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A Reassessment of the Molecular Origin of Cold Denaturation

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The existence of cold denaturation is now firmly demonstrated by its direct observation for several globular proteins in aqueous solution. But the physico-chemical explanation of this intriguing phenomenon is still unsatisfactory. In this paper we deepen our understanding of cold denaturation by taking advantage of the theoretical model developed by Ikegami and using thermodynamic data on the transfer to water of liquid *N*-alkyl amides. The analysis leads to the conclusion that the presence of water is fundamental to determine the existence of cold denaturation due to its strong energetic interaction with the amino acid residues previously buried in the protein's interior.

Key words: protein stability, cold denaturation, peptide-water interaction.

The Gibbs energy change associated with the reversible denaturation of globular proteins is a direct measure of the thermodynamic stability of the native biologically active conformation. Experimental investigations by spectroscopic and calorimetric techniques have led to the conclusion that the denaturation Gibbs energy change shows a parabolic-like profile as a function of temperature (*1, 2*). This finding suggested the existence, beyond the well-known denaturation induced by a temperature increase, of a second conformational transition caused by a temperature decrease, which would usually occur at temperatures lower than room temperature (*3*). This intriguing phenomenon was called "cold denaturation." In 1986, by means of DSC and spectroscopic measurements, Privalov and co-workers achieved the first direct characterization of cold denaturation using myoglobin, for which the transition was expected to occur above 0°C at pH 3.8 (*4*). Now the phenomenon has been observed for several globular proteins dissolved in aqueous solutions (*5, 6*). These results show that cold denaturation is probably a universal feature of protein stability and that the "thermodynamic hypothesis" is the guideline to study the conformational transitions of globular proteins (*7*). Experiments demonstrated that, when the temperature of an aqueous solution of protein is gradually lowered, cold denaturation occurs with heat release and entropy decrease (*4-6*). These findings are surprising, since protein denaturation is a transition from an ordered three-dimensional structure to a disordered one. Indeed, in 1991 Franks and Hatley (*8*) wrote the following: "The exact molecular nature of an exothermic order/disorder transition is still something of a mystery."

In this paper we perform an analysis of the protein thermal stability in order to clarify the molecular origin of cold denaturation by taking advantage of Ikegami's model (*9-11*). This mean-field statistical thermodynamic model is able to account for many peculiar properties of globular proteins. The model is appealing for its simplicity and the possibility to consider the interaction of residues with water. By assuming that the interaction of buried residues with water can be mimicked by the transfer to water of liquid *N*-alkyl amides, cold denaturation readily emerges. The analysis leads to the conclusion that cold denaturation is a general phenomenon for globular proteins dissolved in aqueous solution, because the strong energetic interaction between buried residues and water tends to destabilize the native structure at low temperature.

THERMODYNAMIC ANALYSIS

The simplest thermodynamic approach is to consider that protein denaturation is well represented by the two-state transition model (*12, 13*). The validity of such a model is confirmed by recent DSC studies reviewed by Makhatadze and Privalov (*14*). The denaturation enthalpy and entropy changes, $\Delta_d H^\circ$ and $T\Delta_d S^\circ$, are reported in Fig. 1 as a function of temperature for a simulated two-state transition. The parameters used are: $T_d = 333.15$ K, $\Delta_d H^\circ(T_d) = 350$ kJ·mol^{-1}, and $\Delta_d C_p^\circ = 3.0$ kJ·K^{-1}·mol^{-1}, which is assumed to be temperature-independent. The values are selected only for a better graphical presentation of the two curves. Due to the large positive value of $\Delta_d C_p^\circ$, the denaturation enthalpy and entropy functions are strongly temperature-dependent and intersect each other at two points corresponding to the cold and hot denaturation temperatures. This figure can be divided into three regions by drawing vertical lines across the temperatures T_H and T_S, where the denaturation enthalpy and entropy, respectively, vanish. In region (a), $\Delta_d H^\circ < 0$ (*i.e.*, destabilizing the native structure), and $T\Delta_d S^\circ < 0$ (*i.e.*, stabilizing the native structure). In region (b), $\Delta_d H^\circ > 0$ (*i.e.*, stabilizing), and $T\Delta_d S^\circ < 0$ (*i.e.*, stabilizing). In region (c), $\Delta_d H^\circ > 0$ (*i.e.*, stabilizing) and $T\Delta_d S^\circ > 0$ (*i.e.*, destabilizing).

