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Hydrophobic effect in protein folding and other noncovalent processes involving proteins

(heat capacity differences/hydrocarbon transfer processes)

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ABSTRACT **Large negative standard heat capacity changes ($\Delta C_P^\circ << 0$) are the hallmark of processes that remove nonpolar surface from water, including the transfer of nonpolar solutes from water to a nonaqueous phase and the folding, aggregation/association, and ligand-binding reactions of proteins [Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2236–2240]. More recently, Baldwin [Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8069–8072] proposed that the ΔC_P° of protein folding could be used to quantify the contribution of the burial of nonpolar surface (the hydrophobic effect) to the stability of a globular protein. We demonstrate that identical correlations between the ΔC_P° and the change in water-accessible nonpolar surface area (ΔA_{np}) are obtained for both the transfer of nonpolar solutes from water to the pure liquid phase and the folding of small globular proteins: $\Delta C_P^\circ/\Delta A_{np} = -(0.28 \pm 0.05)$ (where ΔA_{np} is expressed in Å^2 and ΔC_P° is expressed in cal·mol^{-1}·K^{-1}; 1 cal = 4.184 J). The fact that these correlations are identical validates the proposals by both Sturtevant and Baldwin that the hydrophobic effect is in general the dominant contributor to ΔC_P° and provides a straightforward means of estimating the contribution of the hydrophobic driving force (ΔG_{hyd}°) to the standard free energy change of a noncovalent process characterized by a large negative ΔC_P° in the physiological temperature range: $\Delta G_{hyd}^\circ \simeq (80 \pm 10)\Delta C_P^\circ$.**

Nonpolar side chains are removed from contact with water in the process of folding a protein into its native globular state. The thermodynamic consequences of the unfavorable interactions of such nonpolar regions with water are defined as the hydrophobic effect (1). Recent work has focused on quantifying the contribution to protein stability of the removal of nonpolar side chains from exposure to water (2–4), where stability is defined as the difference between the standard chemical potentials of the denatured and the native states at 37°C: $\Delta\overline{G}^\circ = \mu_D^\circ - \mu_N^\circ$ (4–6). Fersht and coworkers (2) find that truncation of certain nonpolar side chains that are buried in the native state destabilizes the enzyme barnase. Matthews and collaborators (3) observe that replacement of isoleucine destabilizes T4 lysozyme by an amount proportional to the reduction in "hydrophobicity" of the substituted residue (where hydrophobicity is proportional to the free energy of transfer from water to ethanol). The contribution of buried nonpolar regions to thermodynamic functions for protein denaturation was examined by Privalov and coworkers (7, 8), who observed that the distinctively large standard heat capacity difference (ΔC_P°) for denaturation correlated with the number of "nonpolar contacts" made in the native structure. [This work has been recently reviewed by Privalov (9) and by Privalov and Gill (10).] Recently, Baldwin (4) proposed that the overall "hydrophobic" contribution to stability (ΔG_{hyd}°) of proteins could be quantified from the ΔC_P° of denaturation. Baldwin's equation for ΔG_{hyd}°, based on thermodynamic data of Gill *et al.* (11) for the transfer of liquid hydrocarbons to water, assumes that ΔC_P° of denaturation results entirely from the hydrophobic effect. However, the thermodynamics of processes involving biopolymers may be more complex. Sturtevant (12) identified six possible nonexclusive origins of the large $|\Delta C_P^\circ|$ seen in protein folding and ligand binding reactions. Although he concluded that the contributions of the hydrophobic effect (≈80%) and changes in internal vibrational modes (≈20%) were of principal importance, his arguments are indirect. We have therefore sought a more direct route to quantify the contribution of the hydrophobic effect to the ΔC_P° of protein folding, based on a comparison of the relationships between heat capacity changes and nonpolar surface area removed from water for the processes of protein folding and of transfer of hydrocarbons from water to the pure liquid phase.

