The Contribution of Hydrophobic Bonds to the Thermal Stability of Protein Conformations*

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In recent discussions of protein structure and reactions, much attention has been paid to hydrophobic bonds, i.e. interactions involving nonpolar amino acid side chains of proteins (1-5). The relative number of amino acids with nonpolar side chains is large, amounting to 35 to 50% of the total number of residues; as a consequence, the contribution of hydrophobic bonds to the stabilization of protein structures may be of considerable magnitude. The purpose of this communication is to point out that, although hydrophobic bonds may be important in stabilizing native proteins through their contribution to the over-all free energy of unfolding, $\Delta F_{\text{unf}}$, other factors may predominate in governing the temperature dependence of stability. This is because the dependence of the equilibrium constant on temperature is determined by the enthalpy of unfolding, $\Delta H_{\text{unf}}$, whereas the greater portion of the contribution of hydrophobic bonds to $\Delta F_{\text{unf}}$ is an entropic one (2, 6).

Aqueous solutions of low molecular weight hydrocarbons can serve as good model systems for the derivation of the thermodynamic properties of hydrophobic bonds in proteins. This was pointed out by Kauzmann (2), who has made some numerical estimates of the strengths of hydrophobic interactions in proteins. In a more detailed treatment, it is necessary to differentiate between the nonpolar side chains in proteins and freely moving small hydrocarbon molecules. We have recently developed the details of a statistical thermodynamic theory of water (7), of aqueous solutions of hydrocarbons (8), and of hydrophobic bonds (6). The results (6) indicate that the formation of a hydrophobic bond consisting of two nonpolar side chains in contact (in aqueous solution) is accompanied by a standard free energy change of approximately 0 to $-1.5$ kcal per mole at $25^\circ$.

The exact value (6) depends on the nature of the side chains and on the extent of contact between them.1 The temperature dependence of the thermodynamic parameters is illustrated by the theoretical values calculated (6) for a leucine-isoleucine bond with the maximal extent of side-chain contact (see Table I). This pair was chosen as an example, since the strength of its interaction is near the middle of the range for various pairs.

The data of Table I can be expressed in calories per mole by an equation of the following form.

$$\Delta F_{\text{H4}} = a + bT + cT^2 = 7290 - 47.8T + 0.0660T^2$$

An equation of this form, with different $a$, $b$, and $c$ values, can also be written for $\Delta F_{\text{H4}}$ for other side-chain pairs (6).

The formation of the hydrophobic bond is endothermic at low temperatures (below $58^\circ$ for this pair), indicating that the strength of the bond increases with increasing temperature until it reaches a maximal value. Bonds involving other side chains exhibit a similar temperature dependence. This conclusion about the temperature dependence of the strength of hydrophobic bonds is independent of the particular method by which the numerical values of Table I were obtained. As long as aqueous solutions of hydrocarbons are accepted as model systems for the treatment of hydrophobic bonds, this conclusion follows immediately, since it is an observed fact that the solution of aliphatic hydrocarbons in water is an exothermic process near room temperature (9, 10). This conclusion has also been pointed out by Kauzmann (2).

If hydrophobic bonds were the only or the predominant stabilizing influence maintaining the native structure of proteins in solution, then protein stability should increase with increasing temperature. In general, proteins have been observed to unfold as the temperature is increased. This indicates that there must be other factors determining the stability of native proteins besides hydrophobic bonds; these factors must have a temperature dependence opposing that of the hydrophobic bond, and predominating over it. Hydrogen bonds may provide this additional interaction, since they are known to become weaker with increasing temperature.

In particular cases, the contribution of various hydrogen bonds to the free energy of unfolding may be close to zero at a certain temperature. In such cases, the hydrophobic bonds would be the predominant factor in determining the stability of the folded protein at this temperature. However, the change in stability with temperature must contain enthalpy terms due to all interactions, and the effect of the hydrogen bonds may still predominate.

Even though they contribute only part of the free energy of unfolding, the enthalpy term is unfavorable, whereas the entropy term is favorable for the formation of the hydrophobic bond.

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stabilization of the folded form, hydrophobic bonds, as well as other possible side-chain interactions, are of great importance in the stabilization of native proteins. Short polypeptide helices without side-chain interactions have a marginal stability (11), depending on chain length and temperature, and a positive contribution to $\Delta F_{\text{unf}}$ from side-chain interactions will raise the transition temperature for unfolding.

