonal ice dominates, here the hydrogen bond network is not optimized. Consequently, the reduction in enthalpy is not enough to compensate for the entropy loss (see Appendix A for a further definition). Hence, at this temperature, exposure of non-polar surface is unfavorable. This is the origin of the hydrophobic effect. In protein folding, this is the driving force for hydrophobic collapse and formation of intermediate states.

Here we use the water structure in a consistent way, to microscopically account for cold denaturation, intermediate states, and the hydrophobic effect. A model proposing that there are two states in water structure, with ice dominating at low temperatures, liquid water at high temperatures, and a rapidly fluctuating bonding-type gradient increasingly becoming liquid type as the temperature climbs, and that non-polar surface promotes order in water, aids in figuring out which folding $\leftrightarrow$ unfolding steps are under entropy, and which are under enthalpy control. We show later that this scheme is also consistently tied to the macroscopic thermodynamic data observed in experiments. Its merit is in providing a clear view as to which are the dominant factors at each step in protein folding at high and at low temperatures, and in protein stability. The curious molten globule state as well as the experimentally observed intermediate states in heat but not in cold denaturation fit perfectly into such a conceptual description.

II. THE HYDROPHOBIC EFFECT SCHEME

A. Folding

The hydrophobic effect, namely, the very limited solubility of non-polar substances in

water, is still not completely understood from a molecular point of view. The well-known empirical rule that "like dissolves like" and *vice versa* has provided an intuitive *macroscopic* explanation of the hydrophobic effect. On the other hand, to describe the hydrophobic effect *microscopically*, the experimental entropy decrease after dissolving non-polar molecules in water was proposed to be accounted for by the formation of an ordered water structure, that is, an iceberg, around the non-polar solute. Recently, Monte Carlo simulations of a simple two-dimensional water model[3] have successfully linked the microscopic water structure and the macroscopic thermodynamic description of the hydrophobic effect.

Although the enthalpic and entropic contributions are markedly temperature dependent, the Gibbs free energy of hydration of nonpolar solutes is relatively insensitive to temperature, owing to their complementary cancellation. Nevertheless, despite this insensitivity, many nonpolar compounds show minimum solubility in water around room temperature.[4] Remarkably, this fact is consistent with the observation that many globular proteins also reach their maximum marginal stabilities around room temperature. The puzzle of why the free energy of transfering liquid hydrocarbons into aqueous solution is most unfavorable around temperature at 400 K instead of room temperature has been resolved by Schellman.[5] Therefore, the minimum solubility and maximum stability on the one hand combined with the invariable existence of a hydrophobic core in globular proteins on the other lead to the inevitable conclusion that the driving force for protein folding is the hydrophobic effect.[4,6] In the second step, further stabilization is imparted by specific interactions determining the native structure.

Hence, protein folding is a two-step process. Starting from a high temperature denatured state (*D*), the first step in a folding reaction is the outcome of the hydrophobic effect. It leads to the molten globule (*MG*) state and is under entropy control.[7] It reflects the marked change in temperature, and therefore in the structure of water. The second rearrangement step leads to specific interactions observed in the native state.[8] Here, in the second step, the water structure can be considered as practically unchanged, playing an insignificant role. This *MG* to native (*N*) step is under enthalpy control. Not every hydrophobic collapse is able to reach the native conformation. In terms of the building block folding model, the combination of the hydrophobic collapse and the rearrangement step is equivalent to the formation of building blocks.[9,10] For a misassembled building blocks intermediate with a high barrier, the native state may be inaccessible.

A two-step process does not imply that protein folding must be a three-state folding. If the population time of the hydrophobic collapsed state (or the *MG* state) is too short for an experimental probe to measure its existence, a reversible two-state folding/unfolding will be observed. Although thermodynamic analysis can be done with a strict two-step process (as seen in Appendix B), a two-step process is not strictly necessarily two distinctive processes, with one following the other. It could be a mixed process falling under the general guidelines in folding: driven by the hydrophobic effect and stabilized by specific interactions.

B. Cold and Heat Denaturation

After further cooling, as a larger fraction of water becomes hexagonal ice, the protein cold-denatures (D′), exposing its non-polar surface to ice-like liquid water. Because a globular protein reaches its maxi-

mum marginal stability around room temperature, protein unfolding at lower temperatures is expected. In cold denaturation, the entropy is reduced. Thus, the major contribution to the Gibbs energy change after cold denaturation derives from the enthalpy change. The favorable exposure of non-polar surface at cold temperatures is consistent with the absence of intermediate states in cold denaturation. Consistently, Grikor[11] has pointed out that the enthalpy increases from D′ to N, and from D to MG. If we consider cold and heat denaturation at the same time, the equilibrium reaction taking place for a polypeptide chain can be written as

Cold denatured (D′) ↔ Folded (N) ↔ Heat denatured (D).

