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Theory of cold denaturation of proteins

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ABSTRACT

A new approach to the problem of cold denaturation is presented. It is based on solvent-induced effects operating on hydrophilic groups along the protein. These effects are stronger than the corresponding hydrophobic effects, and they operate on the hydrophilic groups which are plentiful than hydrophobic groups. It is shown that both heat and cold denaturation can be explained by these hydrophilic effects.

Keywords: Protein Folding; Cold Denaturation; Hydrophobic; Hydrophilic Effects

1. INTRODUCTION

Understanding the Protein Folding Problem (PFP) has been one of the most challenging problems in molecular biology. An even more challenging problem is known as the cold-denaturation process [1-13].

In an excellent review article entitled "Cold Denaturation of Proteins", Privalov makes the following comments [1]:

"…disruption of the native structure upon heating, the heat denaturation of protein, appear to be an obvious effect. By the same argument, a decrease of temperature should only induce processes leading to increasing order."

Indeed for any process in which a molecule ***P*** converts from a state ***F*** having a lower energy and lower entropy to a state ***U*** having a higher energy and higher entropy, we should expect that as we increase the temperature the process will proceeds from ***F*** to ***U***. When the temperature is lowered the reverse process from ***U*** to ***F*** is expected to occur. There is no mystery in this.

The mystery of protein folding upon decreasing the temperature is that the conversion from ***U*** to ***F*** occurs at a range of temperatures at which the protein should have attained the ***U***, rather than the ***F*** state. Thus, the main challenge is to find the factors that cause the folding at relatively higher temperatures.

It is generally believed that water is the main factor that confers stability to the folded state (***F***) [14]. This belief is supported by the fact that the addition of a large quantity of a co-solvent at a fixed temperature, causes denaturation. This means that in the absence of a water-rich environment, the protein would have been in the unfolded state (***U***).

How exactly water molecules help in maintaining the stability of the folded state at temperatures which favor the unfolded state has been the essence of the mystery associated with protein folding.

In 1959, Kauzmann introduced the idea that the hydrophobic ($H\phi O$) effect is probably one of the major factors that confer stability to the native structure of the protein [14]. Since then most people held the view that the $H\phi O$ effect is the dominant factor in maintaining the stability of the native structure of protein [14,15].

The dominance of the $H\phi O$ effect in protein folding was challenged in the 1990s [16-18]. It was found that Kauzmann's model for the $H\phi O$ effect is not adequate in explaining the folding of proteins. Instead, a new and a rich repertoire of hydrophilic ($H\phi I$) effects were discovered. These $H\phi I$ effects provided explanation for both the process of protein folding and protein-protein association. In effect, the discovery of the $H\phi I$ effects has removed the mystery out of the protein folding phenomenon. This aspect of protein folding has been discussed in great detail elsewhere [15,19].

This article is devoted to the phenomenon of cold denaturation (CD) of proteins. As in the PFP, there are many factors that are operative in the process of CD. We shall examine some of these factors which, to the best of the author's knowledge were never considered before.

The main problem of cold denaturation is the following. It is relatively easy to understand the process of denaturation as the temperature increases. This aspect of the problem is briefly discussed in Section 2. When we cool down some solutions of a denatured protein a spontaneous renaturation occurs. The mystery associated with this process is one part of the PFP, and will not be discussed here [19]. Yet, an even greater mystery lurks at lower range of temperatures. In thermodynamic terms, we write the standard Gibbs energy of folding as

$$\Delta G^{\circ}\left(\boldsymbol{U}\rightarrow\boldsymbol{F}\right)=\Delta H^{\circ}-T\Delta S^{\circ} \qquad (1.1)$$

At sufficiently high temperature the entropy term will dominate. Since ΔS° for folding is negative, the standard Gibbs energy of folding at high temperatures is positive, *i.e*. the ***U*** state is favored. When the temperature is lowered, there must be an energetic reason that makes ΔH° negative and large enough to over compensate for the large positive $-T\Delta S^\circ$. This is essentially the PFP, namely what makes the folded structure more stable at lower temperatures.

Accepting whatever explanation for the change in the sign of ΔG° from positive to negative upon lowering the temperature, we expect that as we further lower the temperature, the value of $\left|T\Delta S^\circ\right|$ will become smaller. Therefore, we should expect that ΔG° will become even more *negative* as we lower the temperature. The fact that ΔG° becomes *positive* at lower temperature is therefore more of a mystery than the folding of the protein at higher temperature range

As in the case of protein folding, most theoretical approaches to CD have been based on the $H\phi O$ effects [2-13]. It is well known that both $H\phi O$ solvation and $H\phi O$ interaction increase, in absolute magnitude, as the temperature increases. This is true for temperature range at which the native structure of proteins is stable. Therefore, it is not a surprise that all microscopic theories of CD have been based on the $H\phi O$ effects. Unfortunately, the strength of the $H\phi O$ effects was grossly exaggerated in protein folding as well as in CD [15,19]. To the best of the author's knowledge no one has considered the $H\phi I$ effects in connection with the phenomenon of CD.

In Sections 3 and 4 we show that both heat and cold denaturation can be explained by the $H\phi I$ effects. The $H\phi O$ effects do contribute in the right direction to the CD, but their strength is about an order of magnitude weaker than the corresponding $H\phi I$ effects. Hence, we conclude that the $H\phi I$ effects must play the major role in both heat and cold denaturation.

2. THE UNFOLDING OF PROTEINS AT HIGH TEMPERATURES

Consider the process of folding of a protein

$$\boldsymbol{U} \rightarrow \boldsymbol{F} \tag{2.1}$$

We assume that all the accessible energy levels of the protein ***P*** can be split into two groups, **Figure 1**. The first group denoted ***F*** is characterized by lower energies and a fewer number of states. The second group, denoted ***U*** is characterized by higher energies and very large number of states.

The internal partition function of the protein P in an ideal gas phase is split into two terms;

$$\begin{aligned} q_P &= \sum_{all\ states} \exp\left[-\beta\varepsilon_i\right] \\ &= \sum_{i\epsilon U} \exp\left[-\beta\varepsilon_i\right] + \sum_{i\epsilon F} \exp\left[-\beta\varepsilon_i\right] = q_U + q_F \end{aligned} \tag{2.2}$$

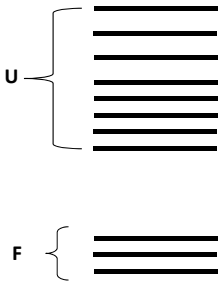

Figure 1. A schematic split of all energy levels of a molecule ***P*** in two groups; ***F*** having lower energies and fewer states, and ***U*** having higher energies and many more states.