In order to illustrate these effects, the treatment of an $\alpha$-helical polypeptide, containing only nonpolar amino acids, is outlined here. The standard free energy of unfolding of such a polypeptide is given by (11, 12)

$$\Delta F_{\text{unf}} = \Delta F^o_{\text{unf}} + \Delta F_{\text{conf}} + \Delta F^\phi$$

where $\Delta F^o_{\text{unf}}$ is the standard free energy of breaking the backbone hydrogen bonds and $\Delta F^o_{\text{conf}}$ is the standard free energy gain due to the conformational entropy of the backbone chain in the random coil. The two terms are usually treated together (11) and are expressed in the following form for a chain of $n$ residues.

$$\Delta F^o_{\text{conf}} = \Delta F^o_{\text{conf}}(n) + \Delta F^o_{\text{conf}}(\text{res}) = (n - 4)\Delta H_{\phi} - (n - 1)\Delta S_{\phi}$$

Equation 3 takes end effects into account. $\Delta F_{\text{conf}}$ is the enthalpy of breaking the hydrogen bond and was estimated by Schellman (11) to be 1500 cal per mole. $\Delta S_{\phi}$ is an effective entropy per residue, including both $\Delta S^o_{\phi}$ and $\Delta S^\phi_{\text{conf}}$, only the second term having been discussed by Schellman (11). $\Delta S_{\phi}$ can be estimated to be approximately 4 to 5 e.u. Both $\Delta H_{\phi}$ and $\Delta S_{\phi}$ are assumed to be independent of temperature over the range considered here.

$\Delta F_{\text{unf}}^o$ is given by Equation 1. The number of hydrophobic bonds (each bond involving two nonpolar side chains) in the $\alpha$-helix and in the random coil are denoted by $p_R$ and $p_{RC}$, respectively. Then

$$\Delta \rho = \rho_R - \rho_{RC}$$

In Equation 2, $\Delta \rho$ is used instead of $p_R$, since some hydrophobic bonds may occur even in the unfolded conformation. $\rho_{RC}$ is not known, but molecular models seem to indicate that not very many side chains can be brought into contact simultaneously in a randomly coiled polypeptide. Thus, with most of the hydrogen bonds broken in the random coil, even a few hydrophobic bonds can be of considerable importance in stabilizing this state to some extent. This effect somewhat diminishes the contribution of hydrophobic bonds to the relative stabilization of the $\alpha$-helix.

By using Equations 1, 2, and 3, the temperature dependence of the stability of the helix can be expressed as

$$\frac{d \ln K_{\text{unf}}}{d (1/T)} = -\frac{1}{R} \left[ (n - 4)\Delta H_{\phi} - (n - 1)\Delta S_{\phi} \right]$$

The temperature of maximal stability, $T_{\text{max}}$, is that at which $\Delta H_{\phi}^o = 0$. If $\Delta H_{\phi}^o$ of Equation 5 is set equal to zero, there is only one solution for $T > 0$. Below $T_{\text{max}}$, the temperature effect of the hydrophobic bonds predominates, resulting in an increase of helix stability with increasing temperature; above $T_{\text{max}}$, the stability of the helix decreases with increasing temperature, being determined by the temperature effect of the hydrogen bonds. The value of $T_{\text{max}}$ for a chain of given $n$ is determined by the strength of the hydrophobic bonds (i.e. $\alpha$ and $\phi$) and by $\Delta \rho$. Thus, we see that an inversion temperature for helix stability exists because of competing effects within the same polypeptide. It may be noted that an inversion temperature can also exist because of other competing effects, i.e. between two solvent components (10); in such cases an increase in temperature (at low temperature) may favor the helical form.

