

Desolvation Penalty for Burying Hydrogen-Bonded Peptide Groups in Protein Folding<sup>†</sup>

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Received: July 29, 2010; Revised Manuscript Received: October 4, 2010

A novel analysis of the enthalpy of protein unfolding is proposed and used to test for a desolvation penalty when hydrogen-bonded peptide groups are desolvated via folding. The unfolding enthalpy has three components, (1) the change when peptide hydrogen bonds are broken and the exposed  $-\text{CO}$  and  $-\text{NH}$  groups are solvated, (2) the change when protein–protein van der Waals interactions are broken and replaced by protein–water van der Waals interactions, and (3) the change produced by the hydrophobic interaction when nonpolar groups in the protein interior (represented as a liquid hydrocarbon) are transferred to water. A key feature of the analysis is that the enthalpy change from the hydrophobic interaction goes through 0 at 22 °C according to the liquid hydrocarbon model. Protein unfolding enthalpies are smaller at 22 °C than the enthalpy change for unfolding an alanine peptide helix. Data in the literature indicate that the van der Waals contribution to the unfolding enthalpy is considerably larger than the unfolding enthalpy itself at 22 °C, and therefore, a sizable desolvation penalty is predicted. Such a desolvation penalty was predicted earlier from electrostatic calculations of a stabilizing interaction between water and the hydrogen-bonded peptide group.

## Introduction

As shown here, the enthalpy of protein unfolding,  $\Delta H_{\text{PR}}$ , at 25 °C is smaller than expected from just one component of the enthalpy change, for breaking peptide hydrogen bonds (HBs) and solvating the newly exposed peptide  $-\text{CO}$  and  $-\text{NH}$  groups, when this component is represented by the unfolding enthalpy of the alanine peptide helix. Moreover, the expected contribution from breaking the van der Waals (vdW) interactions is considerably larger than this. This result immediately suggests that there is a desolvation penalty for burying the peptide HB in protein folding because at 25 °C, the enthalpy contribution from the hydrophobic interaction is close to 0, according to the liquid hydrocarbon model.<sup>4</sup> To test for a desolvation penalty, the factors contributing to  $\Delta H_{\text{PR}}$  are analyzed here. The probable existence of a desolvation penalty was deduced earlier from electrostatic calculations of solvation that showed a stabilizing interaction between water and H-bonded peptide groups.<sup>5–8</sup>

A pioneering analysis of  $\Delta H_{\text{PR}}$  was made in 1995 by comparing force field simulations of protein unfolding in vacuum<sup>1</sup> with quasi-experimental values<sup>2</sup> found by converting measured values of  $\Delta H_{\text{PR}}$  in water (25 °C) to values in vacuum by using empirical calibrations of the heats of desolvating exposed polar and nonpolar groups.<sup>2</sup> The results for four proteins<sup>1,2</sup> indicate there are four main factors contributing to  $\Delta H_{\text{PR}}$ , breaking the peptide HB, breaking vdW interactions, solvating nonpolar (NP) groups (the hydrophobic interaction), and solvating the polar  $-\text{NH}$  and  $-\text{CO}$  groups exposed by breaking the peptide HB.

However, there is a serious practical problem in estimating  $\Delta H_{\text{PR}}$  from these four factors because  $\Delta H_{\text{PR}}$  in water is a small difference between opposing factors;<sup>3</sup> breaking vdW and HB interactions favors folding, while solvating the newly exposed polar and nonpolar groups favors unfolding. Moreover, the impracticality of using these four factors to predict  $\Delta H_{\text{PR}}$  is

evident from comparing the simulated and quasi-experimental values of the unfolding enthalpy in vacuum (Tables I–III in ref 1);<sup>1</sup> the quasi-experimental values are roughly twice as large as the simulated values. The analysis given here assumes that the four main factors contributing to  $\Delta H_{\text{PR}}$  are correctly identified<sup>1,2</sup> but proposes a new route to analyzing  $\Delta H_{\text{PR}}$  in water.