The canonical partition function of a system of N molecules in a volume V and temperature T is

$$Q(T,V,N) = \frac{q_P^N V^N}{\Lambda_P^{3N}} \tag{2.3}$$

where Λ_P^3 is the momentum partition function, $\beta = \left(k_B T\right)^{-1}$, with k_B the Boltzmann constant and the T the absolute temperature.

The equilibrium constant for the reaction (2.1) can be easily obtained by maximizing the Helmholtz energy, or equivalently by finding the most probable distribution of molecules between the two states ***U*** and ***F*** [15].

$$K^{ig} = \left(\frac{\rho_F}{\rho_U}\right)_{eq} = \frac{q_F}{q_U} = exp\left[-\beta\left(\mu_F^* - \mu_U^*\right)\right] \tag{2.4}$$

where μ_F^* and μ_U^* are the pseudo chemical potentials of ***F*** and ***U***, respectively [20]. These are defined by

$$\mu_U^* = -k_B T ln q_U = -k_B T ln\left\{\sum_{i\epsilon U} \exp\left[-\beta\varepsilon_i\right]\right\} \tag{2.5}$$

$$\mu_F^* = -k_B T ln q_F = -k_B T ln\left\{\sum_{i\epsilon F} \exp\left[-\beta\varepsilon_i\right]\right\} \tag{2.6}$$

Note that since the momentum partition functions of ***U*** and ***F*** are equal to each other, the equilibrium constant depends only on the ratio of the internal partition functions of ***U*** and ***F***.

In this system the standard Helmholtz energy, entropy and energy of the system are given by

$$\Delta A^\circ\left(\boldsymbol{U} \rightarrow \boldsymbol{F}\right) = \mu_F^* - \mu_U^* \tag{2.7}$$

$$\begin{aligned} \Delta S^\circ\left(\boldsymbol{U} \rightarrow \boldsymbol{F}\right) = &-k_B \sum_{i\epsilon F} P\left(i/\boldsymbol{F}\right) ln P\left(i/\boldsymbol{F}\right) \\ &+k_B \sum_{i\epsilon U} P\left(i/\boldsymbol{U}\right) ln P\left(i/\boldsymbol{U}\right) \end{aligned} \tag{2.8}$$

$$\Delta E^\circ\left(\boldsymbol{U} \rightarrow \boldsymbol{F}\right) = k_B \sum_{i\epsilon F} P\left(i/\boldsymbol{F}\right)\varepsilon_i - \sum_{i\epsilon U} P\left(i/\boldsymbol{U}\right)\varepsilon_i \tag{2.9}$$

where $P(i/\boldsymbol{F})$ is the conditional probability of finding the molecule in state *i*, given that it is in the group of states ***F***. A similar meaning applies to $(i/\boldsymbol{U})$.

According to our assumptions ΔE° is negative, *i.e.* the average energy level of ***F*** is lower than that of ***U***. Also, ΔS° is negative for this reaction. Therefore, as the temperature increases we must have

$$K^{ig} = \exp\left[-\beta\left(\Delta E^{\circ} - T\Delta S^{\circ}\right)\right] = \exp\left[-\frac{\Delta E^{\circ}}{k_B T} + \frac{\Delta S^{\circ}}{k_B}\right] \to 0 \quad (2.10)$$

We find that as $T \to \infty$, $K^{ig} \to 0$, hence $x_F \to 0$. A simple example is shown in **Figure 2**. Here, we have only two energy levels ***U*** and ***F*** with different degeneracies ω_U and ω_F, respectively. In this case

$$\Delta E^{\circ} = \varepsilon_F - \varepsilon_U < 0 \quad \Delta S^{\circ} = k_B \ln\frac{\omega_F}{\omega_U} < 0 \quad (2.11)$$

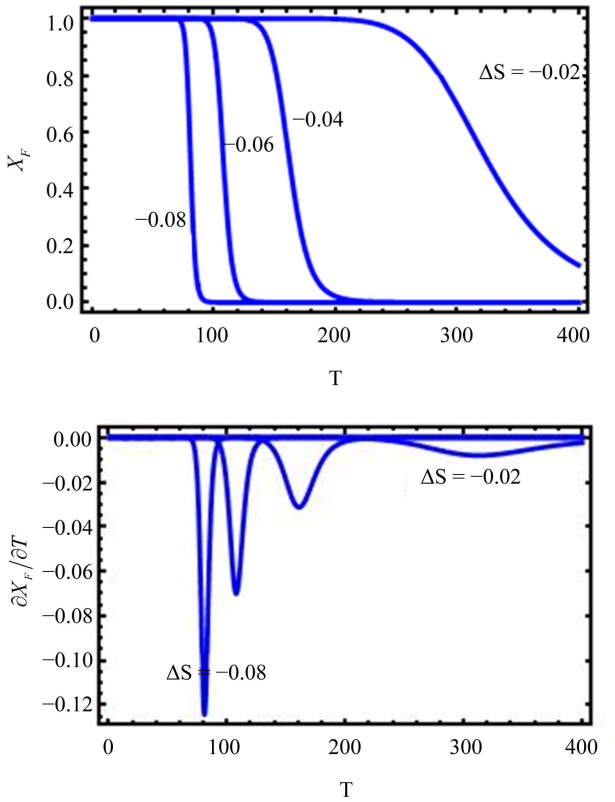

Figure 2. "Denaturation curves" for a system of two energy levels, with $\Delta E^{\circ} = -6.5$ kcal/mol and various values of $\Delta S^{\circ} = R\ln\frac{\omega_F}{\omega_U}$, The computations are based on **Eq.2.11**. These curves correspond to the degeneracy ration $\frac{\omega_U}{\omega_F}$ of about: 3×10^7, 10^{13}, 5×10^8 and 2×10^4. Lower panel shows the derivatives of x_F with respect to T.

Figure 2 shows a series of "denaturation" curves as a function of temperature for a fixed energy difference ΔE°, and varying the ratio of the degeneracies ω_F/ω_U, or ΔS°.

We see that as we increase the ratio ω_F/ω_U, the transition from ***F*** to ***U*** become sharper and occur at lower temperatures. The reason is simple and well understood. At higher temperatures the molecule will favor the state of higher degeneracy. On the other hand, at very low temperatures the molecule will favor the state of lower energy. The reason for the transition from ***F*** to ***U*** in real protein is essentially the same as in the simple case discussed above.

3. THE FIRST MYSTERY: WHY PROTEINS FOLD AS WE LOWER THE TEMPERATURE

We have seen that for any polymer having two macrostates; one having lower average energy and low degeneracy denoted ***F***, and the second having higher average energy and higher degeneracy denoted ***U***, we expect that as $T \to 0$, the system will favor ***F***, whereas as $T \to \infty$, the system will favor ***U***.

