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Hydrophobicity at low temperatures and cold denaturation of a protein

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We elucidate the microscopic mechanism of the weakening of the hydrophobicity at low temperatures by investigating cold denaturation of a protein. We employ an elaborate statistical-mechanical theory combined with a realistic water model. At low temperatures, the ordered structure with enhanced hydrogen bonds of water molecules is formed near nonpolar groups, leading to entropic loss and energy gain which are both quite large. However, they are canceled out and make no contribution to the free-energy change. We argue that a different factor, which is responsible for the weakening of the hydrophobicity at low temperatures, induces cold denaturation.

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The hydrophobicity plays crucially important roles in a variety of phenomena in aqueous environments such as micelle formation, protein folding and aggregation, lipid membrane formation, and molecular recognition [1]. Historically, the concept of hydrophobicity arose in the context of the low solubility of nonpolar solutes in water. One of the complex aspects of the hydrophobicity is its temperature dependence. It is widely believed that the hydrophobicity originates from the inability of the solutes to participate in hydrogen bonds of water. If this is true, the hydrophobicity should be strengthened when the hydrogen bonding is enhanced, for example, by lowering the temperature. However, the hydrophobicity is weakened and the solute solubility becomes higher at low temperatures [2,3]. In this Rapid Communication, we analyze the microscopic mechanism of the weakened hydrophobicity at low temperatures by investigating cold denaturation of a protein [4,5], a striking example phenomenon which should closely be related to the weakening.

A protein folds into a unique native structure in aqueous solution under physiological conditions. However, the native structure becomes unstable at low temperatures (~255 K) as well as at high temperatures (~335 K), leading to the denaturation. The former is referred to as "cold denaturation." The microscopic mechanism of cold denaturation has been studied with the emphasis on the hydrophobicity. For example, it has recently been observed by Dias *et al.* [6] in molecular dynamics simulations for simplified model systems that the ordered structure of water is formed near nonpolar groups by the enhancement of hydrogen bonds at low temperatures. This water structuring leads to entropic loss and energy gain. It is assumed without proof that the energy gain dominates and a free-energy gain occurs. The transition to the denatured state, in which more nonpolar groups are exposed to water than in the native structure, accompanies large entropic loss and energy gain followed by significant lowering of the free energy. Thus, in the recently proposed view [6] the water molecules near nonpolar residues of a protein play key roles for the weakening of the hydrophobicity at low temperatures, which leads to cold denaturation.

In the present Rapid Communication, we reconsider the microscopic mechanism of cold denaturation. Although our basic stance is the same as that in the previous studies in the sense that the emphasis is placed on the hydrophobicity, we employ a more realistic model for water [7] and an elaborate statistical-mechanical theory for molecular liquids [3]. The changes in thermodynamic quantities upon the denaturation and their temperature dependences are actually calculated, and the physical origins of the calculation results are analyzed in detail.

Diluted proteins are considered here. The free-energy difference between the denatured state and the native structure ΔF can be described by

$$\Delta F(T) = \Delta E_{\mathrm{I}} + \Delta\mu(T) - T\Delta S_{\mathrm{C}}(T). \tag{1}$$

Here, E_{I} is the protein intramolecular energy, μ is the hydration free energy, S_{C} is the conformational entropy of the protein, and T is the absolute temperature. $\Delta Z \equiv Z_{\mathrm{D}} - Z_{\mathrm{N}}$ denotes the change in a thermodynamic quantity upon the denaturation. The subscripts "N" and "D" represent the values for the native structure and for the denatured state, respectively. Under the isochoric condition considered here, the hydration free energy is given by $\mu = U_{\mathrm{VH}} - TS_{\mathrm{VH}}$ where U_{VH} is the hydration energy and S_{VH} is the hydration entropy.