According to this division, we can conclude that: (i) cold denaturation, occurring on lowering temperature, is enthalpy-driven: there is a gain in enthalpy for the polypeptide chain on unfolding that is not compensated for by a corre-

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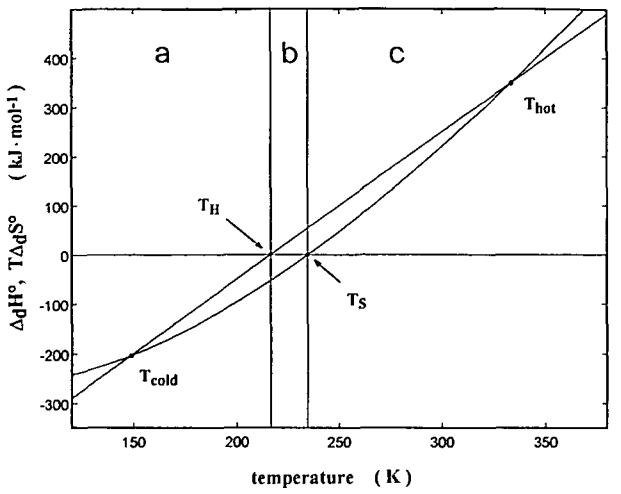

Fig. 1. **Plot of $\Delta_d H°$ and $T\Delta_d S°$ as a function of temperature for a calculated two-state transition.** The thermodynamic parameters used for the calculation are: $T_d = 333.15$ K, $\Delta_d H°(T_d) = 350$ kJ·mol^{-1} and $\Delta_d C_p° = 3.0$ kJ·K^{-1}·mol^{-1}. They are selected for presentation purposes only. See text for further details.

sponding entropy change; (ii) hot denaturation, occurring on increasing temperature, is entropy-driven: there is a gain in entropy for the polypeptide chain on unfolding that is not compensated for by a corresponding enthalpy change.

This thermodynamic analysis does not explain the underlying physical mechanism of the two transitions, but clearly emphasizes that they are not specular (*6*), because the driving forces are different in nature. For hot denaturation, it is straightforward to identify the gain of conformational degrees of freedom on raising temperature as the dominant term in the Gibbs energy balance. For cold denaturation, the dominant term is enthalpic and its molecular origin requires a satisfactory explanation. To reach a deeper understanding of cold denaturation we used as a framework the theoretical model developed by Ikegami.

IKEGAMI'S MODEL

In this model the protein structure is described by the binding state of possible non-covalent bonds in the uniquely folded conformation. Ikegami (*9-11*), setting at 0 K the standard state of energy, derived, in the mean-field approximation, the following expression for the Gibbs energy of the system:

$$G_o(N_b,T) = N_u\varepsilon + (N_bN_uZJ/N_o) - N_u\alpha T - RT\cdot\ln(N_o!/N_u!N_b!) \quad (1)$$

where N_o represents the number of non-covalent bonds which connect the system and which are uniformly arranged on a lattice in topologically similar distribution to that in the native structure at 0 K; N_o can be considered equal to the number of residues in the polypeptide chain; N_b is the number of formed bonds; and N_u is the number of broken bonds. The ε parameter represents the energy required to break one bond; J is the energy loss when two nearest neighbour bonds are not in the same state, assuming that each bond is surrounded by a number of nearest neighbours equal to Z. The α parameter represents the entropy gain associated with the breaking of one bond and is related to the increase of conformational degrees of freedom. Finally, the last term on the right-hand side of Eq. 1 represents the entropy change due to combinatorial effects, accounting for the modes to distribute over a lattice of N_o sites, N_b formed bonds, and N_u broken bonds.

The expression of G_o can be modified by introducing a variable $X \equiv 2(N_b/N_o) - 1$, corresponding to an "order parameter" of the structure:

$$G_o(X,T) = N_o\varepsilon\cdot[(1-X)/2] + N_oZJ\cdot[(1-X^2)/4] - N_o\alpha T\cdot[(1-X)/2] + N_oRT\cdot\{[(1+X)/2]\cdot\ln[(1+X)/2] + [(1-X)/2]\cdot\ln[(1-X)/2]\} \quad (2)$$

The order parameter is equal to -1 when all bonds are broken and to $+1$ when all bonds are formed. From our knowledge of Gibbs energy, it is possible to calculate the ensemble average value, $\langle X\rangle$, of the order parameter:

$$\langle X(T)\rangle = \int_{-1}^{1} X\exp[-G_o(X,T)/RT]\,dX \Big/ \int_{-1}^{1}\exp[-G_o(X,T)/RT]\,dX \quad (3)$$

and, by double differentiation with respect to temperature, the ensemble average heat capacity of the system:

$$\langle C_p(T)\rangle = -(N_o\varepsilon/2)\cdot(\partial\langle X\rangle/\partial T) - (N_oZJ/4)\cdot(\partial\langle X^2\rangle/\partial T) \quad (4)$$