Now suppose that the molecular parameters are such that at about room temperature, say $T = 300K$. We find that $x_F \approx 0$. For instance, if the ratio of the degeneracies is $r = 10^4$, and the energy difference between the two states is of the order of $\varepsilon_{HB} \approx -6.5$ kcal/mol we find that at $T = 300$ K nearly all the molecules will be in the ***U*** state (see right curve in **Figure 2**). In this system one must go to temperatures below freezing $(T \approx 273\text{ K})$ to find any significant concentration of ***F***.

Now, we place the same polymer in water, and for simplicity we assume that the solution is very dilute with respect to the polymer. In this solution, if we find that the majority of the polymer molecules are now in the ***F*** state, then we must conclude that the equilibrium constant has changed, due to solvation effects. We write the equilibrium constant in the liquid state as [20]

$$K^{l} = K^{ig}\exp\left[-\beta\left(\Delta G_F^{*} - \Delta G_U^{*}\right)\right] = K^{ig}\exp\left[-\beta\delta G(\boldsymbol{U}\to\boldsymbol{F})\right] \quad (3.1)$$

where ΔG_{α}^{*} is the solvation Gibbs energy of the species α and $\delta G(\boldsymbol{U}\to\boldsymbol{F})$ is the solvent induced effect for the transition $\boldsymbol{U}\to\boldsymbol{F}$.

The relationship between the solvent-induced quantity δG and the solvation Gibbs energies is shown in **Figure 3**. Note that both ΔG_F^{*} and ΔG_U^{*} are determined by the solvation Gibbs energy of all the specific conformers belonging to the groups ***U*** and ***F***. If we denote by ΔG_i^{*} the Gibbs energy of solvation of a specific conformer *i*, then we have the relationships [20]

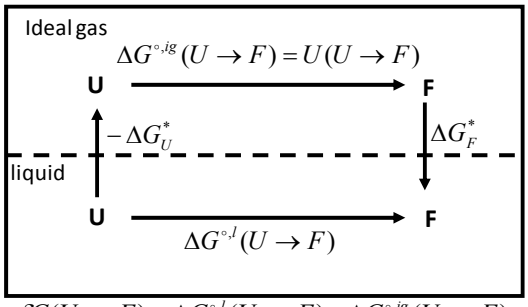

Figure 3. The relationship between the solvent induced effect $\delta G(\boldsymbol{U} \to \boldsymbol{F})$, and the solvation Gibbe energies may be deduced from the cyclic process in the figure.

$$\exp\left[-\beta\Delta G_F^*\right] = \sum_{i\epsilon F} x_i^{ig} \exp\left[-\beta\Delta G_i^*\right] \tag{3.2}$$

$$\exp\left[-\beta\Delta G_U^*\right] = \sum_{i\epsilon U} x_i^{ig} \exp\left[-\beta\Delta G_i^*\right] \tag{3.3}$$

where x_i^{ig} is the mole fraction of the specific conformer *i* in an ideal gas phase.

As Privalov had noted [1], according to Le Chatellier's principle, any process which is induced by increasing temperature should proceed with heat absorption, or equivalently with an increase in enthalpy and entropy.

For the reaction (2.1) we can formulate the Le Chatellier's principle as follows. At equilibrium we have

$$\mu_F = \mu_U \tag{3.4}$$

From the total derivative of $\Delta\mu = \mu_F - \mu_U$, along the equilibrium line, *i.e.* maintaining the condition 3.4, we have

$$\begin{aligned} 0 &= \left(\frac{\partial\Delta\mu}{\partial T}\right)_{P,eq} = \left(\frac{\partial\Delta\mu}{\partial T}\right)_{P,N_F,N_U} \\ &+ \frac{\partial\Delta\mu}{\partial N_F}\left(\frac{dN_F}{\partial T}\right)_{P,eq} + \frac{\partial\Delta\mu}{\partial N_U}\left(\frac{dN_U}{\partial T}\right)_{P,eq} \\ &= \left(\frac{\partial\Delta\mu}{\partial T}\right)_{P,N_F,N_U} + \left(\mu_{FF} - 2\mu_{FU} + \mu_{UU}\right)\left(\frac{\partial N_F}{\partial T}\right)_{P,eq} \end{aligned} \tag{3.5}$$

where N_F and N_U are the number of moles of $\boldsymbol{F}$ and $\boldsymbol{U}$ at equilibrium, and $\mu_{\alpha\beta} = \dfrac{\partial G}{\partial N_\alpha \partial N_\beta}$. From (3.5) we get

$$\left(\frac{\partial N_F}{\partial T}\right)_{P,eq} = \frac{\Delta S}{\left(\mu_{FF} - 2\mu_{FU} + \mu_{UU}\right)} \tag{3.6}$$

or equivalently, since $\Delta S = \bar{S}_F - \bar{S}_U = \left(\bar{H}_F - \bar{H}_U\right)/T$ at equilibrium we have

$$\left(\frac{\partial N_F}{\partial T}\right)_{P,eq} = \frac{\Delta H}{T\left(\mu_{FF} - 2\mu_{FU} + \mu_{UU}\right)} \tag{3.7}$$

The quantity $\mu_{FF} - 2\mu_{FU} + \mu_{UU}$ must be positive at equilibrium [20-22].

At the temperature of heat denaturation $\left(\dfrac{\partial N_F}{\partial T}\right) < 0$, and $\Delta H < 0$, $\Delta S < 0$. On the other hand at the temperature of Cold denaturation $\left(\dfrac{\partial N_F}{\partial T}\right) > 0$, and and $\Delta H > 0$, $\Delta S > 0$. As Privalov had noted it is relatively easy to understand the heat denaturation. The more intriguing question is to understand why ΔH (as well as ΔS) change signs at lower temperatures.

The question that has concerned many biochemists was to identify the part of the solvent induced effect that is sufficiently large and negative, such that it can turn the standard Gibbs energy of the transition $\boldsymbol{U} \to \boldsymbol{F}$ from large positive to large negative.

The answer to this question cannot be given without a detailed examination of *all* the contributions to the solvent induced effect $\delta G(\boldsymbol{U} \to \boldsymbol{F})$. For a long time most people assumed, based on Kauzmann's model for the *HϕO* effect, **Figure 4**, that $\delta G(\boldsymbol{U} \to \boldsymbol{F})$ is mainly determined by the desolvation of the *HϕO* groups, which are known to occupy the interior of the protein. Kauzmann's ideas were ad-hoc solutions to a difficult problem. It was a brilliant idea that captured the imagination of all those who were interested in protein folding. It should be said however, that at the time when Kauzmann suggested his ideas about the *HϕO* effect, it was also believed that intramolecular hydrogen bonding could not contribute significantly to the stability of the protein [15,21,22]. Furthermore, no other *HϕI* effects were known at that time. Hence, the dominance of the *HϕO* effect in protein folding was universally accepted.