At room temperature, the native structure is stabilized and therefore ΔF is positive. For cold denaturation to occur, ΔF must turn negative at low temperatures. Here we argue that $\Delta\mu$ must exhibit a significantly large decrease with decreasing T for $\Delta F < 0$. ΔS_{C}, which is positive, is considered to be almost constant [4] or a slightly increasing function of T [8]. In either case, $-T\Delta S_{\mathrm{C}}$ increases as T becomes lower, shifting ΔF in a more positive direction. Thus, ΔS_{C} is not the factor inducing cold denaturation. Within the framework of classical mechanics, the intramolecular energy for any protein structure remains constant against the change in T. ΔE_{I} is not responsible for cold denaturation, either. Therefore, cold denaturation is induced only by a sufficiently large decrease in $\Delta\mu$.

To exclusively investigate the relation between the hydrophobicity at low temperatures and cold denaturation, we treat a model protein which comprises only nonpolar groups: The protein is modeled as a set of fused hard spheres. The (x,y,z) coordinates of all the protein atoms (hydrogen, carbon, nitrogen, oxygen, etc.) in the backbone and side chains

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are used as part of the input data to account for characteristics of each structure on the atomic level. The diameter of each atom is set at the σ value of the Lennard-Jones potential parameters of AMBER99. We assume that the denatured structure resembles a random coil because it is experimentally known that a protein takes almost completely unfolded structures upon cold denaturation [5]. Thirty-two random coils are generated by assigning random numbers to the dihedral angles [9]. The protein we consider here is protein G [Protein Data Bank code: 2GB1].

The hydration thermodynamic quantities are obtained through the morphometric approach [10,11]. In this approach, any of the hydration thermodynamic quantities Z is expressed using only four geometric measures of a protein with a fixed structure and corresponding coefficients which are independent of the geometric feature of the solute. The resultant expression is

$$Z = C_1 V_{\mathrm{ex}} + C_2 A + C_3 X + C_4 Y. \tag{2}$$

Here, V_{ex} is the excluded volume (EV), A is the water-accessible surface area (ASA), and X and Y are the integrated mean and Gaussian curvatures of the accessible surface, respectively. The water-accessible surface is the surface that is accessible to the centers of water molecules [12]. The EV is the volume that is enclosed by the surface area [10]. The four coefficients are determined from the thermodynamic quantities of hydration calculated for spherical solutes with various diameters d_{U} (with the length scales occurring for the protein considered: $0 \leqslant d_{\mathrm{U}} \leqslant 10 d_{\mathrm{S}}$). Details of the determination are described in our earlier publication [9]. In the calculations for spherical solutes, we employ the angle-dependent integral equation theory [3] applied to the multipolar model for water [7]. The model and theory has been shown to give a quantitatively accurate value of the hydration free energy (HFE) of a nonpolar solute [3]. The HFE μ is calculated using the Morita-Hiroike formula extended to molecular liquids [3,9]. S_{VH} is evaluated through the numerical differentiation of μ with respect to the temperature [3,9]. U_{VH} is obtained from $U_{\mathrm{VH}}=\mu+TS_{\mathrm{VH}}$. Four temperatures, 298, 273, 263, and 258 K, are examined. The number density of the model water is taken to be that of real water (the value at the lowest temperature is estimated by the extrapolation). Once the four coefficients are determined, the thermodynamic quantities of hydration for a protein with a fixed structure are obtained by calculating only the four geometric measures. The high reliability of the morphometric approach is demonstrated in our earlier publications [9,10]. In particular, the experimentally measured changes in thermodynamic quantities upon apoplastocyanin (a protein with 99 residues) folding are quantitatively reproduced by the present approach [9].

Hereafter, we discuss ΔZ (Z is μ, S_{VH}, and U_{VH}) which denotes the change in a thermodynamic quantity of water upon the denaturation. For example, ΔS_{VH} is the change in the water entropy. From Eq. (2), ΔZ is expressed as

$$\Delta Z = C_1 \Delta V_{\mathrm{ex}} + C_2 \Delta A + C_3 \Delta X + C_4 \Delta Y. \tag{3}$$

We decompose $\Delta\mu$, ΔS_{VH}, and ΔU_{VH} into two physically insightful terms. One of them consists of the second, third, and fourth terms in Eq. (3). This term, which is referred to as term 2, depends only on the changes in the area and curvatures of the water-accessible surface and represents the contribution from the water molecules near the protein. The other is the first term in Eq. (3) which is referred to as term 1. Term 1 depends on the change in the EV and includes the contribution from the water molecules which are considerably far from the protein. This term is related to the change in the total volume available to water molecules that coexist with the protein.