Ikegami showed that, on increasing temperature, the model gives rise to a first-order phase transition when $(\varepsilon/ZJ) < (\alpha/2R)$, and otherwise a second-order phase transition. As the denaturation of protein tertiary structure can be assimilated to a first-order phase transition (*15, 16*), in our computations the condition $(\varepsilon/ZJ) < (\alpha/2R)$ is always satisfied. We performed calculations with the following set of parameter values: $N_o = 130$; $\varepsilon = 5{,}900$ J·mol^{-1}; $\alpha = 19.0$ J·K^{-1}·mol^{-1}; $ZJ = 7{,}110$ J·mol^{-1}. When the calculated $\langle C_p\rangle$ is plotted as a function of temperature, a sharp endothermic peak appears at the transition temperature equal to $\varepsilon/\alpha = 310.53$ K, corresponding to the first-order phase transition (see curve a of Fig. 2). This pattern of $\langle C_p\rangle$ contrasts with the DSC measurements of small globular proteins in aqueous solution (*4-6*). Indeed, on increasing temperature, in suitable conditions, from about -10°C to 100°C, two endothermic peaks are present: the first associated with cold renaturation and the second associated with hot denaturation. Furthermore, the calculated $\langle C_p\rangle$ falls to zero after completion of the transition, in contrast with the firmly established large positive heat capacity difference between denatured and native states (*1, 2*).

COLD DENATURATION IN IKEGAMI'S MODEL

For a reliable description of the temperature dependence of protein stability, the interaction of buried residues with water must be taken into account. This is achieved by introducing an extra term in Eq. 2, proportional to ΔG_w, the Gibbs energy change per mole of water, describing the interaction of broken bonds with water:

$$G_a(X,T) = G_o(X,T) + N_u\gamma\Delta G_w \quad (5)$$

where γ represents the number of water molecules interacting with one broken bond (*i.e.*, one residue, remembering that N_o roughly corresponds to the number of residues in the protein). In Ikegami's model, ΔG_w is expressed as a

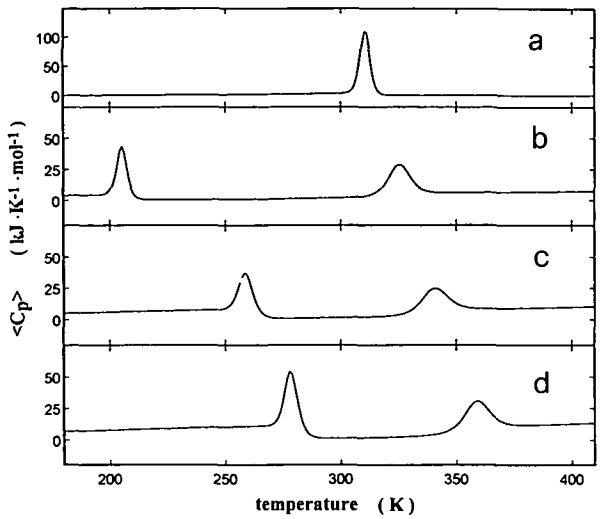

Fig. 2. **Plot of $\langle C_p \rangle$ as a function of temperature on changing the value of γ, the number of water molecules interacting with one broken bond.** $\gamma = 0$ for curve a; $\gamma = 2.5$ for curve b; $\gamma = 3.5$ for curve c and $\gamma = 4.5$ for curve d. Calculations are carried out according to Eq. 11, using the parameter values reported in the text.

quadratic function of temperature:

$$\Delta G_w = a + bT + cT^2 \quad (6)$$

The coefficients a, b, and c can be determined from the relations:

$$\Delta H_w = -T^2[\partial(\Delta G_w/T)/\partial T] = a - cT^2 = 0 \quad \text{at } T = T_H \quad (7)$$

$$\Delta S_w = -(\partial \Delta G_w/\partial T) = -b - 2cT = 0 \quad \text{at } T = T_S \quad (8)$$

$$\Delta C_{p,w} = (\partial \Delta H_w/\partial T) = -2cT \quad (9)$$

We assumed that a reliable process to mimic the interaction of buried residues with water is represented by the transfer to water of liquid *N*-alkyl amides, because these compounds contain the same chemical groups that constitute proteins. The interaction of polar peptide groups with water on unfolding must be accounted for, as Chothia and colleagues (*17*) showed that, on average, 75% of the polar accessible surface area, ASA, is buried in the protein interior. The burial of the polar peptide groups is an unavoidable consequence of the chain-connectivity, in order to obtain a folded globular structure.

The calorimetric data of Wadso and co-workers on a set of *N*-alkyl amides (*18, 19*), reported in Table I, allowed us to calculate the values of T_H, the temperature where the enthalpy change vanishes, assuming that the transfer heat capacity change is temperature-independent. The mean value of $T_H = 376.5$ K. Due to a lack of experimental data on the transfer entropy or Gibbs energy changes, the value of T_S, the temperature where the entropy change vanishes, has to be fixed. We selected $T_S = 386.0$ K, corresponding to that determined for the transfer to water of liquid hydrocarbons by Baldwin (*20*). This choice is validated by the fact that the overall entropy change for the transfer to water of an amphiphilic molecule is almost completely determined by nonpolar groups (*21, 22*). In addition, Oobatake and Ooi pointed out that the transfer enthalpy is the most distinct quantity to distinguish polar from nonpolar groups (*23*).