## Results and Discussion

**Analysis of  $\Delta H_{\text{PR}}$  in Water.** To avoid estimating the four factors separately,  $\Delta H_{\text{PR}}$  is expressed in terms of the enthalpy changes for three sets of coupled reactions. Each of the three components of the overall enthalpy change is a difference between protein–protein interactions made within the native protein and protein–water interactions made after unfolding. (1)  $\Delta H_{\text{HB}}$  is the enthalpy change for breaking the peptide HB and solvating the newly exposed peptide  $-\text{NH}$  and  $-\text{CO}$  groups. (2)  $\Delta H_{\text{vdW}}$  is the enthalpy difference between breaking protein–protein vdW interactions and making protein–water vdW interactions. (3)  $\Delta H_{\text{NP}}$  is the enthalpy change for transferring nonpolar groups from the protein interior (represented here by a liquid hydrocarbon) into water.

The key point in the analysis is that  $\Delta H_{\text{NP}}$  can be represented by the liquid hydrocarbon model (LHM),<sup>4</sup> according to which  $\Delta H_{\text{NP}}$  is strongly temperature-dependent and passes through 0 at 22 °C. Thus,  $\Delta H_{\text{PR}}$  at 22 °C can be compared with the sum of the two remaining components,  $\Delta H_{\text{HB}} + \Delta H_{\text{vdW}}$ . The vdW interactions have been computed for native protein structures by an algorithm developed for this purpose,<sup>1</sup> based on a pairwise Lennard-Jones potential, and these values are used to model  $\Delta H_{\text{vdW}}$ . The enthalpy change expected from breaking the peptide HB is taken provisionally from the known value for unfolding an alanine peptide helix.

**Dividing the Unfolding Reaction into Two Stages.** It is convenient to divide the protein unfolding reaction into two hypothetical stages,  $\text{N} \rightleftharpoons \text{DMG}$  and  $\text{DMG} \rightleftharpoons \text{U}$ , where N = native, U = unfolded, and DMG = dry molten globule, a virtual

<sup>†</sup> Part of the “Robert A. Alberty Festschrift”.

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**TABLE 1: Parameters of the Liquid Hydrocarbon Model<sup>a</sup>**

compound	$T_h$	$T_s$	$k_{CP}$	$k_{ASA}$
benzene	15.8	112.8	86.2	21
toluene	18.4	116.9	86.7	22
ethylbenzene	18.6	111.5	82.3	23
cyclohexane	25.3	114.7	78.1	28
pentane	30.0	111.9	71.2	27
hexane	25.0	108.9	73.9	28

<sup>a</sup> Units:  $T_h$  and  $T_s$  are °C;  $k_{CP}$  is K;  $k_{ASA}$  is cal mol<sup>-1</sup> Å<sup>-2</sup>;  $k_{CP}$  is calculated from eq 2 using these values of  $T_h$  and  $T_s$ ;  $k_{ASA}$  is calculated from eq 1 using  $\Delta G$  values<sup>4</sup> from  $\Delta H$ ,  $\Delta S$  with  $\Delta G = \Delta H - T\Delta S$ , and with ASA values from ref 43 (Richards radii). The values of  $k_{CP}$  and  $k_{ASA}$  are at 25 °C.

intermediate. The purpose of dividing unfolding into two stages is to aid in explaining the results and does not affect the outcome. The DMG is not close-packed and has a liquid-like interior. Unfolding intermediates with DMG-like properties have been reported recently,<sup>9–11</sup> and a DMG-like intermediate has been found by simulating urea-induced unfolding of hen lysozyme.<sup>12</sup> The liquid-like vdW interactions present inside of the hypothetical DMG intermediate are assumed to have nearly the same energies as those made by the unfolded protein with water, as explained below.

**Modeling  $\Delta H_{NP}$  by the Liquid Hydrocarbon Model.** The general properties of the LHM are as follows. Accurate values of the transfer enthalpy,  $\Delta H$ , for a hydrocarbon solute passing between liquid hydrocarbon and water are known between 15 and 35 °C for six hydrocarbons.<sup>13</sup> The results fit a uniform model for the six hydrocarbons, and consequently, the LHM has been used to describe the transfer of nonpolar groups from the protein interior to water when unfolding occurs.<sup>4</sup>

$$\Delta H_{NP} = \Delta C_p(T - T_h) \quad (1)$$

Here,  $T_h$  is the temperature at which  $\Delta H_{NP}$  passes through 0 (for the six hydrocarbons,  $T_h = 22 \pm 5.5$  °C; Table 1).  $\Delta C_p$  is the difference in the heat capacity of the hydrocarbon solute between the liquid hydrocarbon and water;  $\Delta C_p$  has a positive sign for transfer from liquid hydrocarbon to water and for protein unfolding.  $\Delta C_p$  is constant in the temperature range studied (15–35 °C),<sup>13</sup> although it decreases at high temperatures.<sup>14</sup>

