Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–623.
Poretz, R. D., & Goldstein, I. J. (1970) *Biochemistry 9*, 2890–2896.
Reeke, G. N., Jr., Becker, J. W., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1525–1547.
Reisler, E., & Eisenberg, H. (1969) *Biochemistry 8*, 4572–4578.
Roark, D. E., & Yphantis, D. A. (1969) *Ann. N.Y. Acad. Sci. 164*, 245–278.
Robinson, R. A., & Stokes, R. H. (1970) in *Electrolyte Solutions*, pp 476–478, Butterworths, London.
Scheraga, H. A. (1963) *Proteins (2nd Ed.) 1*, 477–594.
Senear, D. F., & Teller, D. C. (1981) *Biochemistry* (preceding paper in this issue).
Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351–371.
So, L. L., & Goldstein, I. J. (1968) *Biochim. Biophys. Acta 165*, 398–404.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Sumner, J. B., Gralén, N., & Eriksson-Quensel, I. B. (1938) *J. Biol. Chem. 125*, 45–48.
Suurkuusk, J. (1974) *Acta Chem. Scand., Ser. B 28*, 409–417.
Tanford, C. (1969) *J. Mol. Biol. 39*, 539–544.
Teller, D. C. (1973) *Methods Enzymol. 27*, 346–441.
Teller, D. C., Horbett, T. A., Richards, E. G., & Schachman, H. K. (1969) *Ann. N.Y. Acad. Sci. 164*, 66–101.
Van Holde, K. E., Rossetti, G. P., & Dyon, R. D. (1969) *Ann. N.Y. Acad. Sci. 164*, 279–293.
Wang, J. L., Cunningham, B. A., & Edelman, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1130–1134.
Wang, J. L., Cunningham, B. A., Waxdall, M. J., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1490–1502.
Wyman, J., Jr. (1964) *Adv. Protein Chem. 19*, 224–394.
Yang, P. H., & Rupley, J. A. (1979) *Biochemistry 18*, 2654–2661.
Yasaka, T., & Kambara, T. (1979) *Biochim. Biophys. Acta 585*, 229–239.

Calorimetric Study of the Interaction of Lysozyme with Aqueous 1-Propanol†

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ABSTRACT: The enthalpies of transfer of hen egg white lysozyme from water to aqueous solutions of 1-propanol were determined by isothermal flow calorimetry at 10, 17, 25, and 40 °C in 0.04 M, pH 2 glycine buffer. Alcohol concentrations up to 3.4 M were employed. Four regions in the dependence of the enthalpy of transfer on alcohol concentration can be discerned: a region of linear increase observable at 10, 17, and 25 °C, an inflection region observable at 17 and 25 °C, a second linear region observable at 17, 25, and 40 °C, and a region of decreasing enthalpies seen at 40 °C. Combination of differential scanning calorimetric data on lysozyme in PrOH–H_2O mixtures [Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180–1186] with the transfer enthalpies reported here shows that the enthalpy in this system can be regarded as a state function and that the apparent specific heat is at first slightly decreased and then strongly increased by the addition of 1-propanol. Comparison of the results for the interaction of lysozyme with 1-propanol with those reported for the interaction with guanidine hydrochloride [Pfeil, W., & Privalov, P. L. (1976) *Biophys. Chem. 4*, 23–50] indicates that the denaturing effects of these two reagents involve very different mechanisms.

We have previously reported (Velicelebi & Sturtevant, 1979) the results of an extensive DSC[1] study of the thermal denaturation of lysozyme in aqueous mixtures of MeOH, EtOH, and PrOH. It was shown that the unfolding of the protein in these solvent systems is reversible and that the calorimetric and van't Hoff enthalpies of denaturation are nearly equal, indicating that the transition under equilibrium conditions is essentially two state in character. In this paper we present the results of isothermal flow calorimetric determinations of the enthalpies of interaction of lysozyme with PrOH at 10, 17, 25, and 40 °C. PrOH was selected as the alcohol to be used in this study since it is much more effective than either MeOH or EtOH in altering the denaturational behavior of the protein.