The Gibbs energy change (ΔG) for the reaction can be defined in terms of the enthalpy change (ΔH) and the entropy change (ΔS),

$$\Delta G = G_D - G_N = \Delta H - T\Delta S$$

with $G_N = H_N - T S_N$, and $G_D = H_D - T S_D$. At the heat denaturation transition temperature (T_G), the Gibbs energy change becomes $\Delta G(T_G) = \Delta H_G - T_G \Delta S_G = 0$. Similar equations can be written for cold denaturation (D′) with the transition temperature at T'_G. The relative entropy and enthalpy of the three states (D′, N, D) are illustrated in Plates 1A, B* for three characteristic temperatures, $T = T_S$, (or $T = T_H$ in 1B), and $T = T'_G$.

At the transition temperatures of heat and cold denaturations, the accompanied positive heat capacity changes indicate that the entropy of the system must increase monotonously from D′ (when $T < T'_G$) to N to D (when $T > T_G$).[12] On the other hand, the enthalpy of the system must accordingly decrease from high to low temperature. For the denatured states to be favorable at the two extreme temperatures (in heat and in cold denaturation), the entropy and enthalpy changes should be in opposite directions. Otherwise, there would be only one favorable denatured state — either at high or at low temperatures, but not at both. Based on the proposed hydrophobic scheme and with the entropy divided into two independent sources (see Appendix B), the relative order of entropy at the different states can be shown as in Plate 1A. From Appendix B, at $T = T_S$, ΔS_D^{MG} (the entropy change from the D to the MG state) is equal to $\Delta S_D^{MG'}$ (the entropy change from N to MG′). As the temperature increases (from $T = T_S$ to $T = T_G$), the entropy is more favorable for the MG state than the N state. Therefore, at $T = T_G$, we see an increase in ΔS_D^{MG}. And, conversely, consistent with MG being more favorable at higher temperatures, there is a decrease in $\Delta S_D^{MG'}$. Plate 1C summarizes the relative order of the free energies in each dominant state at the three temperatures, $T = T'_G$, $T = T_S$, and $T = T_G$. Plate 1D illustrates which factor — entropy or enthalpy — controls a given step at any specified temperature in the folding process.

Figure 1 presents a schematic diagram of the folding/unfolding steps in water, where P1 and P2 are two parts of the protein (P) that interact in the native state, and W1 and W2 two clusters of water molecules. After denaturation the protein parts separate, with each now interacting with a cluster of water molecules. The steps in folding/unfolding relate to temperature, and a two-state model of the water structure can conveniently reflect the effect of the change in the temperature and hence microscopically account for each step.

To relate the change in water to each step in the folding/unfolding process in the heat and in the cold, we first describe the

* Plates appear following page 58.