The transfer free energy of the hydrocarbon solute is found from its solubility in water

$$\Delta G_{NP} = -RT \ln X \quad (2)$$

where  $X$  is mole fraction for liquid–liquid transfer. The transfer entropy is

$$-T\Delta S_{NP} = \Delta C_p \ln(T_s/T) \quad (3)$$

where  $T_s$  is the temperature at which  $\Delta S_{NP} = 0$  for the six hydrocarbons studied ( $112.8 \pm 2.2$  °C or 386 K, the value to be used in eqs 3 and 4) (Table 1). ( $T_s$  is found by linear extrapolation from within the temperature range where  $\Delta C_p$  is constant.) Thus,  $\Delta G_{NP}$  is proportional to  $\Delta C_p$  and depends directly on  $T_h$  and  $T_s$ .

$$\Delta G_{NP} = \Delta C_p(T - T_h) + \Delta C_p T \ln(T_s/T) \quad (4)$$

The uniform pattern of results for the six hydrocarbons refers to the uniform values of  $T_h$  and  $T_s$  (Table 1).

If the hydrocarbon is toluene, which serves as a model for the phenylalanine side chain,  $\Delta G$  is 5.2 kcal/mol; it follows that a large change in free energy occurs when a buried phenylalanine side chain inside of a protein is exposed to water by unfolding, as pointed out originally by Kauzmann.<sup>15</sup> This basic fact about the energetic importance of the hydrophobic interaction in protein folding is widely accepted today, as is the use of solute transfer experiments<sup>15</sup> between water and an organic solvent to quantify the free-energy change for side-chain burial. The aim of the LHM is to go further and quantify not only the free-energy change but also the changes in enthalpy and entropy.

An important property of the LHM is that the vdW interaction energies are approximately the same (at 25 °C) for an alkane interacting with its neighbors in a liquid alkane or with water in aqueous solution.<sup>16,17</sup> This property is the basis for the assumption that the liquid-like vdW interaction energies within the virtual DMG intermediate have roughly the same values as those when the unfolded protein interacts with water.

**Modeling  $\Delta H_{HB}$  and  $\Delta H_{vdW}$ .** To model  $\Delta H_{HB}$  for the coupled processes of breaking the peptide HB and solvating the exposed –NH and –CO groups, consider first the known value of  $\Delta H_{HB}$  for unfolding peptide helices. Accurate enthalpy values for forming peptide helices were obtained in 2002–2005 by titration calorimetry. A sequence taken from an EF-hand protein can initiate helix formation when Ca<sup>2+</sup> or La<sup>3+</sup> is added<sup>18</sup> and the complete helix-forming reaction can be measured. Independent determinations by two groups gave  $-\Delta H_{HB} = -0.9 \pm 0.1$  kcal/mol for forming an Ala peptide helix,<sup>19,20</sup> and no measurable dependence of  $-\Delta H_{HB}$  on temperature was found between 5 and 45 °C. Similar values were found later for other peptide helices, although smaller (negative) values were found for  $\beta$ -branched amino acids, with  $-\Delta H_{HB}$  near  $-0.6$  kcal/mol.<sup>21</sup> Because helix formation is enthalpy-driven and Ala has only a –CH<sub>3</sub> side chain, the helical peptide HB itself is clearly helix-stabilizing, while  $-\Delta H_{HB}$  includes not only the HB but also any other interactions present in the helix and absent in the coil.