Correlations Between Changes in Nonpolar Surface Area Exposed to Water and ΔC_P° of Processes Involving Hydrocarbons and Proteins

Previous work has demonstrated a correlation between the standard heat capacity differences seen in the transfer of a variety of model compounds and their solvent-accessible surface areas (13, 14). Ha *et al.* (15) have recently shown that the thermodynamics of transfer of hydrocarbons from water to the pure liquid state provide the most directly interpretable information about hydrocarbon–water interactions. (Previous correlations have included the transfer of gases and solids in the data set.) Hermann (16) calculated water-accessible surface areas of these hydrocarbons by rolling a water molecule of radius 1.5 Å around the van der Waals radii of the carbons and hydrogens of a given molecule. Table 1 reproduces the entire liquid hydrocarbon data set for which both the amount of nonpolar surface area removed in the transfer (ΔA_{np}) and the calorimetric ΔC_P° of transfer have been reported. These data are plotted in Fig. 1 along with the line determined by assuming that ΔC_P° is proportional to ΔA_{np}, with the average heat capacity increment calculated in Table 1: $d\Delta C_P^\circ/d\Delta A_{np} = (0.28 \pm 0.04)$ cal·mol^{-1}·K^{-1}·Å^{-2} (1 cal = 4.184 J) of nonpolar surface removed.

Lee and Richards (20) and Chothia (18) have calculated water-accessible surface areas of proteins from the arc traced out by a water molecule of 1.4 Å as it rolls around the van der Waals radii of the folded and unfolded forms. Differences exist between the models and methodologies used by Hermann (16) and Lee and Richards (20) for calculating solvent-accessible surface areas. Preliminary calculations suggest that these differences are not significant within the uncertainties cited in Table 1 (J. Livingstone, personal communication). To model the unfolded form, model peptides of the

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Table 1. Correlation between ΔA_{np} and ΔC_P°

	ΔA_{np}, Å^2	$-\Delta C_P^\circ$, cal·mol^{-1}·K^{-1}	$-\Delta C_P^\circ/\Delta A_{np}$, cal·mol^{-1}·K^{-1}·Å^{-2}
Hydrocarbons			
Benzene	240.71*	53.8 ± 1.2†	0.223 ± 0.005
Toluene	273.90*	62.9 ± 3.1†	0.230 ± 0.011
Ethylbenzene	302.27*	76.0 ± 3.1†	0.251 ± 0.010
n-Propylbenzene	334.07*	93.4 ± 6.0†	0.280 ± 0.018
Cyclohexane	279.10*	86.0 ± 7.2†	0.308 ± 0.026
n-Pentane	286.97*	95.6 ± 16.7†	0.333 ± 0.058
n-Hexane	318.77‡	105.2 ± 10.8†	0.330 ± 0.092
		Average	0.28 ± 0.04
Proteins			
Pancreatic trypsin inhibitor (PTI)	3150§	720 ± 110¶‖	0.229 ± 0.085
Ribonuclease	5037**	1220 ± 180¶	0.242 ± 0.036
Lysozyme	6501**	1430 ± 220¶	0.220 ± 0.034
Myoglobin	9108**	2770 ± 420¶	0.304 ± 0.046

*Ref. 16.
†Ref. 11.
‡Ref. 17.
§Ref. 18.
¶Ref. 8. As uncertainties in the ΔC_P° of protein folding were not reported, an estimate of the associated error was made with the experimental values of ΔC_P° in figure 5 of ref. 7.
‖Ref. 19 reports a negligibly small value of ΔC_P° for PTI that disagrees with that in ref. 8. For consistency, we have used the value from ref. 8.
**Ref. 20.

form Gly-Xaa-Gly or Ala-Xaa-Ala were used (18, 20). [This model assumes that the polypeptide chain of these proteins adopts a random coil conformation under the denaturing conditions of the calorimetric experiments. Support for this assumption has been obtained by Privalov and coworkers for lysozyme, RNase, and myoglobin (21, 22). If residual structure is present in the unfolded form, these calculations would overestimate the amount of solvent-accessible surface of the unfolded form.] A sum of these areas over the amino acid content of the protein yields the water-accessible surface of the unfolded form. Lee and Richards (20) defined sulfur and carbon atoms as nonpolar, whereas Chothia (18) considered only carbon atoms as nonpolar. Since only hydrocarbons exhibit the thermodynamic characteristics of the hydrophobic effect, we have used only carbon atoms in the data set and have corrected Lee and Richards' surface areas accordingly. Both calculations of nonpolar surface area of proteins include main-chain and side-chain atoms but do not treat hydrogen atoms separately. Instead, hydrogens are included in the radii used for the carbon atoms. All available values for the nonpolar surface area removed from water in the folding process and the corresponding calorimetric ΔC_P° are given in Table 1 and plotted in Fig. 2. A weighted least-squares fit of the protein data alone yields a heat capacity increment of $-(0.28 \pm 0.05)$ cal·mol^{-1}·K^{-1}·Å^{-2} of nonpolar surface buried on folding and an intercept that is 0 within error (180 ± 230 cal·mol^{-1}·K^{-1}). This correlation is therefore identical to that which describes the hydrocarbons in Fig. 1 and is replotted in Fig. 2 for purposes of comparison. We conclude that ΔC_P° is a universal function of nonpolar surface area removed from water, as exemplified by the transfer of liquid hydrocarbons and the folding of small globular proteins. Several important conclusions can be drawn from this striking result. First, the hydrophobic effect dominates the large negative ΔC_P° observed in the folding of many proteins. Second, model compound studies can be used to model quantitatively complicated macromolecular systems at the level of ΔC_P°. Finally, the area of nonpolar (i.e., hydrocarbon) surface removed from water in the process of folding a protein may be estimated from the ΔC_P° of folding:

$$\Delta A_{np} = -(3.6 \pm 0.6)\Delta C_P^\circ. \quad [1]$$

Use of ΔC_P° to Estimate ΔG_{hyd}°

Empirical relationships between standard thermodynamic functions (including ΔG°, ΔS°, and ΔC_P°) for transfer processes of model solutes and changes in the extent of exposure of nonpolar surface area to water have been reported (13, 14, 16, 17, 23, 24). In particular, using McAuliffe's solubility data (25), both Tanford and coworkers (17) and Hermann (16) found a linear correlation between the standard free energy changes for the transfer of hydrocarbons from water (ΔG_{tr}°) and their cavity surface areas. Tanford and coworkers (17) restricted their analysis to data for the transfer to the pure liquid state [in contrast to Hermann (16), who included transfer data to both the liquid and gaseous states] and observed that

$$\Delta G_{tr}^\circ/\Delta A_{np} = -21 \text{ cal·mol}^{-1}\text{·Å}^{-2}, \quad [2]$$

where ΔA_{np} (in Å^2) is as defined above. For these transfer processes, Fig. 1 demonstrates that

$$\Delta C_{P,tr}^\circ/\Delta A_{np} = -(0.28 \pm 0.04) \text{ cal·mol}^{-1}\text{·K}^{-1}\text{·Å}^{-2}. \quad [3]$$

The question naturally arises as to which of these thermodynamic correlations with nonpolar surface area is the more fundamental at the molecular level of interactions between

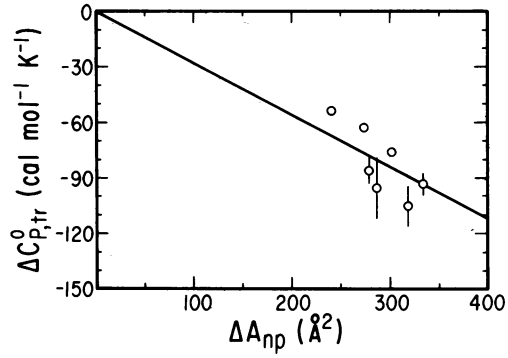

FIG. 1. Complete data set of $\Delta C_{P,tr}^\circ$ as a function of change in water-accessible nonpolar surface area (ΔA_{np}) for transfer of hydrocarbons from water to the pure liquid phase (cf. Table 1). Solid line has the slope calculated from Table 1 and the required intercept of 0.

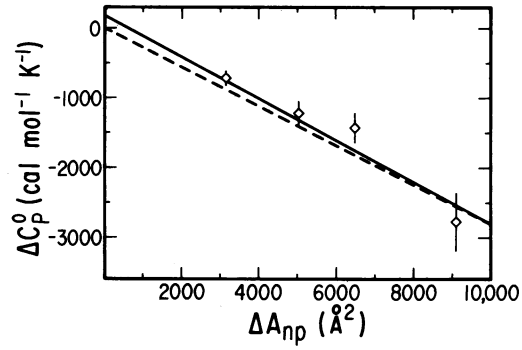

FIG. 2. ΔC_P° as a function of change in water-accessible nonpolar surface area (ΔA_{np}) for folding of the four globular proteins for which these data are available (cf. Table 1). Solid line, weighted linear least-squares fit of the protein data; dashed line, linear analysis of hydrocarbon data from Fig. 1.