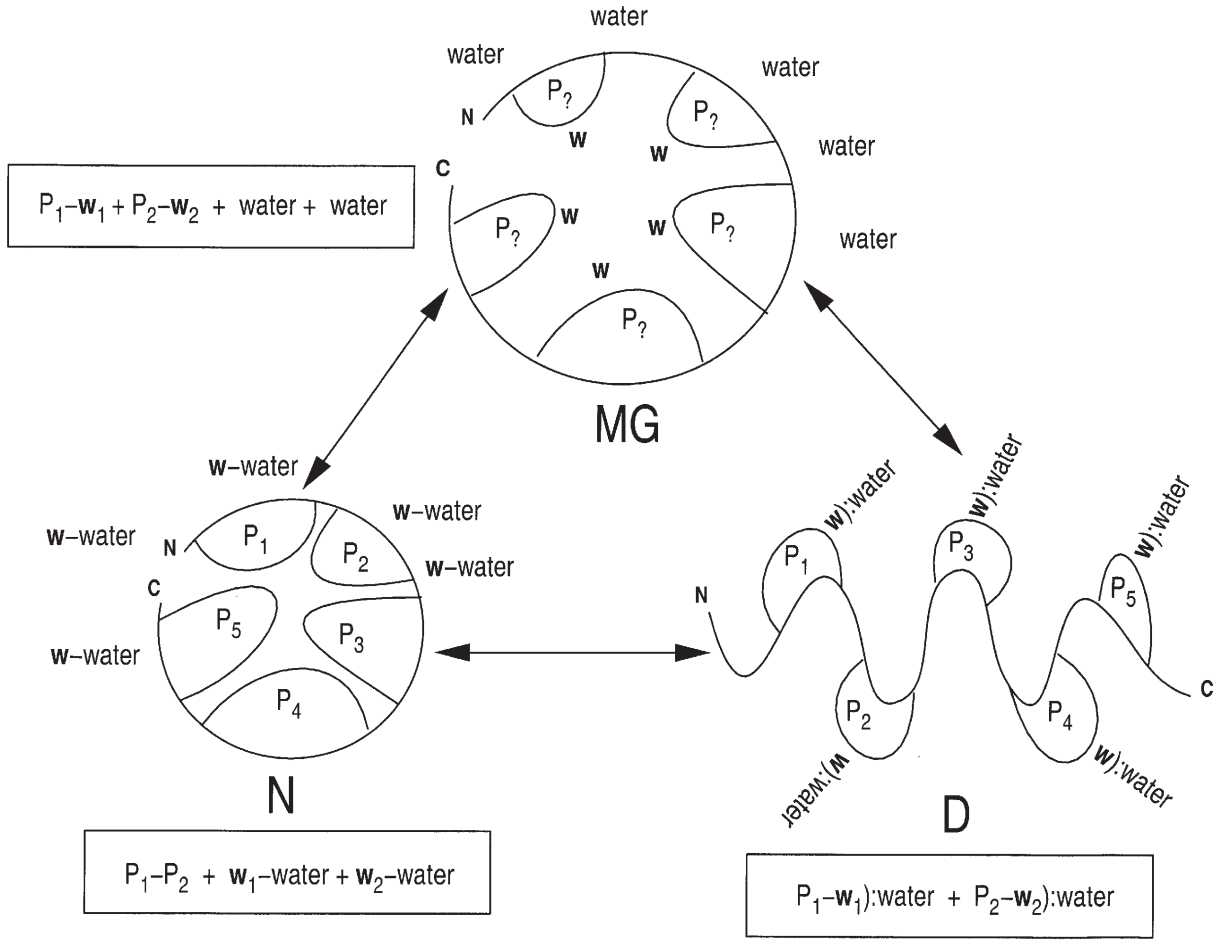

Figure 1. A schematic drawing of the native (N), molten globule (MG), and denatured (D) states observed in the process of protein folding. Three terms, "W", "P", and "Water" are responsible for the presence of these states and are depicted in the figure. These terms account for the changes taking place in the two-step folding/unfolding procedure, D ↔ MG and MG ↔ N. "P" represents a non-polar protein part. "P" parts are backbone-linked, from the amino- (N) to the carboxy (C) terminus. "W" represents the first shell water molecules which is in contact with the protein. The third term is "Water". It represents the surrounding liquid water. In the denatured state, the symbol "):" implies the propagation of the change in the structure of liquid water by the exposed non-polar surface. The question mark "?" in the MG state emphasizes that the outcome of the hydrophobic collapse is a combinatorial assembly process. The interactions responsible for the stability of each (N, MG, D) state are summarized in the respective rectangular boxes. This simplified scheme illustrates clearly the changes in the dominant interactions from one state to the other. Between D ↔ MG, the structure of water governs the changes. The protein-protein interactions determine the changes between MG ↔ N.

water structure at different temperatures, in the absence of the protein. We stress, however, that while here we use a slight variant of the Vedamuthu et al.[2] model, any (fluctuating) two-state water structure model that reproduces the water anomaly and has ice/liquid features, can explain the temperature-dependent contributions of entropy and enthalpy to ΔG.

C. The Fluctuating, Two-State Water Model

Vedamuthu et al.[2] proposed that there are two (or more) general types of intermolecular bonding configurations: the first is the low-density "normal" hexagonal ice, Ice-Ih (Figure 2A). The second (and others) represent denser liquid bonding forms (Ice-II, -III, -IV, etc, Figure 2B). At low temperatures, Ice-Ih prevails. As the temperature rises, Ice-Ih starts breaking down, with an increasingly larger fraction of disordered, fluctuating denser liquid Ice-II type forms. This is *not a mixture-of-ices* model; rather, its essence is a "rapidly fluctuating mixture of *intermolecular bonding types* found in the most stable polymorphs of ice". In hexagonal Ice-Ih, every O atom has four nearest neighbors, with a 4.5 Å next-to-nearest distance and a regular 109.5° tetrahedral angle. As the temperature (or pressure) is raised, this bonding type weakens, and the water structure increasingly fluctuates between energetically similar intermolecular bonding types. In these dense liquid forms, the O…O...O angle is about 80°, resulting in non-hydrogen bonded next-to-nearest O...O neighbors in the 3.24 to 3.51 Å range. Unlike in the model of Vedamuthu et al.[2] who consider Ice-II (-III, -IV, etc states) to possess specific, ordered structures, we view Ice-II (-III, -IV) as disordered, fluctuating states, all conforming to the higher density characteristic. Liquid water resembles Ice-II. The water structure is dynamic, with the hydrogen bonds continuously broken and created on a very short time scale,[13] and the molecules in perpetual motion. Such a two-state water structure model reproduces the density anomaly of water.[2] Figures 2A and 2B adopt the attractive simplified Silverstein et al.[3] representation, with the Mercedes Benz (MB) logo. Ice-Ih has nondistorted H-bonds, with more favorable potential energy. Ice-II has more favorable entropy, less favorable H-bonds, and less favorable ΔH.

D. Entropy Control, Enthalpy Control and the Temperature-Dependent Change in the Water Structure

Now, let us first consider the transition from liquid water to solid ice under atmospheric pressure. Clearly, Ice-Ih is enthalpically favored due to the tetrahedrally coordinated hydrogen bond network, and liquid water is entropically favored because a water molecule has more freedom to move around in liquid water than in ice. The enthalpy and entropy of ice and of liquid water are temperature dependent. Ice-Ih forms as temperature decreases just below 0°C simply because the enthalpy gained from the tetrahedrally coordinated hydrogen bond network is more significant than the entropy loss by the structured water molecules. In contrast, just above 0°C, Ice-Ih melts because the entropy gained from disrupting the lattice is larger than the enthalpy lost from disruption of a perfect hydrogen bond network.