To model  $\Delta H_{vdW}$ , the vdW interaction energy in the native protein is first divided into two parts, a close-packed part and a liquid-like part, because native proteins are close-packed while unfolded proteins make liquid-like (i.e., not close-packed) interactions with water after unfolding. As pointed out above, the vdW interactions made by an alkane in liquid alkane have approximately the same energies as those made with water in aqueous solution. This is the basis for the simplifying assumption made here that the liquid-like part of the vdW interactions cancels out upon unfolding and only the close-packed part contributes to the unfolding enthalpy. When alkane crystals melt to a liquid form, the close-packed part of the vdW interaction energy is approximately one-half of the total energy,<sup>22</sup> and similar behavior is assumed here for the close-packed part of protein vdW interactions. The energy needed to break the vdW interactions when hen lysozyme unfolds in vacuum has been found by simulation to be 5.7 kcal/mol per residue,<sup>1</sup> and the close-packed part is assumed to be  $5.7/2 = 2.8$  kcal/mol per residue.

Large-to-small mutations of buried nonpolar side chains (e.g., Val to Ala) in two protein systems show large changes in  $\Delta H_{PR}$  at 25 °C.<sup>23,24</sup> If any structural changes caused by a mutation can be neglected, the observed enthalpy change should result from a change in  $\Delta H_{vdW}$ , the contribution from vdW interactions, because the contribution from  $\Delta H_{NP}$  should be negligible at 25

°C. The change in  $\Delta H_{PR}$  caused by a large-to-small mutation was measured as a function of the unfolding transition temperature ( $T_m$ ) between 30 and 80 °C for three mutations in ubiquitin, and linear plots of  $\Delta\Delta H_{PR}$  versus  $T_m$  were found.<sup>23</sup> Extrapolated to 25 °C, the changes in  $\Delta H_{PR}$  were (G. I. Makhatadze, personal communication) V5A, 2.4 kcal/mol; V17A, 6.7 kcal/mol; and L67A, 8.6 (total) or 2.9 kcal/mol per  $-\text{CH}_2$  group. Structural changes caused by the mutations may explain why different values of  $\Delta\Delta H_{PR}$  per  $-\text{CH}_2$  group were found.

In a related calorimetric study of  $\Delta\Delta H_{PR}$  values at 25 °C for large-to-small mutations in RNase S, the X-ray structures of the mutants were determined, and a new variable, the occluded surface (OS), was calculated from the mutant structures and used as an index of the total vdW interaction energy.<sup>24</sup> A good linear correlation between  $\Delta\text{OS}$  and  $\Delta\Delta H_{PR}$  was found, suggesting that the enthalpy changes are indeed caused by changes in the vdW interaction energy. Significant changes in  $\Delta H_{PR}$  were caused by mutations such as norleucine to leucine or isoleucine that do not change the number of  $-\text{CH}_2$  groups,<sup>24</sup> suggesting that structural changes caused by the mutations also produce a loss of vdW interaction energy. The ratio  $(\Delta H_{\text{vdW}})/(\text{OS})$  was computed for the four proteins with known vdW energies<sup>1</sup> and compared with the ratio  $(\Delta\Delta H_{PR})/(\Delta\text{OS})$  for the large-to-small mutations, and surprisingly, the mutations gave the larger ratio (R. Varadarajan, personal communication). Thus, both studies of large-to-small mutations<sup>23,24</sup> suggest that the vdW energies are at least as large as those indicated by calculated values for native proteins.<sup>1</sup>

**Comparison of  $\Delta H_{HB}$  and  $\Delta H_{HB}$  with Measured Enthalpies of Protein Unfolding.** In 1988, the unfolding enthalpies of 12 proteins were measured by scanning calorimetry and discussed;<sup>25</sup> tabulated values at 25 and 110 °C were presented. The difference in  $\Delta H_{PR}$  between 22 and 25 °C is small and is neglected here. The increase in  $\Delta H_{PR}$  between 25 and 110 °C is striking; for some proteins, the increase is  $\sim 6$ -fold. The values at 25 °C are small; individual values vary from 0 to 0.6 kcal/mol per residue, and the average value is 0.26 kcal/mol per residue. For hen lysozyme, which is discussed below, the value is 0.48 kcal/mol per residue. To compare this value with  $\Delta H_{HB}$  for the Ala peptide helix (0.9 kcal/mol), the latter value should be multiplied by 0.7 to give 0.6 kcal/mol because only  $\sim 70\%$  of residues in globular proteins make network HB.<sup>26</sup> The desolvation penalty is calculated for hen lysozyme as  $\Delta H_{HB} + \Delta H_{\text{vdW}} - \Delta H_{PR} = 0.6 + 2.8 - 0.5 = 2.9$  kcal/mol, which agrees well with earlier predictions; see below.