We consider the isochoric condition while the experiments are performed under the isobaric condition. Under the isobaric condition, a slight system-volume change occurs upon the protein denaturation. We note, however, that $\Delta\mu$ is the same under the two conditions [13]: $\Delta\mu=\Delta U_{\mathrm{VH}} - T\Delta S_{\mathrm{VH}}=\Delta H - T\Delta S_{\mathrm{PH}}$ ($\Delta H \sim \Delta U_{\mathrm{PH}}$ at 1 atm). As for the change in water entropy or energy, term 2 remains the same but term 1 becomes different. Under the isobaric condition, only the small part of term 1 of the water-entropy change which should occur under the isochoric condition is simply converted to term 1 of the water-enthalpy change [9]. Our conclusions are not altered under the isobaric condition.

It is experimentally known that the changes in entropy and enthalpy upon cold denaturation are both negative [4,5]. These signs are determined by the thermodynamic quantities of water for the following reason. The entropy change upon cold denaturation consists of the water-entropy change ΔS_{VH} and the conformational-entropy change for the protein ΔS_{C}. Since the latter is positive, cold denaturation accompanies a large, negative change in ΔS_{VH}. The enthalpy change arises from ΔE_{I} and ΔU_{VH}. The former is positive because the native structure has more intramolecular hydrogen bonds and lower van der Waals energy. Therefore, ΔU_{VH} must take a large negative value. Below we show that these characteristics as well as a sufficiently large decrease in $\Delta\mu$ at low temperatures can be reproduced by our theoretical method.

We first discuss the temperature dependences of $\Delta\mu$, $-T\Delta S_{\mathrm{VH}}$, and ΔU_{VH} shown in Fig. 1. Each thermodynamic quantity of the denatured state is the average of the values calculated for the 32 random coils. $\Delta\mu$ is $\sim$465 kJ/mol at 298 K but $\sim$350 kJ/mol at 258 K [Fig. 1(a)]. This decrease seems to be sufficiently large to induce cold denaturation for the following reason. It is experimentally known for a number of proteins that ΔF at 298 K is approximately +50 kJ/mol [14]. For our model protein, a fused hard spheres, E_{I} in Eq. (1) is zero. Assuming that ΔS_{C} is independent of T and applying Eq. (1) to the cases of 298 K and 258 K, we can estimate that ΔF at 258 K is ~ -10 kJ/mol: The denatured state becomes more stable.

We find that the hydrophobicity is weaker at a lower temperature in the sense that μ becomes smaller with decreasing T for both the native structure and the denatured state. This finding is consistent with the observation for a simple nonpolar solute such as methane [3]. Since the reduction in μ of the denatured state is much larger than that of the native structure with lowering T, the decrease in $\Delta\mu$ occurs at low temperatures.

$-T\Delta S_{\mathrm{VH}}$ [Fig. 1(b)], which is positive, increases further as T becomes lower. ΔU_{VH} [Fig. 1(c)], which is negative, decreases further as T becomes lower. The increase and the