TABLE I. **Number of nonpolar hydrogen atoms, enthalpy and heat capacity changes associated with the transfer of *N*-alkyl amides from pure liquid phase to water at 298.15 K.** The data are from Refs. *18* and *19*. The temperature values where the transfer enthalpy is zero are reported in the last column.

Substance	N_{CH}	$\Delta_{tr}H^*$ (kJ·mol^{-1})	$\Delta_{tr}C_p^*$ (J·K^{-1}·mol^{-1})	T_H[a] (K)
N-Methyl-acetamide	6	−13.10±0.02	106.6±3.0	421.0
N-Ethyl-acetamide	8	−15.48±0.02	163.0±4.0	393.1
N-Methyl-propanamide	8	−14.87±0.02	155.0±3.0	394.1
N-Propyl-acetamide	10	−15.76±0.02	230.0±4.0	366.7
N-Isopropyl-acetamide	10	−17.24±0.02	230.0±4.0	373.1
N-Methyl-butanamide	10	−16.02±0.02	227.0±4.0	368.7
N,2-Dimethyl-propanamide	10	−15.79±0.03	222.0±5.0	369.3
N-Butyl-acetamide	12	−14.72±0.03	280.0±1.4	350.7
N-Methyl-pentanamide	12	−15.03±0.02	285.6±4.0	350.8

[a]The average value of T_H is 376.5±22.7 K.

In order to mimic the behaviour of amino acid residues on unfolding, it has to be noted that the denaturation heat capacity change, normalized per residue, proves to be limited in the range 30-90 J·K^{-1}·mol^{-1} for a large set of globular proteins (*24*). Actually, the positive contribution from nonpolar groups is larger, but is partially offset by the negative contribution from polar groups, especially CONH groups (*14*). Graziano and Barone (*25*) showed that the heat capacity change associated with the transfer to water of a nonpolar hydrogen atom, CH, is a constant, regardless of the molecular species and originating phase, and is equal to 30.0 J·K^{-1}·mol^{-1} at 298.15 K. Since there are 1.6 water molecules around a CH group (*25*), the heat capacity change per water molecule, $\Delta C_{p,w}$ in Eq. 9, can be set equal to 18.75 J·K^{-1}·mol^{-1} at 298.15 K. By allowing the number of water molecules γ to vary in the range 1.5-5.5, we indirectly take into account both the polar and nonpolar contributions, and the heat capacity change per broken bond (*i.e.*, per residue on unfolding) lies in the range 28-100 J·K^{-1}·mol^{-1}. Solving Eqs. 7-9 gives the following expression of ΔG_w:

$$\Delta G_w(\text{amides}) = -4457 + 24.27 \cdot T - 0.03144 \cdot T^2 \quad \text{J·(mol water)}^{-1} \quad (10)$$

This includes contributions from both the polar and nonpolar groups constituting the *N*-alkyl amides.

From the expression of $G_a(X,T)$, Eq. 5, it is possible to derive the ensemble average heat capacity of the system as a function of temperature:

$$\langle C_p(T) \rangle = -[N_o h(T)/2] \cdot (\partial \langle X \rangle/\partial T) - [N_o ZJ/4] \cdot (\partial \langle X^2 \rangle/\partial T) - N_o \gamma\, cT \cdot (1 - \langle X \rangle) \quad (11)$$

where $h(T) = \varepsilon + \gamma a - \gamma c T^2$. The calculations are carried out with the following set of parameter values: $N_o = 130$; $\varepsilon = 5{,}900$ J·mol^{-1}; $\alpha = 19.0$ J·K^{-1}·mol^{-1}; $ZJ = 7{,}110$ J·mol^{-1}; $\gamma = 2.5$, 3.5, and 4.5. When the calculated $\langle C_p \rangle$ is plotted as a function of temperature (see curves b, c, and d of Fig. 2), two endothermic peaks are present, corresponding to two phase transitions, the cold renaturation and the hot denaturation. Indeed, at increasing temperature, the ensemble average order parameter passes from $\langle X \rangle \approx -1$ to $\langle X \rangle \approx +1$, and then returns to $\langle X \rangle \approx -1$ (data not shown). Taking into account the interaction of buried residues with water, mimicked by the transfer to water of liquid *N*-alkyl amides, the $\langle C_p \rangle$ patterns calculated with

Ikegami's model qualitatively resemble the experimental ones.