nonpolar surface and water. As discussed by Ha *et al.* (15), this question cannot be resolved by a purely thermodynamic analysis, because both ΔG°_{tr} and $\Delta C^\circ_{P,tr}$ are relatively temperature invariant over a broad range and proportional to one another, where the "constant" of proportionality is determined primarily by the characteristic temperatures T°_S and T°_H for the transfer process (4, 10, 15):

$$\Delta G^\circ_{tr}/\Delta C^\circ_{P,tr} = (T - T^\circ_H) - T\ln(T/T^\circ_S)$$
$$\simeq (T^\circ_S - T^\circ_H) - (T^\circ_S - T)^2/2T^\circ_S + \ldots, \quad [4]$$

where T°_S and T°_H are the temperatures where $\Delta S^\circ_{tr} = 0$ and $\Delta H^\circ_{tr} = 0$, respectively. [T°_S is relatively constant for the hydrocarbons examined, whereas T°_H is somewhat variable (4).] Consequently, both ΔG°_{tr} and $\Delta C^\circ_{P,tr}$ correlate equally well with nonpolar surface area. From Eq. **4** (or from Eqs. **2** and **3**) one obtains a simple proportionality of ΔG°_{tr} to $\Delta C^\circ_{P,tr}$ valid near 25°C:

$$\Delta G^\circ_{tr} \simeq (80 \pm 10)\Delta C^\circ_{P,tr}. \quad [5]$$

(The uncertainty estimate in Eq. **5** is based on both the lack of constancy of T°_H and the residual temperature dependence of ΔG°_{tr} near 25°C.) As demonstrated above, the correlation between ΔC°_P and nonpolar surface area for the transfer of hydrocarbons is identical to that for protein folding. Therefore, Eq. **5** may be generalized to estimate the magnitude of the hydrophobic driving force involved in protein folding:

$$\Delta G^\circ_{hyd} \simeq (80 \pm 10)\Delta C^\circ_P. \quad [6]$$

Eq. **6** is mathematically equivalent to Baldwin's (4) expression for ΔG°_{hyd} and provides a straightforward method for obtaining the contribution of the hydrophobic effect to the overall free energy change for protein folding and for other macromolecular processes accompanied by a large ΔC°_P of hydrophobic origin.

The Temperature Dependence of ΔC°_P Is Small in the Temperature Range of Interest

Recent work by Privalov and coworkers over a very wide temperature range indicates ΔC°_P of processes involving the hydrophobic effect varies with temperature. Using applied pressure (≈6 atm) to extend the temperature range where water is a liquid, Makhatadze and Privalov (26) observe that ΔC°_P of transfer of benzene and toluene from water to the pure liquid phase becomes less negative with increasing temperature. Similarly, for the denaturation of several small globular proteins above atmospheric pressure, Privalov and coworkers (21) find that the partial molar heat capacity of the denatured state is a nonlinear function of temperature, while that of the native state appears to be linear in temperature. This variation causes a plot of ΔH° of denaturation versus T to depart significantly from linearity above 80°C. However, from 0°C to 80°C, the ΔC°_P of unfolding is temperature independent within experimental uncertainty. For transfer of benzene, ΔC°_P varies systematically by 10% between 5°C and 25°C and by 18% between 25°C and 80°C. As the errors in determining ΔC°_P of protein unfolding are typically 10–20% (6, 7), the observed small temperature dependence of ΔC°_P of transfer should not significantly affect its use as a reference to interpret ΔC°_P of protein unfolding.

Changes in Exposure of Polar (Uncharged) Macromolecular Surface Are Unlikely to Contribute to ΔC°_P