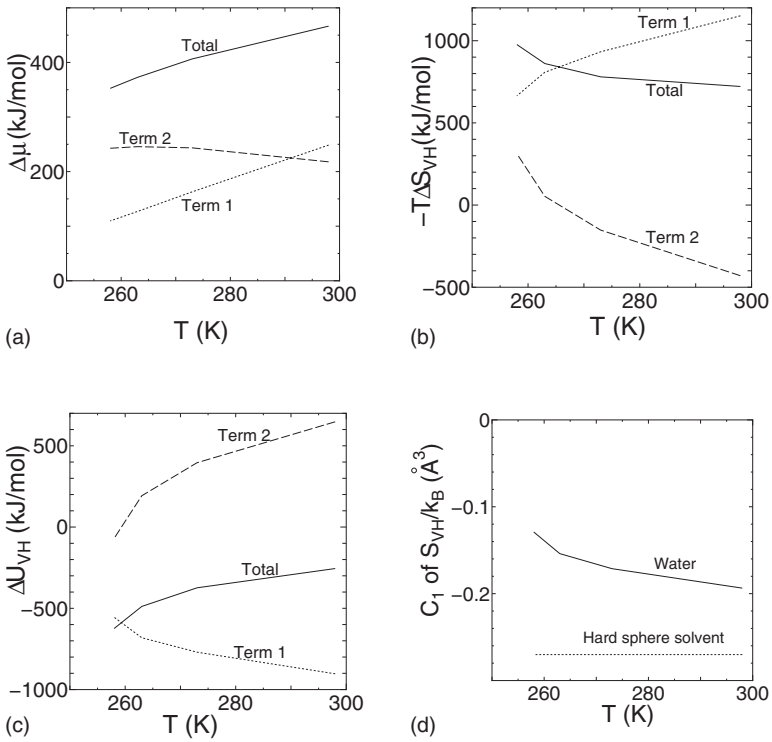

FIG. 1. Temperature dependence of changes in thermodynamic quantities of water upon protein denaturation: (a) free energy, (b) entropy multiplied by $-T$, and (c) energy. Term 1 is the first term in the right-hand side of Eq. (3) and term 2 comprises the other three terms. The sum of terms 1 and 2 is indicated by "total." (d) Temperature dependence of C_1 of S_{VH} for the hard-sphere solvent and water.

decrease are quite large at low temperatures. These observations are consistent with the experimental results showing that cold denaturation accompanies negative entropy and enthalpy changes.

The decomposition of $\Delta\mu$, $-T\Delta S_{\mathrm{VH}}$, and ΔU_{VH} into terms 1 and 2 is also shown in Fig. 1. The temperature dependence of $-T\Delta S_{\mathrm{VH}}$ [Fig. 1(b)] or that of ΔU_{VH} [Fig. 1(c)] is governed by term 2. Term 2 of $-T\Delta S_{\mathrm{VH}}$ and that of ΔU_{VH}, respectively, increase and decrease sharply at low temperatures, which implies large entropic loss and energy gain of the water near the protein surface. This result, which indicates that hydrogen bonds of the water molecules near nonpolar groups are strongly enhanced when the temperature is lowered, is in accord with the recently proposed view [6]. However, the entropic loss and energy gain arising from the enhanced hydrogen bonding (or equivalently, the formation of ordered structure of water), which occur upon the denaturation, are compensating. They are canceled out almost completely and make no significant contribution to the free-energy change as the line for term 2 in Fig. 1(a) shows.

The decrease in $\Delta\mu$ with decreasing temperature is ascribed to term 1. Therefore, cold denaturation cannot be induced by the formation of the ordered structure of water near nonpolar groups, which conflicts with recently proposed view [6]. Term 1 of $-T\Delta S_{\mathrm{VH}}$ decreases by $\sim$600 kJ/mol upon the temperature lowering from 298 K to 258 K, while term 1 of ΔU_{VH} increases by $\sim$400 kJ/mol. The increase in term 1 of ΔU_{VH} prevents cold denaturation. Therefore, the factor which weakens the hydrophobicity and induces cold denaturation is term 1 of $-T\Delta S_{\mathrm{VH}}$. This result indicates that the weakening of the hydrophobicity originates not from the change in hydrogen-bonding properties of the water molecules near the solute surface but from another factor.