In denatured proteins, the non-polar surface area is exposed to the solvent. At low temperatures exposed non-polar surface area promotes the restriction of the orientation of the first shell of water molecules, and the propagation of an optimized

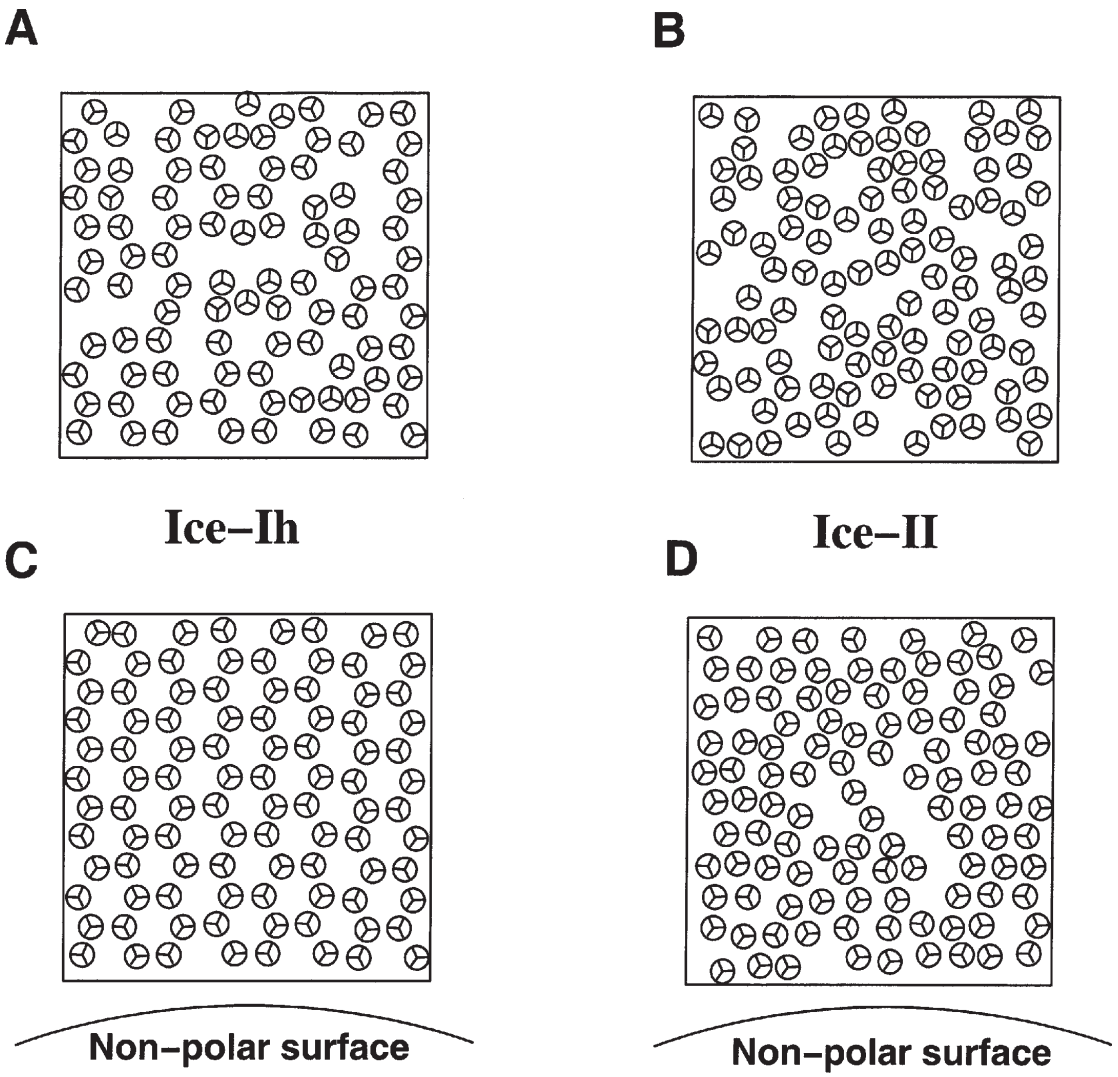

FIGURE 2. The two-state fluctuating water structure model, and the effect of exposure of non-polar surface. The figure adopts the simplified, two-dimensional attractive MB (Mercedes-Benz) logo model for water, proposed by Silverstein et al.[3] Figures A and B depict a snapshot of the water structure at correspondingly low and high temperatures. (A) Ice-Ih; (B) Ice-II. Ice-Ih is "normal", low density enthalpically favorable hexagonal ice. Clearly, the Ice-Ih state does not imply that solid ice populates the entire system. Ice-II is the high density disordered fluctuating entropically favorable forms of liquid water, increasingly existing at high temperatures. Figures C and D provide snapshots of the effect of exposed non-polar surface on the water structure around the respective T'_G (cold) and T_G(heat) transition temperatures. In both cases, the non-polar surface promotes ordered water structure, with optimal hydrogen bonds. A comparison of Figure A with Figure C illustrates the enhancement of order, and of optimized H-bonds in ice, resulting in reduced entropy and enthalpy. Comparison of Figure B with Figure D illustrates that in liquid Ice-II forms order is also enhanced, although to a substantially lesser extent. As can be seen in the figure, around the transition temperature, the H-bonds are not as optimal. Hence, here too there is an unfavorable reduction in entropy. However, the favorable reduction in enthalpy is not significant enough to counterbalance the unfavorable entropy. This drives the burial of the non-polar surface, and is the origin of the hydrophobic effect.