Another choice is to consider that the interaction of buried residues with water can be mimicked by the transfer to water of liquid hydrocarbons (*26*). The process is characterized by $T_H = 295.0$ K and $T_S = 386.0$ K, as pointed out by Baldwin (*20*), and a Gibbs energy change that is large, positive, and not strongly temperature-dependent (*27*). The enthalpy change associated with the transfer to water of liquid aliphatic hydrocarbons is nearly zero at room temperature, because there is an almost perfect balance between water-hydrocarbon and hydrocarbon-hydrocarbon van der Waals interactions. By adopting the same type of constraints of Eqs. 7-9, the following expression of ΔG_w results:

$$\Delta G_w(\text{hydrocarbons}) = -2736 + 24.27 \cdot T - 0.03144 \cdot T^2 \quad \text{J} \cdot (\text{mol water})^{-1} \qquad (12)$$

The temperature dependence of this function is reported in Fig. 3, curve b, along with that of Eq. 10, curve a. The two curves have the same temperature derivative and concavity because we assumed that the transfer to water of liquid *N*-alkyl amides differs from that of liquid hydrocarbons for the enthalpy change but not the entropy change. In addition, the heat capacity change per mole of water is equal in both cases, in order to mimic the behaviour of amino acid residues (see above). As is well known, the solubility of liquid hydrocarbons increases on lowering temperature, but the transfer process is always non-spontaneous from a thermodynamic point of view. We inserted ΔG_w(hydrocarbons) in Eq. 5 and computed the transition temperatures using the same set of parameters as reported above. The temperatures are calculated by simply solving the following second-degree equation, which is analytically derived from Ikegami's model:

$$\gamma c T^2 + (\gamma b - \alpha) T + (\varepsilon + \gamma a) = 0 \qquad (13)$$

The values are collected in Table II along with those calculated with ΔG_w(amides). The data demonstrate that, by considering the transfer to water of liquid hydrocarbons, the two transitions happen in an unreasonable temperature range for a globular protein (*i.e.*, $T < 110$ K for cold denaturation, and $T > 450$ K for hot denaturation).

The validity of these results is grounded on the reliability of the parameter values used in the calculations. It would be necessary to show that the values selected for ε, ZJ, and α are physically reliable. The strength of a hydrogen bond in a polypeptide chain amounts to 5.4 kJ·mol^{-1}, according to the DSC data of Baldwin and co-workers on the temperature-induced helix-coil transition of a *de novo* designed α-helical C-50 alanine-rich peptide (*28*). The van der Waals interactions amount to 1.3 kJ·mol^{-1} per CH group, according to the analysis of the melting of aliphatic hydrocarbons by Honig and co-workers (*29*). On the average, an amino acid residue in globular proteins possesses 1.1 hydrogen bonds (*30*), and about 5.7 nonpolar CH groups (*24*). The value $\varepsilon = 5.9$ kJ·mol^{-1} selected by us corresponds well with the strength of a hydrogen bond, while the value $ZJ = 7.11$ kJ·mol^{-1} agrees with the product of the average number of CH groups and the strength of van der Waals interactions. Thus, on the basis of Ikegami's model, it seems that hydrogen bonds give rise to a network interconnecting the residues of the chain, whereas van der Waals interactions between side-chains provide the close-packed interior responsible for the strong cooperativity characteristic of the tertiary structure. On the other hand, the conformational entropy change per residue (*i.e.*, both the backbone and side-chain contributions) associated with protein unfolding falls in the range 18-25 J·K^{-1}·mol^{-1}, according to theoretical estimates (*31, 32*). The value $\alpha =$ 19.0 J·K^{-1}·mol^{-1} selected by us agrees with these estimates. In addition, from an analysis of existing thermodynamic data, it has been suggested that globular proteins share a common temperature, reflecting the melting of the solid-like protein core, equal to 324.7±8.0 K (*33*). This value is close to that used in our calculations, *i.e.*, 310.53 K.

Having verified the reliability of the parameters employed, we can conclude that Ikegami's model qualitatively reproduces the experimental findings only when coupled with the transfer to water of liquid *N*-alkyl amides. The amphiphilic nature of amino acid residues plays an important role in determining the temperature dependence of native conformation stability, because globular proteins in no respect resemble soluble hydrocarbon polymers.