However, a detailed study of all the ingredients that contribute to δG reveals that the answer to the ques-

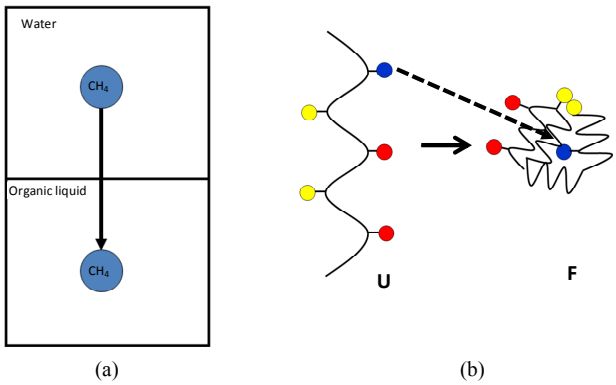

Figure 4. Kauzmann's model for the *HϕO* effect. The Gibbs energy of transferring a non-polar molecule, say methane from water to an organic liquid (a) is assumed to be similar in magnitude to the Gibbs energy change of a transferring a non-polar group from water into the interior of the protein (b).

tion is far from trivial. First, it was found that Kauzmann's model, *i.e*. the Gibbs energy of transferring of a $H\phi O$ solute from water to an organic liquid does not feature in $\delta G(\boldsymbol{U}\to\boldsymbol{F})$. The Gibbs energy of transferring a $H\phi O$ *group* attached to the protein from being exposed to water in the $\boldsymbol{U}$ conformer into the interior of the protein was found to be one or even two orders of magnitudes *smaller* than the estimated values of the Gibbs energy changes based on Kauzmann's model [15].

On the other hand, a host of solvent induced effects due to $H\phi I$ groups were found to be much larger than the corresponding $H\phi O$ effects [22]. Therefore, it was concluded that the $H\phi I$ effects are more likely to be the dominant contributor to the stability of the $\boldsymbol{F}$ conformer than any of the $H\phi O$ effects.

Thus, when comparing a specific $H\phi O$ effect with a specific $H\phi I$ effect, one finds that the magnitude of the latter is much larger than the former. Moreover, in real proteins what determines the standard Gibbs energy of the reaction is the combined effects of all the $H\phi O$ groups and all the $H\phi I$ groups. If there are roughly 30% of $H\phi O$ side chains and 50% $H\phi I$ side chains (the other 20% are "neutral"), then a protein of M amino acids have about M/3 $H\phi O$ groups, and about (M+M/3) $H\phi I$ groups, the additional 2 M of $H\phi I$ groups are the C=O and NH groups contributed by the backbone of the protein.

Therefore, even if each of the $H\phi O$ effect had the same magnitude as the corresponding $H\phi I$ effect, then we should expect that the combined effects of all the HϕI groups will be larger than the combined effects of all the $H\phi O$ groups. This conclusion is a fortiori true when each of the $H\phi I$ effect is an order magnitude *larger* than the corresponding $H\phi O$ effect. For more details see references [15,19]. We shall demonstrate this effect in a simple model in Section 5.

4. THE SECOND MYSTERY: WHY PROTEINS UNFOLD AS WE FURTHER LOWER THE TEMPERATURE

Having given a plausible argument, based on $H\phi I$ effects, for the folding of a protein in spite of the multitude of conformations belonging to the unfolded form, answers one of the most challenging problems of protein folding [19]. Yet, an even more challenging problem is lurking when we face the phenomenon of cold denaturation.

If $H\phi I$ interactions are the dominant factors that stabilize the 3D structure of the folded form, how can we explain the denaturation of the protein at lower temperatures.

Superficially, one would be tempted to embrace the $H\phi O$ effect to explain the cold denaturation. It is known that the strength of the $H\phi O$ effects, both solvation and pair wise interactions increase with temperature. Therefore, accepting the $H\phi O$ effect as the dominant one in the folding of protein offers a plausible explanation of the cold denaturation. Namely, as we decrease the temperature, the $H\phi O$ becomes weaker, hence the folded form becomes destabilized. This is the main argument given in all the theoretical approaches to the problem of CD.

Unfortunately, all the $H\phi O$ effects are too weak to explain folding in the first place. Therefore, one cannot rely on the temperature dependence of the $H\phi O$ to explain the unfolding of a protein at low temperatures.

A superficial argument based on $H\phi I$ effect seems to lead to the conclusion that as we lower the temperature, the $H\phi I$ effect will become stronger, and therefore causing further stability to the folded form. Indeed, this conclusion is true, had we only one type of $H\phi I$ effect. In reality, there is a host of $H\phi I$ effects, having different temperature dependence. Therefore, the answer to the question of why proteins unfold at a lower temperature is to be found in the *difference* in the rate of change of the various $H\phi I$ effect with increasing the temperature. In the next section, we shall demonstrate this effect in a simple model. Here, we present the general argument.

First, note that one type of $H\phi I$ effect operates mainly to stabilize the folded form. This is the direct intramolecular HBs between $H\phi I$ groups. Others are pair wise, triple-wise, etc. $H\phi I$ effects operate both on the folded and on the unfolded form. For simplicity let us assume that only one intramolecular HB is formed between two "arms" of two $H\phi I$ groups (say between NH and C=O). The formation of such a HB contributes to $\Delta G(\boldsymbol{U}\to\boldsymbol{F})$ about [15]

$$\begin{aligned}\delta G_2^{HB} &= \varepsilon_{HB} - 2\Delta G_1^*(one\ arm)\\ &\approx -6.5 - 2(-2.25) \approx -2\ \text{kcal/mol}\end{aligned} \tag{4.1}$$

i.e. we form one HB involving energy ε_{HB}, and we lose the solvation Gibbs energy of two arms $\left[\Delta G_1^*(one\ arm)\right]$, which were solvated in the $\boldsymbol{U}$ form **Figure 5**.

The second $H\phi I$ effect is between two $H\phi I$ groups at a distance of about 4.5 Å, and at the correct orientation so that they can be bridged by a water molecule, **Figure 6**. In this case, the contribution to the solvent-induced

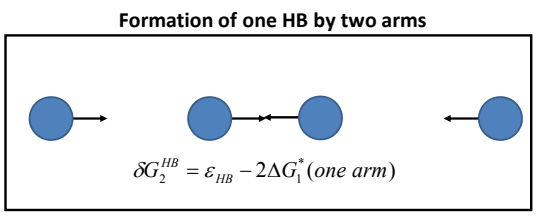

Figure 5. Formation of one intramolecular HB by two "arms" of $H\phi I$ groups involve the hydrogen bond energy ε_{HB} and the loss of solvation Gibbs-energies of the two arms.