hydrogen bond network to the next ice-like shells (Figure 2C). Here, the hydrophobic effect is gone, explaining the absence of intermediate states. On the other hand, at very high temperatures there is no propagation of such order, as practically no hexagonal ice bonding type is present (Figure 2D). Hence, for a two-part (P1, P2) protein unfolding in Water with W1, W2, two water clusters,

$$P1 - P2 + W1 - Water + W2 - Water \rightarrow P1 - W1 - Water + P2 - W2 - Water.$$

At higher temperatures ($T > T_G$),Water is largely liquid Ice-II. This process is under *entropy control*. In contrast, at lower temperatures ($T < T_G'$), Water is largely ice-like. Hence the *enthalpy controlled* cold denaturation promotes ice formation (Figure 2C). In agreement with such a microscopic view, in the MB model[3] the first shell water molecules are ordered around small inert solutes, and consequently have strengthened hydrogen bonds relative to bulk water molecules. Similar clathration mobile-order,[13] water-shell effects may hold for larger molecules, such as proteins. At low temperatures this effect resembles a cascade of propagating water structure, still dynamic but with diminishing fluctuations reducing the entropy (and enthalpy) of the system. At high temperatures ($T > T_G$), the fluctuating water molecules are increasingly disordered and no propagation of order takes place. Consistently, around the (T_G) transition temperature, the unfolding is still incomplete, with residual structure persisting in the denatured state.

At the $T'_G < T < T_G$ temperature zone, exposure of non-polar surface leads to ordering the water molecules (Figure 2D) lowering the entropy, with no compensating favorable enthalpy. This is the *hydrophobic effect,*[4-6] the first step in the folding reaction. In the second step, specific optimized interactions are formed between the protein parts. Figure 1 illustrates the two steps, the hydrophobic collapse and the combinatorial assembly of the protein parts.

In our hydrophobic scheme, which states that protein folding is a two-step folding process, enviromental water is not involved in the $N \leftrightarrow MG$ process. At $T = T_S$, the $N \leftrightarrow MG$ process is under enthalpy control because the free energy of the N state is lower than the MG state at this temperature. Hence at $T = T_S$, the enthalpy gain from specific interactions is more important than the entropy loss in the MG to N step. As shown in Plate 1C, the MG state is higher in energy (more unfavorable) than the N state at $T = T_S$. On the other hand, as expected from the observed hydrophobic effect (and reflected in Plate 1C), the D state at $T = T_S$ is expected to have higher energy than the MG state. Due to the entropy term, elevation of the temperature favors the MG state more than the N state. Hence, the free energy gap between N and MG decreases as the temperature increases. In contrast, the free energy gap between these states increases as the temperature decreases. When the elevated temperature reaches $T = T_G$, the N and D states are at equal free energy levels. However, the MG state is likely to be above the N state because N is the most favorable state in the $T'_G < T < T_G$ temperature range. As temperature decreases from $T = T_S$ toward $T = T'_G$, at which the N and D′ states are at equal energy levels, the free energy gap between N and MG′ enlarges as shown in Plate 1C.

III. EXPERIMENTAL EVIDENCE: THE CASE OF α-LACTALBUMIN

α-lactalbumins (LA) belong to a family of mammalian Ca^{2+}-binding proteins involved in lactose transport.[8,14,15] α-LAs have

123 residues and four disulfide bridges. Their structure can be divided into two domains. At acidic pH (A-state) α-LA typically has a compact structure, with native-like secondary structure. However, it lacks well-defined tertiary interactions. The α-domain appears to form specific disulfide bonds and to have a native-like fold. In contrast, the β-domain appears more "disordered".

A. The Relative Contributions of Enthalpy and Entropy to the Free Energy

Griko[11] has studied the energetic basis of the structural stability of the continuously changing heterogeneous population of conformational states representing the MG. His results present a framework for a definition of the energetics of the structural stability of the MG states. Following Griko, Plates 1A and 1B depict the entropy and enthalpy behavior in the unfolding (D′) ↔ folding (N) ↔ unfolding (D).