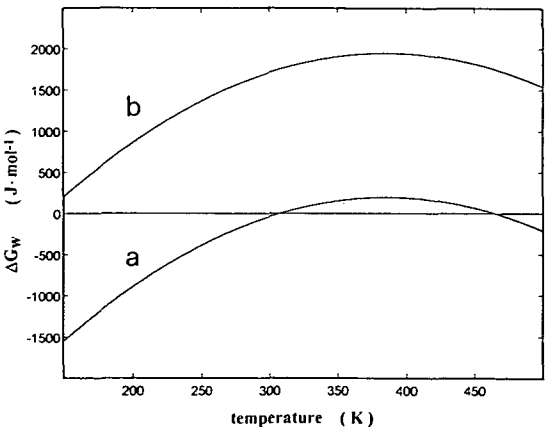

Fig. 3. **Plot of the Gibbs energy change due to the water interaction, ΔG_w, as a function of temperature for liquid *N*-alkyl amides, Eq. 10, curve a, and for liquid hydrocarbons, Eq. 12, curve b.**

INTERPRETATION OF THE RESULTS

According to the proposal by Bello (*34*) and Herzfeld (*35*), the denaturation of globular proteins can be divided into two sub-processes: (a) fusion of the protein interior; (b)

TABLE II. **Values of cold and hot denaturation temperatures, in Kelvin degrees, calculated by Ikegami's model coupled with the transfer to water of liquid *N*-alkyl amides, Eq. 10, and of liquid hydrocarbons, Eq. 12, as a function of γ, the number of water molecules interacting with one broken bond.** The parameter values used in the calculations are: $\varepsilon = 5{,}900$ J·mol^{-1}; $ZJ = 7{,}110$ J·mol^{-1}; $\alpha = 19.0$ J·K^{-1}·mol^{-1}.

γ	*N*-Alkyl amides		Hydrocarbons	
	T_{cold}	T_{hot}	T_{cold}	T_{hot}
0	—	310.53	—	310.53
1.5	52.64	316.42	—	453.11
2.5	205.24	324.97	23.61	506.61
3.5	259.15	340.13	62.20	537.08
4.5	278.95	358.70	81.49	556.16
5.5	286.97	375.10	92.96	569.11

interaction of previously buried residues with water. Thus the following denaturation Gibbs energy results:

$$\Delta_d G^\circ = \Delta_{s\to l} G + \Delta_{l\to w} G \qquad (14)$$

where s→l stands for the solid to liquid phase transition, and l→w stands for the transfer from liquid phase to water. This factorization is not an unfolding pathway but a thermodynamic division suitable for isolating the stabilizing and destabilizing factors, and it is not contradictory to the two-state transition model.

The solid-like nature of globular proteins is strongly supported by experimental results. In fact, detailed analyses pointed out that globular proteins have packing densities and adiabatic compressibilities comparable to those of molecular solids (*36-38*). Thus globular proteins are to be considered "crystal molecules," as suggested by Liquori about thirty years ago (*39*). Ikegami's model perfectly matches the scheme of Eq. 14, as the G_o term represents the fusion of the system, while the $N_u\ \gamma\ \Delta G_w$ term describes the water interaction [*i.e.*, Ikegami defined his model a "crystal approximation" (*11*)].

It is worth noting that Oobatake and Ooi (*23*), and Makhatadze and Privalov (*14*) separated the overall denaturation process as: (a) sublimation of the native structure; (b) hydration (*i.e.*, transfer from gas phase to water) of previously buried residues:

$$\Delta_d G^\circ = \Delta_{s\to g} G + \Delta_{g\to w} G \qquad (15)$$

where s→g stands for the solid to gas phase transition and g→w stands for the transfer from gas phase to water. As the Gibbs energy is a state function of the system, and the initial and final conditions are always the native and denatured conformations in aqueous solution, both the factorizing schemes are thermodynamically correct. There is no conceptual reason to prefer one scheme to the other, and it would be significant if both approaches led to the same results (see the "APPENDIX").

The native structure destabilization at low temperature, leading to cold denaturation, is caused by the ΔG_w term, modeled with the transfer to water of liquid *N*-alkyl amides. ΔG_w(amides) is depicted in Fig. 3 (curve a) as a function of temperature. The transfer to water of liquid *N*-alkyl amides is unfavourable at high temperature, but favourable at low temperature, as $\Delta G_w = 0$ at 301.0 K. The strongly attractive energetic interaction between amides and water is the dominant contribution at low temperature. In fact, as emphasized by the values of Table I, at room temperature and below, the transfer process is strongly exothermic. On the other hand, as temperature rises, the dominant contribution is the unfavourable entropy change due to an excluded volume effect (*40*). Thus, the analysis leads to the conclusion that the interaction of buried residues with water at low temperature destabilizes the native structure, because, from an energetic point of view, these residues prefer water rather than the protein interior.