Two qualifying points should be noted. First, water interacts more strongly with the H-bonded peptide group of an Ala residue than with residues that have bulkier side chains,<sup>7</sup> and this effect will reduce the desolvation penalty for the bulkier residues. Second, the predicted desolvation penalty has been calculated for complete burial with no contact to water, while many protein residues have partial exposure to water, and this effect will also reduce the predicted desolvation penalty.

**Earlier Prediction of a Desolvation Penalty.** The probable existence of a desolvation penalty was predicted from finding an interaction with water that stabilizes a H-bonded dimer of *N*-methylacetamide;<sup>5,6</sup> a similar interaction was found for H-bonded peptide groups in both a peptide helix<sup>7</sup> and a  $\beta$ -hairpin.<sup>8</sup> Solvation of polar groups by water is known to be primarily an electrostatic interaction, and the DelPhi algorithm has been used to estimate the solvation free energy,  $\Delta G_{\text{sol}}$ ,<sup>27</sup> and to determine esf values (electrostatic solvation free energies) of polar groups.<sup>7,8</sup> The PARSE parameters of DelPhi (atomic partial charges and radii) are calculated so that they will

reproduce the experimental values of  $\Delta G_{\text{sol}}$  for a library of polar molecules.<sup>27</sup> The contribution to  $\Delta G_{\text{sol}}$  from the nonpolar moiety of an amide is estimated separately, either by a calibration that assumes proportionality to ASA (water-accessible surface area)<sup>27</sup> or by subtracting the known value of  $\Delta G_{\text{sol}}$  for the hydrocarbon that corresponds to the nonpolar moiety.<sup>28</sup> The experimental  $\Delta G_{\text{sol}}$  is based on the Ben-Naim standard state (1 M in both the gas and liquid phases)<sup>29</sup> and uses the ideal gas law to express the pressure in the gas phase<sup>16</sup> (note the different standard states used for liquid–liquid (mole fraction) and gas–liquid transfer). Because amides have extremely low vapor pressures, experimental values of  $\Delta G_{\text{sol}}$  are available only for a few amides, obtained by radioisotope labeling of the amide.<sup>30</sup>

The esf values of the H-bonded peptide groups in a peptide helix<sup>7</sup> and  $\beta$ -hairpin<sup>8</sup> have been found by using DelPhi. The basic assumptions of the electrostatic calculations are (1) fixed partial charges and (2) a continuum solvent. Although the solvent is assigned a uniform dielectric constant (the known value for water), the access of the solvent to the peptide group is limited by the diameter (3.0 Å) assigned to the water molecules. The same esf value,  $-2.5$  kcal/mol, was found for the interaction with water of a H-bonded peptide group in both an Ala peptide helix<sup>7</sup> and  $\beta$ -hairpin.<sup>8</sup> Most of the interaction ( $-2.0$  kcal/mol) occurs between water and the peptide  $-\text{CO}$  group, and only  $-0.5$  kcal/mol occurs with the  $-\text{NH}$  group.<sup>7,8</sup> The esf value is a free energy, and the corresponding enthalpy is needed for the desolvation penalty discussed here. They are related by an empirical correlation ( $\Delta H_{\text{sol}} \approx 1.1$  esf)<sup>31</sup> based on experimental data for the solvation free energies and enthalpies of the polar amide group in some amides. In contrast to the limited values of  $\Delta G_{\text{sol}}$  available for amides, ample experimental data are available for  $\Delta H_{\text{sol}}$ .<sup>28</sup> The heat of sublimation of the crystalline amide is combined with its heat of solution in water to give  $\Delta H_{\text{sol}}$ . A simple correction ( $RT = 0.55$  kcal/mol at 25 °C) is applied to the experimental data to obtain the value for the Ben-Naim standard state.<sup>2,16,29</sup> The desolvation penalty  $\Delta H_{\text{sol}}$  predicted from esf values of H-bonded peptide groups is  $(1.1)(2.5) = 2.8$  kcal/mol (positive sign for the folding direction), which agrees well with the desolvation penalty estimated from protein unfolding enthalpies.