1. Entropy

Proceeding from the cold denatured (D′) to the native (N) state, the conformational entropy decreases. It increases monotonously from N to MG and subsequently to the heat-denatured (D) state. If, however, we consider the whole system including the water structure in the D′ to N to D there is a monotonous increase in entropy (Plate 1A). The MG state with buried non-polar surface has a higher entropy than D. On the other hand, the water structure plays an insignificant role in the N to MG step. This step reflects the higher conformational entropy of MG. The D′ to N step reflects the effect of the changing water structure.

2. Enthalpy

Plate 1B illustrates the changes in the enthalpy. Proceeding from D′ to N, the enthalpy increases, clearly showing that cold denaturation is under enthalpy control. From N to MG the enthalpy also increases, illustrating that the rearrangement in the MG → N step is under enthalpy control. From MG to D the enthalpy decreases, putting the D → MG folding step under entropy control. This reflects the increase in the fraction of normal ice. Had the non-polar surface been exposed at T_{MG}, order could have propagated to a larger extent than at T_D. For two-state folding proteins, the enthalpy increases monotonously, from D′ to N to D. It is the presence of the MG that enables a clear illustration of the hydrophobic effect. In the absence of a collapsed MG state, the two curves for enthalpy and entropy behave similarly.

B. Molten Globule State Explained: Two Steps from D → N

MG can be depicted as a broad shallow well on the potential energy landscape. The conformers possess a hydrophobic core and are relatively stable. However, reaching the bottom of the funnel to settle down into a native conformation depends on specific protein-protein interactions, including a number of terms, such as vdW (compactness), disulfide bonds, Ca^{2+} binding (to overcome electrostatic repulsion), H-bonds, as well as the hydrophobic effect. Thus, there are two steps in the α-LA folding reaction (Figure 1): hydrophobic collapse and combinatorial search to optimize electrostatic, disulfide, and vdW interactions. At pH 2, the MG state of α-LA prevails, owing to electrostatic repulsion. The penalty paid in

this MG to N step is reduction in conformational entropy. Because the search initiates with the hydrophobically collapsed structure, the Levinthal paradox does not arise.

C. No MG′ detected at Low Temperature

As Privalov[1] has long argued, the hydrophobic effect can be understood by comparing cold with heat denaturation (Plates 1A,B). Plotting the temperature dependence of the heat capacity of β-lactoglobulin, a broad curve, with no intermediate states is obtained for cold denaturation. On the other hand, an intermediate is clearly observed in heat denaturation.[16] The disruption after cooling is accompanied by cooperative changes in heat capacity, whereas in heating the heat capacity changes more gradually. The residual structure in cold denaturation is more extensive than in heat denaturation, probably in regions largely stabilized by interactions other than the hydrophic effect.

The population of the α-LA MG state is strongly temperature dependent, undergoing cold denaturation just like the native state.[17] The hydrophobic effect decreases with temperature and is a principal reason why the native and the MG states of α-LA are destabilized at low temperatures. Consistent with Griko,[11] the increase in heat capacity after cold denaturation of the MG is positive, owing to the decrease in ΔH. The transition temperature for cold denaturation of the native state (240.5 K) is lower than that of the MG (267.5 K), consistent with the major stabilizing source of the MG state being the hydrophobic effect. Why, then, is there no observable MG′ state, between the native and the D′ states? As Plates 1A and 1B illustrate, such a state may hypothetically exist. A hypothetical MG′ would have had both higher entropy (Plate 1A) and enthalpy (Plate 1B). From Plate 1C, the free energy gap between D and MG or between D′ and MG′ reaches its maximum at $T = T_S$, in favor of the MG state. This observation is consistent with the general view of the hydrophobic effect. At $T = T_G$ and at $T = T'_G$, the relative free energies reverse and both favor the denatured state. However, at $T = T_G$, as the free energy gap between the D and the MG states is marginal, the MG state is still accessible. In contrast, at $T = T'_G$, the free energy gap is so large that the MG′ state is impossible to reach. This explains why it has not been observed experimentally.[17]

D. Additional Supporting Evidence

Consistent with our scheme, the *E. coli* HPr (histidine-containing phosphocarrier protein) has been shown to undergo cold denaturation.[18] It is interesting that its $\Delta H/\Delta C_P$ ratio is small. HPr has low ΔH and among the highest ΔC_P. Because ΔC_P correlates with ΔASA, the smaller contribution of the specific interactions and higher contribution of the hydrophobic effect to its stability lead to a higher, and hence observable cold denaturation transition temperature. Further, cold denaturation has been observed for helical peptide oligomers with an appreciable hydrophobic core, but not for monomers. However, in aqueous HFIP (hexafluoroisopropanol) strengthening the H-bonds, cold denaturation of the helical peptide monomers has been observed.[19] Finally, a scheme with some similar features has been described.[20] In that scheme, water has been considered fundamental to the understanding of cold denaturation. However, as the temperature dependence of the water structure is lacking, the authors came

to the conclusion that it is the interaction of the polar surface with the water that drives the denaturation in the cold. Another recent study has also suggested that polar groups rather than nonpolar surfaces promote the formation of hydrogen bonding network at low temperatures.[21]