The hydration entropy consists of the translational and orientational components which represent, respectively, the losses of the translational and orientational freedoms of water molecules caused by the solute insertion. We have recently shown that C_1 of the orientational component is 0 [9]. Thus, term 1 of $-T\Delta S_{\mathrm{VH}}$ possesses the translational component alone. Since the contribution from the water molecules near the protein surface is entirely in term 2, term 1 of $-T\Delta S_{\mathrm{VH}}$ is related to water molecules that coexist with the protein in the system. As shown in our earlier works [9,11,15], the EV of the native structure is much smaller than that of the denatured structure. Upon protein folding, the total volume available to the translational displacement of water molecules in the system increases and the water crowding reduces, leading to a large gain in the EV-dependent term of the translational component. Conversely, the denaturation causes a large loss of the EV-dependent term. This loss represented by term 1 of $-T\Delta S_{\mathrm{VH}}$ becomes much smaller at low temperatures, inducing cold denaturation.

The reduction in term 1 of $-T\Delta S_{\mathrm{VH}}$ at low temperatures is not only because T becomes lower but also because the EV-dependent term of the translational component of ΔS_{VH} itself, which is negative, also becomes smaller especially in the low temperature region. This implies that negative C_1 of S_{VH} increases with decreasing T [see Fig. 1(d)] because ΔV_{ex} is independent of T. Here we compare the hard-sphere solvent and water sharing the same number density and molecular diameter. As shown in Fig. 1(d), the absolute value of C_1 of the hard-sphere solvent is considerably larger and remains almost unchanged against the temperature change. Therefore, the temperature dependence of C_1 for water is ascribed primarily to the strong attractive interactions (i.e., hydrogen bonds). At lower T, the effect of the attractive interactions becomes larger, and C_1 departs more from that for the hard-sphere solvent.

Since the temperature dependence of term 2 of $-T\Delta S_{\mathrm{VH}}$ or ΔU_{VH} is larger than that of term 1, the change in the heat capacity of water upon the denaturation ΔC_{VH} is determined mainly by the temperature derivative of term 2. As observed in Fig. 1(b) or Fig. 1(c), ΔC_{VH} is positive, which is in agreement with the experimental result [4,5].

Polar and charged groups of a protein are not considered in the present analysis. However, we have recently found that they become more hydrophilic in the sense that the hydration free energy of a polar solute or an ion further decreases at lower temperatures. They are promoted to be exposed to water and the denatured state is more favored. Thus, the presence of polar and charged groups is expected to facilitate cold denaturation.

At the large-solute limit, C_1 and C_2 in the morphometric form for μ are the pressure P and the surface tension γ, respectively. However, this does not hold for small solutes such as methane and a protein with hydrophobic regions of widely varying length scales. If $C_1=P=1$ atm, the EV term would be extremely small and could be neglected. If this was true, μ would be scaled by the ASA. Since $C_2=\gamma(>0)$ increases as T becomes lower, μ would be larger at a lower temperature. This clearly contradicts the weakening of the hydrophobicity which is experimentally known and should be a cause of cold denaturation of a protein. Therefore, in our study, Eq. (2) is used as a reasonable fit in which the formulation of the morphometric form is borrowed. The fit is justified by our recent study [9] showing that the morphometric approach can well reproduce the results from the three-dimensional integral equation theory applied to the same model protein immersed in a simple solvent (the solvent particles interact through strongly attractive potential such as water molecules).

In conclusion, we have elucidated the microscopic mechanism of the weakening of the hydrophobicity at low temperatures which leads to cold denaturation of a protein. At low temperatures the ordered structure of water with strongly enhanced hydrogen bonds is formed near nonpolar groups. The exposure of more nonpolar groups to water accompanies entropy loss and energy gain. These are both quite large and responsible for the observed negative changes in entropy and enthalpy upon the denaturation. However, the entropic loss and energy gain are almost completely canceled out and make no significant contribution to the free-energy change. The effect of the translational displacement of water molecules in the system, which stabilizes the native structure, becomes considerably less powerful when the temperature is lowered, leading to the weakening of the hydrophobicity and cold denaturation.

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