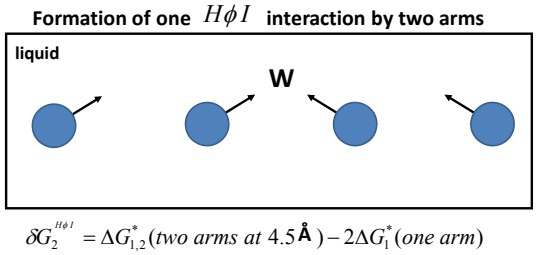

Figure 6. Formation of one $H\phi I$ interaction by two "arms" of $H\phi I$ groups at a distance of 4.5 Å and in the right orientation to be bridged by a water molecule.

part of the Gibbs energy is about [15]

$$\delta G_2^{H\phi I} = \Delta G_{1,2}^*\left(4.5\text{Å}\right) - 2\Delta G^*\left(one\ arm\right) \approx -2.5\ \text{kcal/mol} \quad (4.2)$$

Thus, if both $\left|\delta G_2^{HB}\right|$ and $\left|\delta G_2^{H\phi I}\right|$ decrease upon increasing the temperature we could not expect that these two effects will cause both a stabilization and a destabilization of the 3D structure. However, from a simple model discussed in the next section, we find that these two effects have different temperature dependence, **Figure 7**. In this particular case $\left|\delta G_2^{HB}\right|$ is larger than $\left|\delta G_2^{H\phi I}\right|$ at higher temperatures. Therefore, at these temperatures δG_2^{HB} stabilizes preferentially the folded form. On the other hand, at lower temperatures the $\left|\delta G_2^{H\phi I}\right|$ become the stronger effect. This $H\phi I$ effect can act on patterns on $H\phi I$ groups in the ***U*** form, while the δG_2^{HB} has the relatively smaller effect.

Thus, the fact that different $H\phi I$ effect operates on different patterns of $H\phi I$ groups, and these have different temperature dependence can explain both the heat and the cold denaturation. This is demonstrated in the next section.

5. A SIMPLE MODEL SHOWING BOTH HEAT AND COLD DENATURATION

We construct a "minimal" model for demonstrating both phenomena of heat and cold denaturation. This is a highly simplified model but it has enough real features, so as to show both transitions from ***U*** to ***F***, then from ***F*** to ***U*** upon cooling the system.

In **Figure 8** we focus on a small segment of the protein. We show here some representatives of solvent induced effects:

1) Desolvation of a $H\phi O$ group in the ***U*** state.

2) Van der Waals interaction between the $H\phi O$ groups and its surrounding in the ***F*** state (dashed lines in **Figure 8**).

3) Desolvation of a $H\phi I$ group in the ***U*** state.

4) An intramolecular HBing of two arms or two $H\phi I$ groups.

5) Pairwise $H\phi O$ interaction (double dashed lines).

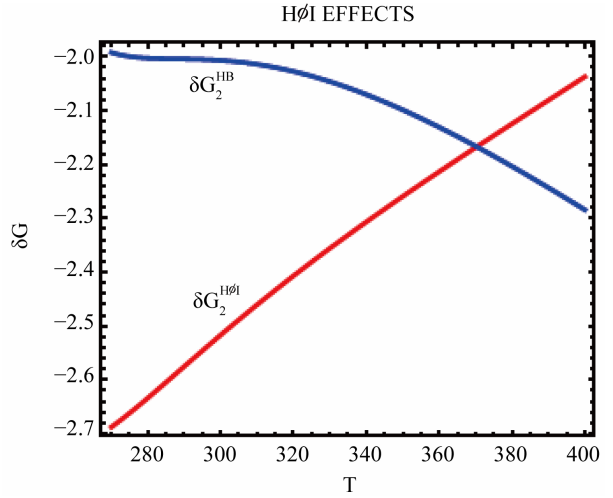

Figure 7. The temperature dependence of the two $H\phi I$ effects; the formation of intramolecular HB, δG_2^{HB} and pairwise $H\phi I$ interaction, $\delta G_2^{H\phi I}$. See Section 4 for details.

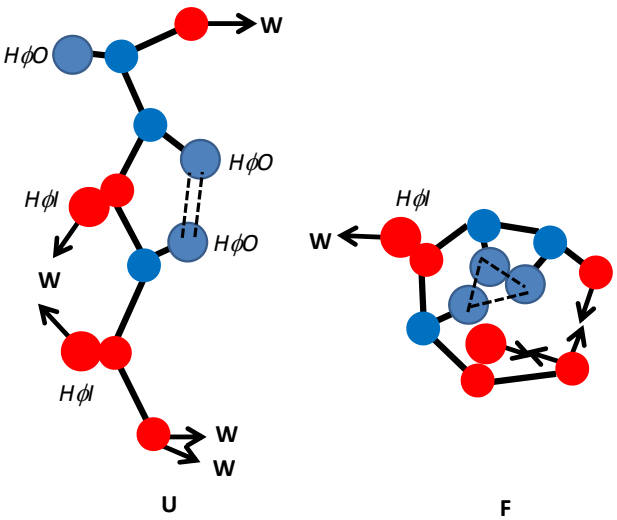

Figure 8. Illustration of a segment of a protein with three $H\phi O$ side chains (blue), and five $H\phi I$ arms; two belonging to side chains, and three belonging to the backbone (red). The configuration on the rhs represents the folded form ***F*** and on the lhs represent the unfolded form ***U***. The intramolecular HBs are represented by two arrows pointing towards each other. Van der Waals interactions are represented by dashed lines and hydrophobic interaction by double dashed doube lines. A non-bonded arrow represents a solvated arm of a $H\phi I$ group. Two arms pointing towards a water molecule (W) represents a pair-wise $H\phi I$ interaction. This drawing does not represent a model for protein. It only serves to show the types of interactions which are taken into account in the calculations discussed in Section 5.

6) Pairwise $H\phi I$ interaction (arrows pointing towards W).

A more complete inventory of all solvent-induced effect is discussed in reference 15.

The parameters we used for the following calculations

are as follows:

We take the HB energy as $\varepsilon_{HB} = -6.5$ kcal/mol . Each van der Waals interaction contributes about −0.5 kcal·mol. These two energies are presumed temperature independent. These are the only energies that contribute to the internal partition function of ***F***.

For the solvation Gibbs energy of a HϕO group we take the value of the conditional solvation Gibbs energy of methane next to a hydrocarbon [15,22] which is about 0.35 kcal/mol at room temperature.

From the experimental date available, we take the temperature dependence of the $H\phi O$ solvation to be

$$\Delta G^*_{H\phi O} \approx 0.3 + 0.0003T \tag{5.1}$$

Later we shall vary the values of these solvation Gibbs energies.