Other evidence indicates that a desolvation penalty is likely. The peptide  $-\text{CO}$  group is able to make two HBs to water. An ab initio study (gas phase) of the HB interactions between NMA and three water molecules shows three equivalent HBs, each with  $\Delta H \approx -5$  kcal/mol; two HBs are made to the  $-\text{CO}$  group, and one HB is made to the  $-\text{NH}$  group.<sup>32</sup> The  $-\text{CO}$  stretch frequency (amide I' band) is different between solvent-exposed and buried helices in proteins, indicating that the solvent-exposed  $-\text{CO}$  group interacts with water.<sup>33</sup>

**Issues.** There are three questions that need to be discussed: (1) are  $\Delta H_{HB}$  and  $\Delta H_{\text{vdW}}$  modeled reliably, (2) does  $\Delta H_{NP}$  in fact pass through 0 at 22 °C, and (3) is there some unknown additional component that contributes significantly to the protein unfolding enthalpy,  $\Delta H_{PR}$ ? The second question is deferred to the following section on tests of the validity of the liquid hydrocarbon model. Regarding the first question, the peptide helix value is used for  $\Delta H_{HB}$  only provisionally to show that protein unfolding enthalpies at 22 °C are not consistent with this value unless a desolvation enthalpy is included. As regards the value of  $\Delta H_{\text{vdW}}$ , the basic question is whether the typical protein value is really as large as the 2.8 kcal/mol per residue estimated here. The two calorimetric studies of large-to-small mutations<sup>23,24</sup> discussed above suggest that this is a minimum estimate.



The third question (has some component been overlooked?) arises especially because of the  $\sim 2$ -fold difference between the simulated<sup>1</sup> and quasi-experimental<sup>2</sup> values of protein unfolding enthalpies in vacuum; see Table II in ref 1.<sup>1</sup> However, a major reason for the  $\sim 2$ -fold difference is now understood, as explained earlier.<sup>31,34</sup> The largest of the four factors contributing to the protein unfolding enthalpy in vacuum (eqs 1 and 2) is the heat of solvation of the polar  $-\text{CO}$  and  $-\text{NH}$  groups produced by breaking the peptide HBs (see Table II of ref 1).<sup>1</sup> The calculated values<sup>2</sup> of  $\Delta H_{\text{soln}}$  are based on a fixed value for the peptide group,  $-14.2$  kcal/mol, taken from experimental data for monoamides. However, it is known today that addition of neighboring peptide groups causes this value to decrease drastically because there are electrostatic interactions between peptide group dipoles of neighboring residues, and a calorimetric study of  $\Delta H_{\text{soln}}$  for dipeptide analogues<sup>35</sup> demonstrates this decrease directly. This effect explains a large part of the 2-fold difference. Future work may of course reveal some significant new component of protein unfolding enthalpies, but until that happens, the present argument for a desolvation penalty appears valid.

**Validity of the Liquid Hydrocarbon Model.** The proportionality constant  $k_{\text{Cp}}$  between  $\Delta G$  and  $\Delta \text{Cp}$  in eq 4 depends directly on the value of  $T_{\text{h}}$  as well as on  $T_{\text{s}}$ .

$$\Delta G_{\text{hyd}} = k_{\text{Cp}} \Delta \text{Cp} \quad (5a)$$

$$k_{\text{Cp}} = (T - T_{\text{h}}) + T \ln(T_{\text{s}}/T) \quad (5b)$$

Consequently, one test of the LHM is to ask whether  $k_{\text{Cp}}$  has the same value in hydrocarbon transfer experiments as that in protein unfolding experiments when  $k_{\text{Cp}}$  is measured from the ratio  $(\Delta G_{\text{hyd}})/(\Delta \text{Cp})$ . However, in protein unfolding experiments,  $\Delta G_{\text{hyd}}$  cannot be observed directly. The standard solution to this problem is to calculate the change in buried nonpolar ASA ( $\Delta \text{ASA}$ ) in the unfolding reaction and to assume proportionality.

$$\Delta G_{\text{hyd}} = k_{\text{ASA}} (\Delta \text{ASA}) \quad (6)$$

The value of  $k_{\text{ASA}}$  is then determined from solute transfer experiments like those on which the LHM is based (see Table 1), which give values of  $\Delta G_{\text{hyd}}$  directly.