For a single helix, $p_R < n/2$. The limiting case of $p_R = n/2$ could be reached only for a $\alpha$-helical polypeptide in which all side chains are oriented in such a way that they can participate in hydrophobic bonds. Even with the maximal extent of hydrophobic bonding possible under such circumstances, and with the assumption of $p_{RC} = 0$ (most probably an underestimate), $T_{\text{max}} < 273^\circ$ K. Thus, the stability of single helices in water will decrease continuously with increasing temperature in the temperature range in which water is liquid at 1 atm. However, in a polypeptide or a protein containing several helices packed in a parallel array, $p_R$ can be higher than $n/2$ because of interchain hydrophobic bonding; this may result in $T_{\text{max}} > 273^\circ$ K. Even in such a molecule, it is fairly likely that $p_{RC} > 0$; this reduces the likelihood of attaining the condition, $T_{\text{max}} > 273^\circ$ K, although this possibility cannot be excluded.

Even though the effect of the hydrophobic bonds on the inversion of the temperature dependence may not be observable, their contribution to the stabilization of the helical form is of great importance and will result in an increase of the transition temperature of the helix-random coil equilibrium. This temperature, $T_{\text{tr}}$, is defined by the condition

$$\Delta F_{\text{unf}}^o = 0$$

and can be obtained from Equations 1, 2, and 3. As an illustration, $T_{\text{tr}}$, will be calculated for poly-$\alpha$-alanine with $n = 30$ and $p_R = 7$, these values corresponding to a polypeptide that has been studied by Berger and Lindström-Lang (14). The following equation holds for alanine-alanine hydrophobic bonds

$$\Delta F_{\text{unf}}^o = 3800 - 24.3 T + 0.0330 T^3$$

in calories per mole. Taking $\Delta S^o_{\text{conf}} = 4.8$ e.u., the value of $T_{\text{tr}}$ was calculated for $\Delta \rho = 0$ (no hydrophobic bond contribution), $\Delta \rho = 4$ (corresponding to $p_R = 3$), and $\Delta \rho = 7$ (maximal contribution with $p_{RC} = 0$). The transition temperatures are $-4, +21$, and $+35^\circ$, respectively. These results illustrate that even a moderate degree of hydrophobic bonding can make a significant difference in the stability of helical structures.

\[\text{Table I}\]

<table>
<thead>
<tr>
<th>$t$</th>
<th>$\Delta F_{\text{unf}}^o$</th>
<th>$\Delta F_{\text{unf}}^\phi$</th>
<th>$\Delta S_{\phi}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$-0.8$</td>
<td>$2.3$</td>
<td>$11.5$</td>
</tr>
<tr>
<td>25</td>
<td>$-1.1$</td>
<td>$1.5$</td>
<td>$8.6$</td>
</tr>
<tr>
<td>50</td>
<td>$-1.2$</td>
<td>$0.4$</td>
<td>$5.1$</td>
</tr>
<tr>
<td>70</td>
<td>$-1.3$</td>
<td>$-0.6$</td>
<td>$2.1$</td>
</tr>
</tbody>
</table>

The assumption of a different value for $\Delta S^o_{\text{conf}}$ would change the values of $T_{\text{tr}}$. However, the differences between the $T_{\text{tr}}$ values for various values of $\Delta \rho$ do not depend on $\Delta S_{\phi}$. Therefore, the
It is pertinent to note that the above discussion is by no means limited to transitions between the helical and randomly coiled conformations. For example, molecular models show that there may be only limited hydrophobic bonding in \( \alpha \)-helical structures of poly-L-lysine. However, \( \beta \)-structures of this polypeptide allow quite extensive hydrophobic bonding between the side chains. The observation (16) that \( \alpha \)-helical poly-L-lysine in aqueous solution at pH 10.8 is converted into the \( \beta \) form as the temperature is increased illustrates a possible influence of hydrophobic bonding also in the \( \alpha \rightarrow \beta \) conversion.

**SUMMARY**

The strength of a hydrophobic bond increases with increasing temperature up to approximately 60\(^\circ\). The observation that most proteins become unstable as the temperature is raised indicates that there must be some other factors besides hydrophobic bonds, e.g. hydrogen bonds, that contribute to the stabilization of proteins. The stability of an \( \alpha \)-helical polypeptide carrying side chains that participate in hydrophobic bonding is discussed. It is shown that the simultaneous presence of hydrogen bonds and hydrophobic bonds may lead to an inversion of the curve of helix stability versus temperature at low temperatures. The effect may be observable only for systems having a large number of hydrophobic bonds, e.g. between several parallel helices. The temperature of unfolding of the helix is shown to increase in the presence of hydrophobic bonds.

**REFERENCES**

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