For the pairwise $H\phi O$ interaction and its temperature dependence we take the values [14]

$$\delta G_2^{\mathrm{H\Phi O}} \approx -0.3 - 0.0003T \tag{5.2}$$

For the solvation Gibbs energy of one arm of a $H\phi I$ group at room temperature we take the value of about −2.25 kcal/mol [15]. Its temperature dependence is calculated by estimating the probability of finding a water molecule in the right location and configuration to form a HB with one arm, from the equation [15,22]

$$\begin{aligned}&\Delta G_1\left(one\ arm\right)\\ &\approx -k_B T ln\left[P_{HB}\exp\left[-\beta\varepsilon_{HB}\right]+\left(1-P_{HB}\right)\right]\end{aligned} \tag{5.3}$$

In (5.3) P_{HB} is the probability that a water molecule will be found in the right position and orientation to form a HB with the arm. From the experimental values of $\Delta G_1\left(one\ arm\right)$ and the choice of ε_{HB} we can get the temperature dependence of the probability P_{HB}.

These values are also used for the calculations of the pairwise $H\phi I$ interactions between two arms [15]. **Figure 7** shows the temperature dependence of the two quantities δG_2^{HB} and $\delta G_2^{H\phi I}$ as defined in Section 4. Note the crossing of these two curves at about 370 K.

For the following calculations we assume that the segment of the protein has three $H\phi O$ groups and 12 $H\phi I$ groups. In real proteins the relative numbers of $H\phi I/H\phi O$ groups is even larger than 4:1. We also assume that in the ***F*** form there are two intramolecular HBs, and three van der Waals interactions. We shall later change the values of the various interactions in order to examine the influence of each of these on the heat and cold denaturation.

The internal partition function for this system in an ideal gas phase is

$$\begin{aligned}q_P &= \sum_{i\epsilon F}\exp\left[-\beta\varepsilon_i\right]+\sum_{i\epsilon U}\exp\left[-\beta\varepsilon_i\right]\\ &= q_P + q_U\end{aligned} \tag{5.4}$$

where

$$\begin{aligned}q_F &= \exp\left[-\beta\left(2\varepsilon_{HB}+3\varepsilon_{VDW}\right)\right]\\ q_U &= \sum_{i=1}^{N}\exp\left[-\beta_0\right] = N_c\end{aligned} \tag{5.5}$$

In the ideal phase we assume that the lower energy level is non-degenerated, and N_c is the degeneracy of the ***U*** form. Here, we choose $N_c = 10^{12}$.

The equilibrium constant in the ideal gas phase is

$$K^{ig} = \left(\frac{\rho_F}{\rho_U}\right)_{eq} = \frac{q_F}{q_U} \tag{5.6}$$

and the mole fraction of the folded form is

$$x_F = \frac{K^{ig}}{1+K^{ig}} \tag{5.7}$$

Figure 9 shows the standard Gibbs energy and the mole fraction x_F as a function of T, for an ideal gas phase. As expected we see that the standard Gibbs energy in monotonically increasing function of T. We also see "folding" at temperatures of about $T^{ig} \approx 260K$.

We next introduced the solvent. The equilibrium constant is changed according to Equation (3.1). In this particular calculation we have

$$\begin{aligned}K^l &= K^{ig}\exp\left[-\beta\left(\Delta G_F^* - \Delta G_U^*\right)\right]\\ &= K^{ig}\\ &\cdot\frac{\exp\left[-\beta\left(8\Delta G_1^* + 8\delta G_2^{H\phi I}\right)\right]}{N_C\exp\left[-\beta\left(12\Delta G_1^* + 3\Delta G_{H\phi O}^* + \delta G_2^{H\phi O} + 2\delta G_2^{H\phi I}\right)\right]}\end{aligned} \tag{5.8}$$

In the ***F*** form we have eight solvation Gibbs energies of the $H\phi I$ arms and eight pairwise $H\phi I$ interactions. In the ***F*** form we have 12 solvation Gibbs energies of the $H\phi I$ arms, 3 solvation Gibbs energies of the $H\phi O$ groups, one pairwise $H\phi O$ interaction and two pairwise $H\phi I$ interactions.

This particular choice was chosen for illustration of both the folding and the cold denaturation. In reality, different proteins will have different numbers of $H\phi O$ and $H\phi I$ groups, as well as different numbers of interactions. The following calculation is for a "typical" protein. Of course, one can multiply all these numbers by M for the whole protein and increase the degeneracy of the ***U*** form accordingly.

Figure 9 shows the results for the mole fraction of the ***F*** form

$$x_F = \frac{K^l}{1+K^l} \tag{5.9}$$

and the Gibbs energy change

$$\Delta G^*\left(\boldsymbol{U}\rightarrow\boldsymbol{F}\right) = -RTlnK^l \tag{5.10}$$

where R is the gas constant.

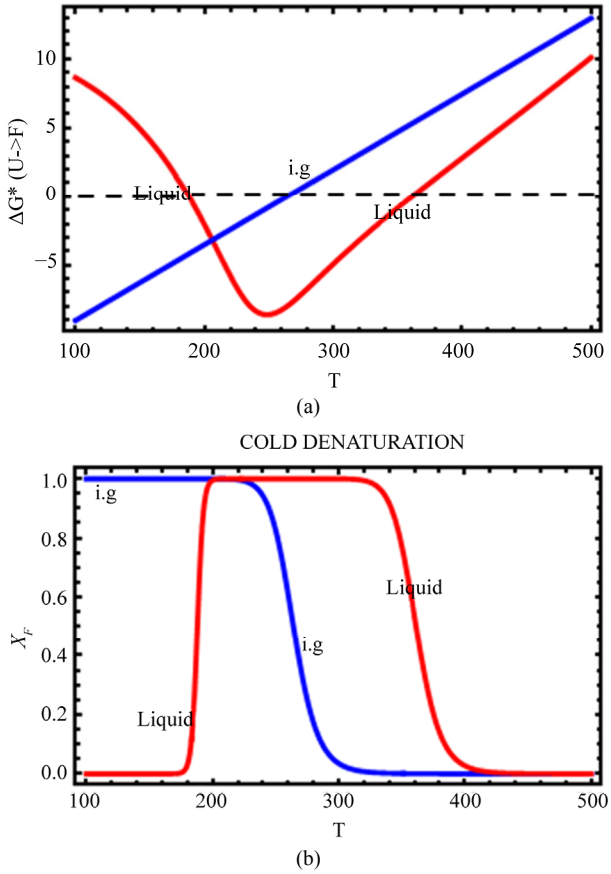

Figure 9. The Gibbs energy of folding (a), and the mole fraction of the ***F*** form (b) for the ideal gas (blue) and the liquid phase (red). Based on **Eqs.5.8** and **5.10**.