In addition to nonpolar surface, significant amounts of polar (uncharged) surface are buried when a protein folds. Does the dehydration of these surfaces contribute to the net observed ΔC°_P? The unusual large change in heat capacity seen when nonpolar (liquid) solutes are dissolved in water indicates that the interactions of water of hydration of nonpolar surface are more thermolabile than those of bulk water (27). However, we expect that water of hydration of polar surface is similar in thermolability to bulk water since its interactions (hydrogen bonding) with that surface and with itself should be similar to those in bulk water. Consequently, we do not expect the dehydration of polar surface upon folding to contribute to ΔC°_P. Thermodynamic studies of $\Delta C^\circ_{P,tr}$ for the transfer of small polar solutes from water to the pure liquid phase support this expectation (28–31). For example, $\Delta C^\circ_{P,tr}$ ($HCONH_2$) = 6 cal·mol^{-1}·K^{-1}; $\Delta C^\circ_{P,tr}$ ($HOCH_2CH_2OH$) = −10 cal·mol^{-1}·K^{-1}; $\Delta C^\circ_{P,tr}$ (CH_3OH) = −16 cal·mol^{-1}·K^{-1}; $\Delta C^\circ_{P,tr}$ ($H_2NCH_2CH_2NH_2$) = −3 cal·mol^{-1}·K^{-1}, whereas $\Delta C^\circ_{P,tr}(C_6H_6)$ = −54 cal·mol^{-1}·K^{-1}. In addition, Ooi *et al.* (14) have analyzed the contribution of various functional groups to the free energy of protein folding by assuming that group contributions are additive and then examining the relationship between solvent-accessible functional group surface area and the ΔG° or ΔC°_P of transfer of various small organic solutes. Although interpretation of their results is complicated by the inclusion of solids and gases as well as liquids in their data set, they observe contributions to ΔC°_P per unit surface area of 0.008 cal·mol^{-1}·K^{-1}·Å^{-2} for hydroxyl groups and −0.012 cal·mol^{-1}·K^{-1}·Å^{-2} for amide and amine groups, compared to 0.296 cal·mol^{-1}·K^{-1}·Å^{-2} for aromatic groups. Thus, any contribution to the heat capacity change from removal of polar surface from water should be quite small in comparison to the contribution from burial of nonpolar surface. It is in fact possible that the minor contributions from the different polar functional groups may to an extent compensate each other, since they are of similar magnitude but of opposite sign.

The Free Energy of Removal of Polar Surface from Water Should Not Exhibit the Same Proportionality to Surface Area as Observed for Nonpolar Surface

Eq. **2** is well established as a means of applying liquid hydrocarbon transfer data to model the contribution to protein stability from the burial of nonpolar surface. This quantitative relationship has been extended to estimate the contribution to protein stability from the removal of total (polar plus nonpolar) surface area of amino acid side chains from water (cf., e.g., refs. 32 and 33). Total surface area has been considered relevant by arguing that polar groups that are hydrogen-bonded in the interior of a protein are effectively "nonpolar" and therefore contribute to "hydrophobic bonding" (33). However, the hydrophobic effect and its characteristic thermodynamic contributions to folding arise from the unfavorable interaction between water and nonpolar surfaces that cannot hydrogen bond. The hydrophobic driving force for protein folding originates in the removal of nonpolar surface from its unfavorable interaction with water. Consequently, it is the nonpolar surface and not the total surface removed from water that determines the contributions of the hydrophobic effect in Eqs. **1** and **6**. The extension of these relationships to include polar surfaces cannot be justified by the hydrophobic effect.

Relevance of Transfer Processes for Modeling Protein Folding

The correlation found here does not imply or assume anything about the state of the protein interior. We have previously reviewed the thermodynamics of transfer of nonpolar solutes from water to the pure liquid phase in detail and have shown that these standard thermodynamic functions are determined entirely by the interaction between the nonpolar solute and water (15). Consequently, use of liquid hydrocarbon transfer data to analyze the role of the hydrophobic effect in protein folding does not imply that we model the interior of the protein as a liquid hydrocarbon: the liquid hydrocarbon transfer data model only the contribution to protein stability arising from the removal of nonpolar surface from contact with water. Thus, we do not model any thermodynamic contributions from changes in the amount of water-accessible polar surface, nor do we directly address the currently controversial question of the extent (if any) of favorable (e.g., van der Waals) attractive interaction between nonpolar surfaces that are removed from water. In a recent review, Privalov and Gill (10) argue that such attractive interactions between nonpolar groups (which they consider part of a "hydrophobic interaction") provide a major contribution to stability of globular proteins and furthermore state that one component of the thermodynamics of exposure of these groups to water ("solvation") is in fact favorable and hence destabilizing to the native state. The results of the present analysis, however, demonstrate that the removal of nonpolar surface from water provides a massive driving force for folding a protein into its native state. Although the correlation does not address the question of the state of the protein interior, or the nature of the interactions in that interior, it contains no inherent assumptions and provides quantitative estimates of the contribution of the hydrophobic effect to the process and of the amount of nonpolar surface area removed from water.