IV. POTENTIAL APPLICATIONS

A. Protein Stability and Design

The scheme presented here illustrates that the hydrophobic effect is insufficient for protein stabilization. The outcome of the hydrophobic collapse is the MG state. In the next folding step, specifically, largely electrostatic interactions and vdW packing lower the enthalpy, leading to the native state. To increase protein stability, a decrease in electrostatic repulsion is essential, as observed in α-LA at low pH and in the absence of Ca^{2+}. Similarly, the isolated adenine binding domain of dihydrofolate reductase exists in an MG-like state owing to electrostatic repulsion. It is overcome by the binding of a charged cofactor (NADPH). The importance of electrostatic interactions is also observed in the higher ratio of polar surface area in the α-domain in hen egg white lysozyme when compared with α-LA (D. Raleigh, personal communication). It is further consistent with the frequently observed "disordered" states in DNA-binding proteins.

This proposition is consistent with our recent comprehensive analysis of macroscopic thermodynamic parameters.[22] These indicate that in thermophiles higher T_G correlates with higher $\Delta G(T_S)$, the maximum stability of proteins. In turn, $\Delta G(T_S)$ correlates with ΔH_G, the slope of the stability curve at the denaturation temperature. Larger ΔH_G results in an upshift of the stability curve. As expected, there is also a correlation between T_G and ΔH_G. In contrast, no correlation is observed between T_G and ΔC_P, the heat capacity change. As ΔC_P correlates with ΔASA, clearly the hydrophobic effect is insufficient for stabilizing proteins at higher temperatures. Furthermore, because higher T_G is obtained largely via an upshift of the stability curve, lower T'_G is frequently observed, illustrating that in the cold-specific interactions, reflected in the enthalpy, are critical for stability. Consistently, psychrophilic proteins are apparently more similar to thermophilic than to mesophilic, despite the more closely related living temperatures.

B. Cold Denaturation and Molecular Simulations

Cold denaturation has not been observed in molecular simulations. Entropy and enthalpy are functions of temperature observed in the opposite signs in heat and in cold denaturation. However, in implicit water simulations, if the force field has no polarization term (the dielectric term is constant), it is temperature independent. Here, despite the cooling, the simulations are still consistent with positive ΔH and ΔS, precluding implicit water simulations for cold denaturation. Explicit water simulations at low temperatures can be carried out. However, currently no force field is able to reconstruct the ice-liquid structural change.

V. CONCLUSIONS

Here we have attempted to provide a new insight into the hydrophobic effect. Our scheme is able to predict cold denaturation,

and the existence of intermediate (molten globule) states in heat, but not in cold denaturation, consistent with the experiment. The different contributions of entropy and enthalpy to the Gibbs energy change at low and at high temperatures leading to these predictions can be conveniently explained by a two-state model of the water structure. Exposure of non-polar surface reduces the entropy and the enthalpy of the system, whether at low or at high temperatures. At low temperatures, the low density structured enthalpically favorable, entropically unfavorable ice prevails. Cold denaturation is driven by the favorable enthalpy decrease. As the temperature rises, the fluctuating fraction of entropy favorable, enthalpy unfavorable liquid water intermolecular bonding types increasingly dominate. At such high temperatures, the reduction in entropy on exposure of non-polar surface would not be compensated by a large enough favorable enthalpy term. Hence, the burial of the non-polar surface and the hydrophobic effect observed in the intermediate (molten globule) state. In the next MG to N step, the water structure plays an insignificant role. The two-state model enables predicting the absence of a molten globule state in cold denaturation. In such a hypothetical MG' state, the conformational entropy increases. However, at these lower temperatures, it is overcome by the larger increase in the conformational enthalpy, leading to unfavorable Gibbs energy change.

Here the microscopic view of the hydrophobic effect is usefully accounted for by a fluctuating two-state water structure model. This scheme is consistently tied to the macroscopic thermodynamic data observed in experiments. The merit of the scheme is in providing a clear view as to which are the dominant factors in the two folding/unfolding steps at high temperature, in the single observed cold denaturation step, and in protein stability. The experimentally observed intermediate states in heat but not in cold denaturation fit nicely into our general, conceptual description. It may further provide guidelines for protein design, consistent with protein stability plots.

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APPENDIX A

In the literature, the language used to describe a reaction is sometime unclear as to whether a specified change is favorable or unfavorable. Because it is critical to our paper, we attempt to clarify it here. First, we define the free energy change of the two-state protein folding, D→N, as $\Delta G^N_D = \Delta H^N_D - T\Delta S^N_D$, with the subscript being the initial state and the superscript the final state. If ΔG is negative, the direction of the reaction from the initial state to the final state is favorable. Consequently, a negative change in ΔH, and a positive change in ΔS, are both favorable for the reaction from the initial to the final state. Otherwise, they are unfavorable for the reaction. Here, "positive" and "negative" do not imply that the reaction is favorable or unfavorable, but rather, they refer to the value change in enthalpy or entropy. Therefore, "positive" implies the change is increasing and "negative" decreasing in value. On the other hand, the gain or loss in enthalpy or entropy means it is favorable for "gain" and unfavorable for "loss". Thus, a gain in enthalpy means the change is negative and a loss in entropy means the change is also negative.