A thermodynamic database for the unfolding reactions of 49 different proteins<sup>36</sup> is used here; the database also contains the corresponding values of  $\Delta \text{ASA}$ . The amount of buried nonpolar ASA in the native protein structure is calculated straightforwardly. The amount of nonpolar ASA exposed to solvent in the unfolded protein is found more indirectly by using values assigned to the 20 types of amino acid residues.<sup>37,38</sup> In the database,<sup>36</sup> the ASA value for each residue type in the unfolded protein was found by the tripeptide (AXA) method. The values given by this method are commonly regarded today as upper bound values, while lower bound values are given by averaging data from 17-residue fragments of known protein structures,<sup>37,38</sup> and an average of the upper and lower bound values is reported. The ratio of upper- to lower-bound values is  $\sim 1.5$ .<sup>37,38</sup> Different values of  $k_{\text{ASA}}$  have been proposed, based on experiments with different solvent transfer systems.<sup>17,39,40</sup> The value used here is  $25 \text{ cal mol}^{-1} \text{ \AA}^{-2}$ , which is a commonly used value<sup>17</sup> and which agrees satisfactorily with the individual values of  $k_{\text{ASA}}$  for the six hydrocarbons in Table 1. Note that the largest differences

in Table 1 among  $k_{\text{ASA}}$  values occur between aromatic and aliphatic hydrocarbons.

The nonpolar ASA exposed upon protein unfolding is proportional to  $N$ , the number of residues.<sup>36</sup> An average value of  $(\Delta \text{ASA}/N) = 50 \text{ \AA}^2$  (AXA method) is taken from Figure 2 of the database<sup>36</sup> and used as the upper bound. The lower-bound is  $(50/1.5) = 33 \text{ \AA}^2$ , and the average,  $42 \text{ \AA}^2$ , is used here to compute  $\Delta G_{\text{hyd}} = 1.05 \text{ kcal/mol}$  per residue, with  $k_{\text{ASA}} = 25 \text{ cal mol}^{-1} \text{ \AA}^{-2}$ .  $\Delta \text{Cp}$  for unfolding is proportional to  $N$ , with  $(\Delta \text{Cp}/N) = 13.9 \pm 0.5 \text{ cal mol}^{-1} \text{ K}^{-1}$ ,<sup>36</sup> and the LHM gives  $\Delta G_{\text{hyd}} = 1.11 \text{ kcal/mol}$  per residue with  $k_{\text{Cp}} = 79.7 \text{ K}$  (the average value from Table 1). The good agreement between the values of  $\Delta G_{\text{hyd}}$  from the ASA method and from the LHM may be fortuitous because any temperature dependence of  $\Delta H_{\text{HB}} + \Delta H_{\text{vdW}}$  will contribute to  $\Delta \text{Cp}$  and is not included here. A scanning calorimetry study of  $\Delta H_{\text{HB}}$  versus temperature for peptide helix unfolding found a significant value of  $\Delta \text{Cp}$ .<sup>41</sup> Earlier, there was considerable interest in determining  $\Delta G_{\text{hyd}}$  from  $\Delta \text{Cp}$  for reactions that involve protein folding or unfolding,<sup>42,43</sup> and the present database comparison between values found by the two methods is interesting in that context.

## Concluding Comments

The novel analysis of protein unfolding enthalpies proposed here needs more work. In particular, a better understanding is needed for the enthalpy contribution made by breaking protein–protein vdW interactions and replacing them with protein–water vdW interactions. A possible approach is to improve the present understanding of the enthalpy changes that occur when large-to-small mutations are made. It is also important to understand better whether the enthalpy contribution from the hydrophobic interaction (changing the solvent environment of the protein nonpolar groups from organic to aqueous) in fact passes through 0 at  $22^\circ \text{C}$ .

**Acknowledgment.** I thank Franc Avbelj, Pehr Harbury, George Makhatadze, George Rose, and Raghavan Varadarajan for their help and discussion. I thank Bob Alberty for introducing me to scientific research in 1950, not just for showing me how it is done but also for communicating his exuberant enthusiasm for the scientific life.

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JP107111F