Application to Other Macromolecular Processes Involving the Hydrophobic Effect

We have recently shown that the thermodynamics of site-specific binding to DNA of *Eco*RI endonuclease, *lac* repressor, and the two other proteins for which data are available bear a striking resemblance to the thermodynamics of protein folding: both exhibit a large negative ΔC_P° and its characteristic thermodynamic consequences (15). Owing to the lack of structural information, we cannot demonstrate directly that the ΔC_P° of complexation is determined entirely by the hydrophobic effect. In particular, we cannot deduce the importance of changes in vibrational modes of the protein accompanying complex formation. However, the present study demonstrates that such contributions are not important in determining the ΔC_P° of protein folding and therefore provides indirect support for the hypothesis that the removal of nonpolar surface area from water also dominates the ΔC_P° of other processes involving proteins, including site-specific binding to nucleic acids. Thus, we propose that Eqs. **1** and **6** can be used to estimate the amount of nonpolar surface area buried and the hydrophobic driving force in noncovalent processes involving proteins that are accompanied by a large negative ΔC_P°.

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1. Tanford, C. (1980) *The Hydrophobic Effect* (Wiley, New York).
2. Kellis, J. T., Jr., Nyberg, K., Sali, D. & Fersht, A. R. (1988) *Nature (London)* **333,** 784–786.
3. Matsumura, M., Becktel, W. J. & Matthews, B. W. (1988) *Nature (London)* **334,** 406–410.
4. Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8069–8072.
5. Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem.* **16,** 115–137.
6. Becktel, W. J. & Schellman, J. A. (1987) *Biopolymers* **26,** 1859–1877.
7. Privalov, P. L. & Khechinashvili, N. N. (1974) *J. Mol. Biol.* **86,** 665–684.
8. Privalov, P. L. (1979) *Adv. Protein Chem.* **33,** 167–241.
9. Privalov, P. L. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18,** 47–69.
10. Privalov, P. L. & Gill, S. J. (1988) *Adv. Protein Chem.* **39,** 191–234.
11. Gill, S. J., Nichols, N. F. & Wadsö, I. (1976) *J. Chem. Thermodyn.* **8,** 445–452.
12. Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2236–2240.
13. Gill, S. J., Dec, S. F., Olofsson, G. & Wadsö, I. (1985) *J. Phys. Chem.* **89,** 3758–3761.
14. Ooi, T., Oobatake, M., Nemethy, G. & Scheraga, H. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3086–3090.
15. Ha, J. H., Spolar, R. S. & Record, M. T., Jr. (1989) *J. Mol. Biol.*, in press.
16. Hermann, R. B. (1972) *J. Phys. Chem.* **76,** 2754–2759.
17. Reynolds, J. A., Gilbert, D. B. & Tanford, C. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2925–2927.
18. Chothia, C. (1976) *J. Mol. Biol.* **105,** 1–14.
19. Moses, E. & Hinz, H.-J. (1983) *J. Mol. Biol.* **170,** 765–776.
20. Lee, B. & Richards, F. M. (1971) *J. Mol. Biol.* **55,** 379–400.
21. Privalov, P. L., Tiktopulo, E. I., Venyaminov, S. Yu., Griko, Yu. V., Makhatadze, G. I. & Khechinashvili, N. N. (1989) *J. Mol. Biol.* **205,** 737–750.
22. Pfeil, W. & Privalov, P. L. (1976) *Biophys. Chem.* **4,** 33–40.
23. Harris, M. J., Higuchi, T. & Rytting, J. H. (1973) *J. Phys. Chem.* **77,** 2694–2702.
24. Gill, S. J. & Wadsö, I. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2955–2958.
25. McAuliffe, C. (1966) *J. Phys. Chem.* **70,** 1267–1275.
26. Makhatadze, G. I. & Privalov, P. L. (1988) *J. Chem. Thermodyn.* **20,** 405–412.
27. Hvidt, A. (1983) *Annu. Rev. Biophys. Bioeng.* **12,** 1–20.
28. Edsall, J. T. (1935) *J. Am. Chem. Soc.* **57,** 1506–1507.
29. Kusano, K., Suurkuusk, J. & Wadsö, I. (1973) *J. Chem. Thermodyn.* **5,** 757–767.
30. Nichols, N., Sköld, R., Spink, C. & Wadsö, I. (1976) *J. Chem. Thermodyn.* **8,** 993–999.
31. Sköld, R., Suurkuusk, J. & Wadsö, I. (1976) *J. Chem. Thermodyn.* **8,** 1075–1080.
32. Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H. & Zehfus, M. H. (1985) *Science* **229,** 834–838.
33. Chothia, C. (1974) *Nature (London)* **248,** 338–339.