APPENDIX B

In the present study, protein folding is a two-step process (depicted in Figure 1), first driven by the hydrophobic effect (D→MG) with subsequent stabilization by specific interactions within the protein (MG → N). Therefore, the entropy of protein folding can be expressed in terms of these two steps as

$$\Delta S^N_D = \Delta S(D\rightarrow N) = \Delta S(D\rightarrow MG) + \Delta S(MG\rightarrow N).$$

Also, as suggested by our hydrophobic scheme, we can divide the folding entropy into two independent sources

$$\Delta S(D\rightarrow N) = \Delta S_{conf}(D\rightarrow N) + \Delta S_{water}(D\rightarrow N)$$

where ΔS_{conf} refers to the protein conformational entropy (plus its direct interactions with water molecules). The second source, ΔS_{water}, is the entropy change due to the propagation of hydrogen bond networks in water when nonpolar surfaces are exposed. From Figure 1, it is clear that there is change in water entropy, ΔS_{water}, only in the first hydrophobic process D→MG. This implies $\Delta S_{water}(MG\rightarrow N)$ or $\Delta S_{water}(N\rightarrow MG)$ is zero. Hence, the entropy change of the two processes in terms of the two independent sources is

$$\Delta S(D\rightarrow MG) = \Delta S_{conf}(D\rightarrow MG) + \Delta S_{water}(D\rightarrow N)$$

$$\Delta S(MG\rightarrow N) = \Delta S_{conf}(MG\rightarrow N)$$

Next, we identify the positive terms and determine their relative order. The fact that the protein conformational entropy change in folding or unfolding is always favorable for the denatured state (D), makes the unfolding entropy, $\Delta S_{conf}(N\rightarrow D)$ or $\Delta S_{conf}(N\rightarrow MG)$ or $\Delta S_{conf}(MG\rightarrow D)$, always positive. From our scheme, the water entropy is always favorable for the native state (or the hydrophobic collapsed state such as the MG state). Therefore, the water entropy of folding, $\Delta S_{water}(D\rightarrow N)$ is positive.

At temperature $T > T_G$, the denatured state is favored due to the entropy term. Therefore, the unfolding entropy, ΔS^D_N ($T > T_G$) is positive. Together with the following two equations:

$$\Delta S^D_N\ (T > T_G) = \Delta S_{conf}(N\rightarrow D) + \Delta S^{heat}_{water}(N\rightarrow D)$$

ΔS_N^D ($T > T_G$) = ΔS_{conf}(N→D) – ΔS_{water}^{heat} (D→N)

deduced at temperature $T > T_G$, the protein conformational entropy change is greater than the water entropy (ΔS_{conf}(N→D) > ΔS_{water}^{heat} (D→N)).

As $\Delta S_N^D(T = T_S)$ is zero by definition at temperature $T = T_S$, the condition ΔS_{conf}(N→D) > ΔS_{water}^{heat}(D→N) extends to the temperature range ($T_s < T < T_G$). So at temperature $T > T_S$, we have

ΔS_{conf}(N→D) > ΔS_{water}^{heat}(D→N)
(when $T > T_S$)

On the other hand, at temperature $T < T'_G$, the folding entropy, ΔS_D^N ($T < T'_G$) is positive. With similar operations, we obtain ΔS_{water}^{cold}(D′→N) is greater than ΔS_{conf}(N→D′) at both $T < T'_G$ and $T'_G < T < T_s$. So at temperature $T < T_s$, we have

ΔS_{water}^{cold}(D′→N) > ΔS_{conf}(N→D′)
(when $T < T_s$)

Note that at temperature $T = T_S$, ΔS_{water}(D→N) = ΔS_{water}^{cold}(D′→N) = ΔS_{water}^{cold}(D→N).

The Relative Order of MG State

We first determine entropy change of D→MG at the temperature $T = T_S$. The entropy change of D→MG in terms of the protein and water terms is written as

$\Delta S_D^{MG} = \Delta S_{conf}$(D→MG) + ΔS_{water}(D→MG).

Because ΔS_{water}(MG →N) is zero, we get

$\Delta S_D^{MG} = \Delta S_{conf}$(D→MG) + ΔS_{water}(D→N)

At $T = T_S$, we know ΔS_{water}(D→N) is equal to ΔS_{conf}(N→D). Therefore, we have

$\Delta S_D^{MG}(T = T_S) = \Delta S_{conf}$(N→D) + ΔS_{conf}(D→MG)

$\Delta S_D^{MG}(T = T_S) = \Delta S_{conf}$(N→MG)

On the other hand, since ΔS_{water}^{cold}(N→MG′) is zero, the entropy change of N→MG′ at any temperature is given as

$\Delta S_D^{MG'}(T) = \Delta S_{conf}$(N→MG′)

Hence, we conclude that at temperature $T = T_S$, $\Delta S_D^{MG} = \Delta S_D^{MG'} = \Delta S_{conf}$(N→MG) as drawn in Plate 1A.