In that cold denaturation is enthalpy-driven, it is significant to separate the transfer enthalpy change into a contribution from the polar CONH group and a contribution from the nonpolar CH groups. The values reported in Table I show that the enthalpy change is practically not affected by an increase of the nonpolar moiety, probably because the van der Waals interactions between nonpolar groups have the same strength in water and liquid amides. Thus, from the numerical average of the values in Table I, the contribution of the CONH group would roughly amount to -15.3 ± 2.0 kJ·mol^{-1} at 25°C. This quantity is very large, especially if one considers that for the transfer to water of liquid hydrocarbons the enthalpy change is nearly zero at room temperature. This simple comparison suggests that the energetic interaction of polar CONH groups with water is the main factor responsible for cold denaturation. It is to be noted, however, that a net and complete separation between polar and nonpolar contributions may be meaningless, because in the polypeptide chain the CONH groups feel the close presence of nonpolar groups and *vice versa*. In such conditions, the use of the additivity principle is probably wrong. In any case, it is almost certain that the energy cost associated with the desolvation and burial of polar peptide groups plays an important role in inducing cold denaturation in conjunction with the contribution arising from the interaction of nonpolar groups with water.

This conclusion is at odds with the traditional explanation (*41, 42*) that cold denaturation is an exclusive consequence of the increased solubility in water of nonpolar groups on lowering temperature. On the other hand, our conclusion agrees with that of Oobatake and Ooi (*23*), and Privalov and Makhatadze (*14*), who, independently, emphasized the fundamental role played by the interaction of water with both the polar and nonpolar groups. Actually, Makhatadze and Privalov (*14*) wrote the following: "the role of polar groups hydration in inducing cold denaturation is even more significant than that of nonpolar groups because hydration of polar groups has a Gibbs energy of negative sign." It is worth noting that different approaches to the problem of protein stability as a function of temperature lead to the same conclusion about the origin of cold denaturation.

EXPLANATION OF EXPERIMENTAL FINDINGS

In Ikegami's model, cold and hot denaturations shift to higher temperatures on increasing the value of γ, but the effect is greater for cold denaturation (see Table II). This agrees with the empirical observation that globular proteins with larger values of $\Delta_d C_p^\circ$ show cold denaturation at higher temperatures (*5*). An increase of γ corresponds to an increase of $\Delta_d C_p^\circ$, which can be taken as a hydrophobic index of the protein interior, since nonpolar groups on unfolding make positive contributions to the heat capacity change, while polar groups make negative contributions (*14, 24*). This proves that more hydrophobic proteins have higher cold denaturation temperatures. This finding can be rationalized as follows. The features of the environment from which the residues are transferred to water influence the strength of their interaction with water molecules. The energy cost associated with the desolvation and burial of polar peptide groups increases and becomes more destabilizing in the delicate Gibbs energy balance when the hydrophobicity of protein interior increases. The behaviour of polar groups is affected by the closeness and number of the surrounding nonpolar groups. Thus, changes in the ratio between polar and nonpolar contributions are reflected in shifts of cold denaturation temperature.

The calorimetric measurements of Wadso and coworkers (*43*) on the transfer of *N*-alkyl amides to various

solvents, collected in Table III, support this explanation. On increasing the solvent polarity (*i.e.*, its dielectric constant), the transfer of amides ceases to be endothermic and becomes exothermic. The transfer enthalpy from pure amide phase is strongly positive to CCl_4, zero to MeOH and strongly negative to H_2O. In addition, for the transfer of *N*-methyl acetamide from CCl_4 to H_2O, Klotz and coworkers (*44*) found, at 25°C, $\Delta_{tr}G^\circ = -17.2$ kJ·mol^{-1}, $\Delta_{tr}H^\circ = -32.6$ kJ·mol^{-1} and $\Delta_{tr}S^\circ = -51.9$ J·K^{-1}·mol^{-1}. The transfer process is enthalpy-driven due to the strong energetic interaction between *N*-methyl acetamide and water.

In this respect, it is important to remark that Fink and Painter (*45*) did not observe the cold denaturation of RNase A in an aqueous solution of 70% methanol at pH 3.0, since the protein showed substantial enzymatic activity even at 203 K, where the protein should be unfolded on the basis of its stability curve (*12*). This puzzling finding has not been explained, even though Privalov stated that alcohols do not aid in the observation of cold denaturation (*5*). This experiment can be rationalized in thermodynamic terms. The calorimetric data in Table III show that the transfer of liquid *N*-alkyl amides to methanol has an associated enthalpy change that is nearly equal to zero at room temperature. This means that the energetic interaction of amides with MeOH is much weaker than with H_2O. Therefore, holding the parallelism between amides and amino acid residues, it can be stated that in MeOH the destabilization of native conformation due to the ΔG_w term is strongly reduced and cold denaturation is likely to be shifted to lower temperature.

CONCLUSION

We have performed a thermodynamic analysis in order to clarify the molecular origin of cold denaturation, emphasizing that the process is enthalpy-driven. By inserting into Ikegami's model the Gibbs energy change associated with the transfer to water of liquid *N*-alkyl amides, we devised a model of globular protein thermal stability that qualitatively reproduces the experimental findings. It must not be forgotten that the validity of a model is difficult to establish. Nevertheless, a model that is able to explain the experimental results in a physically reasonable manner has to be taken seriously. This is the case of the model developed in this paper. It is simplistic and requires some empirical parameters, but it leads to a reliable picture of protein thermal stability.