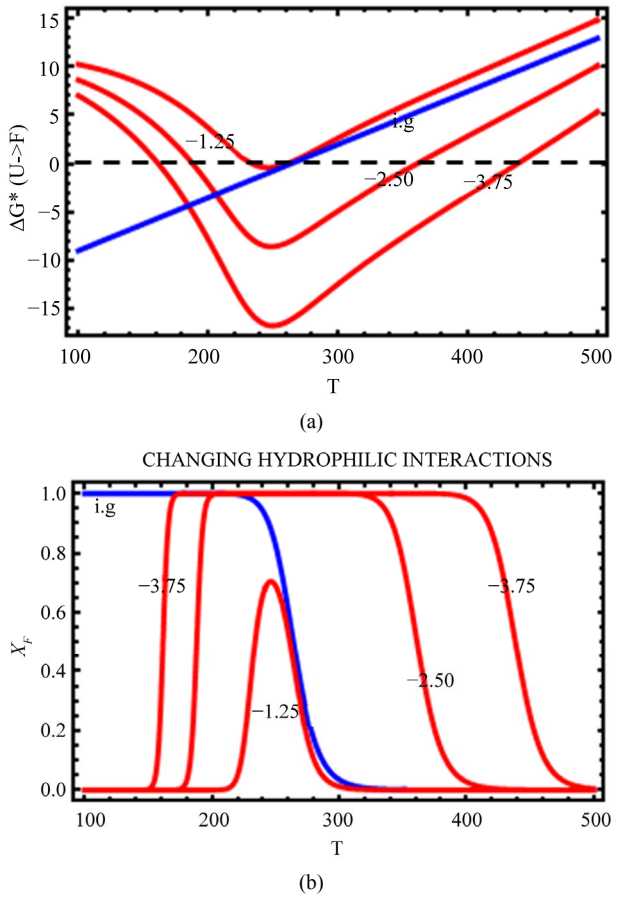

Figure 10. Same as **Figure 9** but with changing $H\phi I$ interaction (as indicated in the figure) above and below the values chosen in **Figure 9**.

In **Figure 9(a)**, we see that the standard Gibbs energy for the conversion $\boldsymbol{U \to F}$ goes through a minimum at about 250 K, but its values are negative in a large temperature range from about 180 K to 360 K. **Figure 9(b)** shows the "denaturation" curve in an ideal gas phase and in the liquid phase. In the liquid phase the folding of the same protein occurs at a considerable higher temperature compared with the transition in an ideal gas phase. At about 180 K we find a steep cold denaturation which, as expected does not occur in the ideal gas phase. Note in particular the large temperature range at which the mole fraction of the ***F*** form is nearly one.

In **Figure 10**, we change only the $H\phi I$ interactions by a factor of 0.5 and 1.5 leaving all the $H\phi O$ effects unchanged. We see that as we increase the $H\phi I$ interactions we get a folding at higher temperatures, and the range of temperatures at which the ***F*** form is stable increases. On the other hand, the temperature at which cold denaturation occurs is less sensitive to the $H\phi I$ interactions. It occurs at slightly lower temperatures as we increase the $H\phi I$ interactions. The most important finding is that when the $H\phi I$ interaction is about half of its estimated value, we get folding at almost the same temperature as in the ideal gas phase, but there is no range of temperatures at which the ***F*** form is stable (*i.e.* $x_F \approx 1$).

In **Figure 11**, we further decrease the $H\phi I$ interaction, we see that the standard Gibbs energy is everywhere positive. We do not observe folding, and there exists no range of temperatures at which the ***F*** form is stable.

Figure 12 shows the effect of changing the $H\phi O$ solvation and the $H\phi O$ interactions by a factor of 5, 9, 13 and 17 (see **Eqs.4.3** and **4.4**). We see that one has to increase the two $H\phi O$ effects by an order of magnitude or more to get folding and cold denaturation.

Figure 13 shows the values of the standard enthalpy of the folding ($\boldsymbol{U \to F}$), and the corresponding change in the heat capacity. It is clearly seen that the standard enthalpy changes from large positive to large negative values as we increase the temperature. This is consistent with the expected values of ΔH according to Equation (3.7). A detailed examination of the various contributions to the enthalpy change shows that the main contribution is the $H\phi I$ effect. **Figure 13(b)** shows a sharp increase of

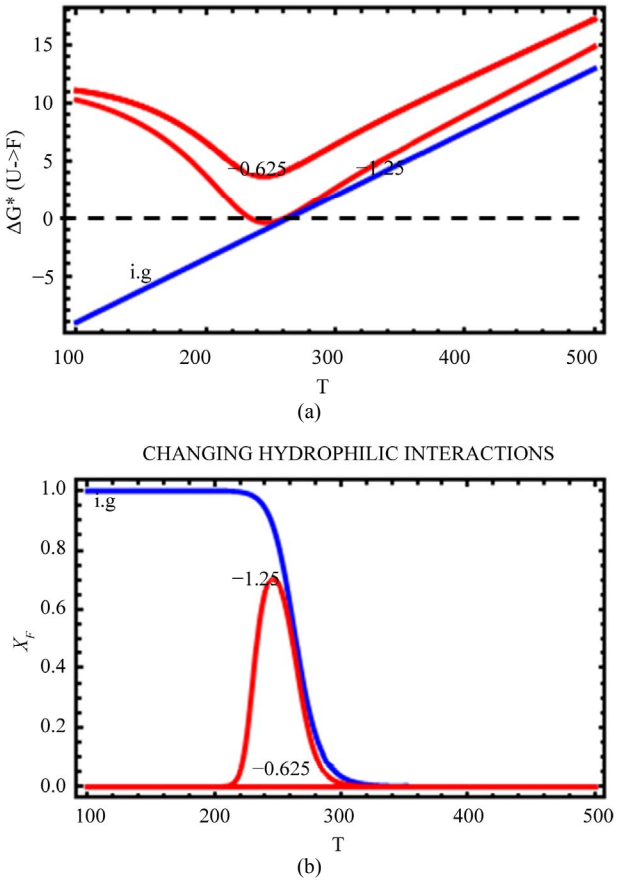

Figure 11. Further decreasing the $H\phi I$ interactions by factors of 0.5 and 0.25 relative to the values in **Figure 9**, (as indicated in the figure).