TABLE III. **Transfer enthalpies at infinite dilution of several liquid and solid *N*-alkyl amides in different solvents at 25°C.** The data are from Ref. *43*. The values of dielectric constant for each solvent at 25°C are reported in the second row.

ε (25°C)	H_2O 78.54	MeOH 32.63	EtOH 24.30	PrOH 20.10	CCl_4 2.23
	$\Delta_{tr}H^\circ$ (25°C) in kJ·mol^{-1}				
MeCONHPr (l)	−15.76	−0.79	−0.09	−0.30	14.36
MeCONHiPr (l)	−17.24	−1.38	−0.53	−0.86	15.44
MeCONHBu (l)	−14.72	−0.32	0.14	—	16.74
EtCONHMe (l)	−14.87	−0.23	0.53	—	15.10
iPrCONHMe (l)	−15.79	0.10	0.48	0.30	16.47
MeCONHtBu (s)	−2.54	10.51	10.85	10.47	27.20
tBuCONHMe (s)	−6.26	9.98	10.48	10.38	24.82

The results indicate that the strong energetic interaction of water molecules with buried residues is the main driving force of cold denaturation. Additionally, the polar peptide groups, largely buried in the native structure as a consequence of chain-connectivity, seem to play a pivotal role. Therefore, the amphiphilic nature of the polypeptide chain and the presence of water are fundamental for the thermodynamic stability of globular proteins and the existence of two-conformational transitions.

APPENDIX

We tried also to use Ikegami's model according to the factorizing scheme of Eq. 15. For this purpose, the parameters of the model have to be re-selected. Theoretical and experimental studies showed that the strength of a hydrogen bond in the solid phase with respect to the gas phase amounts to 20-30 kJ·mol^{-1} (*46, 47*). The van der Waals interactions correspond to about 2.0 kJ·mol^{-1} per CH group, from the sublimation enthalpy of hydrocarbons (*48*), whereas the conformational entropy per residue amounts to 40-50 J·K^{-1}·mol^{-1} (*14, 23*). Thus, we fixed $\varepsilon =$ 24 kJ·mol^{-1}, $ZJ = 13$ kJ·mol^{-1} and $\alpha = 40$ J·K^{-1}·mol^{-1}. In this manner, the condition $(\varepsilon/ZJ) < (\alpha/2R)$ for a first-order transition holds and the transition temperature of Ikegami's model without water is $\varepsilon/\alpha = 600$ K. This value is close to that obtained by Oobatake and Ooi (see Table 10 in Ref. *23*). In fact, the mean value over 14 proteins of the chain unfolding enthalpy to the chain unfolding entropy ratio is 524.6 K.

The hydration of aliphatic hydrocarbons is characterized by $T_H = 354.2$ K and $T_S = 395.2$ K (*14*), and, according to the analysis by Graziano and Barone (*25*), the same condition selected for the transfer from liquid phase to water holds for the hydration heat capacity change. Solving Eqs. 7-9 gives the following function for ΔG_w:

$$\Delta G_w = -3944 + 24.85 \cdot T - 0.03144 \cdot T^2 \quad \text{J·(mol water)}^{-1} \qquad \text{(A1)}$$

Solving Eq. 13 with this function and $\gamma = 4.5$, only one transition temperature emerges, $T_{hot} = 583.4$ K; with $\gamma =$ 7.0, two transition temperatures emerge, $T_{cold} = 28.3$ K and $T_{hot} = 580.4$ K, but the values are unreasonable for a globular protein. On the contrary, by fixing the following function for ΔG_w:

$$\Delta G_w = -8560 + 29.15 \cdot T - 0.03144 \cdot T^2 \quad \text{J·(mol water)}^{-1} \qquad \text{(A2)}$$

and solving Eq. 13 with $\gamma = 4.5$, one obtains two transition temperatures, $T_{cold} = 287.7$ K and $T_{hot} = 356.8$ K, whose values are reasonable for a globular protein. The main difference between Eqs. A1 and A2 is in the enthalpic contribution: $\Delta H_w = -1.15$ kJ·mol^{-1} and -5.77 kJ·mol^{-1}, respectively, at 298.15 K. This finding confirms that it is necessary to consider the strong energetic interaction of buried residues with water, mostly due to polar peptide groups, to qualitatively reproduce cold denaturation. Therefore, also considering the factorizing scheme of Eq. 15, the conclusions reached using Ikegami's model do not change. Finally, a word of caution: in these conditions very small changes in γ can render the roots of Eq. 13 not real.

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