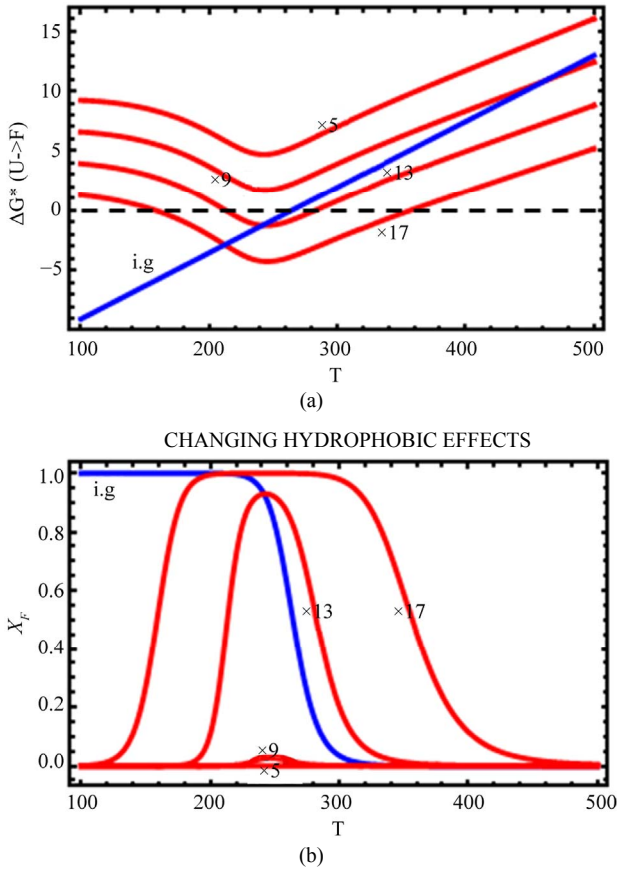

Figure 12. Changing the values of the $H\phi O$ solvation, and pair-wise $H\phi I$ interaction, and no $H\phi I$ interactions, (as indicated in the figure).

the heat capacity change $\left|\Delta C_p^*\right|$ in agreement with the experimental findings [1].

6. DISCUSSION AND CONCLUSION

The problem of cold denaturation (CD) is not a lesser mystery than the heat denaturation. As in the case of the protein folding problem, the search for a solution to the problem of CD has been derailed mainly because of the adherence to the myth that the $H\phi O$ effects are the most important effect in protein folding [19,22].

In the highly simplified model described in section 4 we have included both $H\phi O$ and $H\phi I$ effects. We have the desolvation of $H\phi O$ groups upon being transferred into the interior of the protein. We have pairwise $H\phi O$ interaction arising from the correlation between the (conditional) solvation of the two $H\phi O$ groups. We also have pairwise $H\phi I$ interaction, and an intramolecular HB.

An analysis of the contribution of the various effects clearly shows that the $H\phi I$ effects are the more important ones in the process of CD. One must realize that different $H\phi I$ effect operates on different patterns of $H\phi I$ groups. Therefore, the magnitude of the contribution of each type of $H\phi I$ effect would depend not only on the particular sequence of amino acids, but also on the particular conformation of the protein.

In real proteins there are many more factors that contribute to the Gibbs energy of the process of folding. There are pair-wise, triple-wise, etc. of the $H\phi O$ effects between different $H\phi O$ groups, and there are many $H\phi I$ effects between different $H\phi I$ groups. Thus, for a protein of M amino acids we might need to consider 20 different kinds of solvations, about 20^2 kinds of different pairwise correlations, and more triplets and quadruplets correlations. Clearly, it is not simple to make any general statement about the main factors that determines either the folding or the unfolding of any specific protein. All we can say at the moment is that each type of $H\phi I$ effect is larger than the corresponding $H\phi O$ effect. Considering that a protein of M amino acids might have about M/3$H\phi O$ groups, and more than 2M + M/3$H\phi I$ groups, we should conclude that the combined $H\phi I$ effects must be more important than the combined effects of all the $H\phi O$ groups.

For the particular cases computed in Section 5 we can conclude that the explanation of both heat and cold de-

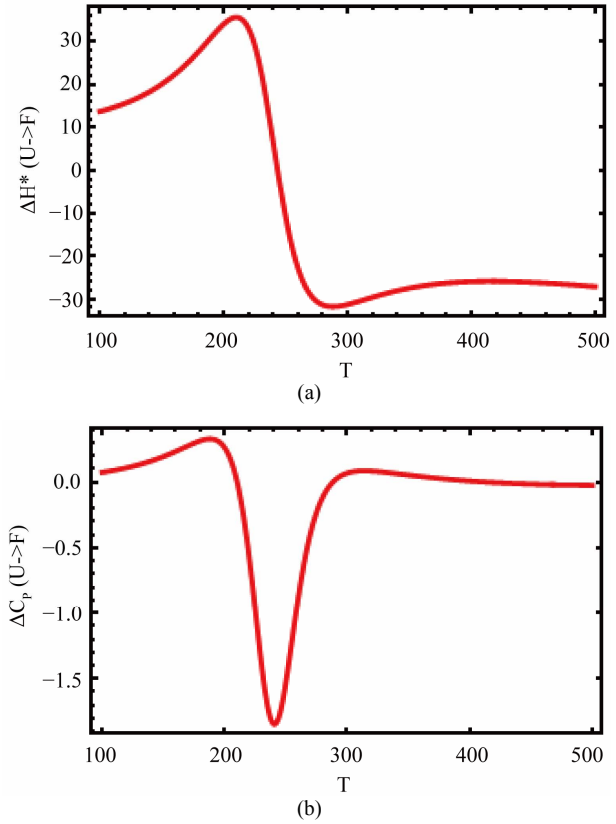

Figure 13. (a) The standard enthalpy change for the reaction $\boldsymbol{U \to F}$ as a function of the temperature; (b) The heat capacity change for the reaction $\boldsymbol{U \to F}$ as a function of the temperature. Calculated for the same parameters as in **Figure 9**.

naturation is as follows: At high temperatures the dominating interaction is δG_2^{HB} (**Figure 7**). This effect works to stabilize the $\boldsymbol{F}$ form. On the other hand, at lower temperatures the solvation Gibbs energy of the hydrophilic groups is larger, hence the tendency to form intramolecular HBs become weaker. This effect works to destabilize the $\boldsymbol{F}$ form. In addition, $\left|\delta G_2^{H\phi I}\right|$ becomes the larger effect at low temperatures (**Figure 7**). This effect operated mainly on the $\boldsymbol{U}$ form, simply because in this form there are more $H\phi I$ groups exposed to the solvent. Therefore, we can conclude that the variation of both δG_2^{HB} and $\delta G_2^{H\phi I}$ with temperature can explain both heat and cold denaturation.

Having said this we might speculate on which of the $H\phi I$ effects might be more important or most important in a real protein. The answer to this question is, of course sequence dependent. There are sequences for which intramoleular HB are the more important, yet there might be other sequences for which the pairwise or triplewise correlations might be more important.

Therefore, any general statement on which kind of $H\phi I$ effects are the dominant ones for all proteins is at present unwarranted and perhaps an even irresponsible statement. This is a fortiori true of statements claiming that the $H\phi O$ effects are the dominant ones in either protein folding or unfolding.

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