Materials and Methods

Hen egg white lysozyme (*N*-acetylmuramide glycanohydrolase; EC 3.2.1.17) was obtained from Sigma Chemical Co. as grade 1, 3 times crystallized, dialyzed, and lyophilized powder. This product was further treated by dissolution in and dialysis against 0.05 M ammonium formate buffer at pH 4.0 and subsequent lyophilization. All buffer solutions were prepared from analytical grade reagents. Protein solutions were prepared by dissolving the lyophilized protein in degassed buffer, the concentrations being determined spectrophotometrically by using $E_{280}^{1\%} = 26.5$ in aqueous solution (Bjurulf & Wadsö, 1972). The absorbance measurements were carried out at 10 °C where the protein was native in all the alcohol solutions employed as judged by DSC denaturation profiles. All the calorimetric experiments were carried out in 0.04 M glycine buffer adjusted in the presence of PrOH to pH 2.0 as

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[1] Abbreviations used: DSC, differential scanning calorimetry; MeOH, methanol; EtOH, ethanol; PrOH, 1-propanol; Gdn·HCl, guanidine hydrochloride.

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Table I: Enthalpies of Interaction of Aqueous Lysozyme with PrOH[a]

PrOH concn,[b] M_1	final PrOH concn, $M_f = {}^1/_2(M_1 + M_2)$	$\Delta q_{prot-alc}$ (mcal min^{-1})	$\Delta q_{alc\ diln}$ (mcal min^{-1})	$\Delta q_{prot\ diln}$ (mcal min^{-1})	$\Delta H_{trans(0,M_f)}$ [kcal (mol of protein)$^{-1}$]
0	0.34	−16.97	−18.46	−0.14	9.7 ± 0.4
0.34	0.67	−24.27	−25.69	−0.17	19.7 ± 0.5
0.67	1.07	−46.06	−47.17	−0.12	27.2 ± 2.9
1.07	1.34	−24.14	−24.85	−0.13	32.4 ± 3.0
1.34	1.75	−70.00	−71.86	−0.19	44.3 ± 3.3
1.75	2.01	−37.68	−38.68	−0.17	51.3 ± 3.3
2.01	2.42	−106.80	−107.80	−0.17	58.2 ± 3.8
2.42	2.69	−53.44	−54.04	−0.17	63.2 ± 3.9
2.59	2.96	−42.08	−42.49	−0.20	66.6 ± 4.0

[a] Protein concentration 5.2–5.9 mg mL^{-1}; 10.13 °C. [b] In solution containing protein.

Table II: Enthalpies of Interaction of Aqueous Lysozyme with PrOH[a]

PrOH concn,[b] M_1	final PrOH concn, $M_f = {}^1/_2(M_1 + M_2)$	$\Delta q_{prot-alc}$ (mcal min^{-1})	$\Delta q_{alc\ diln}$ (mcal min^{-1})	$\Delta q_{prot\ diln}$ (mcal min^{-1})	$\Delta H_{trans(0,M_f)}$ [kcal (mol of protein)$^{-1}$]
0	0.33	−16.18	−18.15	−0.07	13.3 ± 5.0
0.33	0.67	−23.80	−24.59	−0.10	18.9 ± 5.2
0.67	1.07	−46.48	−47.93	−0.07	29.1 ± 5.7
1.07	1.34	−24.39	−24.91	−0.15	33.1 ± 5.7
1.34	1.74	−72.26	−73.78	−0.17	42.6 ± 5.8
1.74	2.01	−36.67	−37.16	−0.18	46.9 ± 5.8
2.01	2.41	−106.08	−106.41	−0.21	50.1 ± 5.9
2.41	2.68	−45.61	−45.74	−0.12	51.8 ± 6.0
2.68	2.95	−37.46	−39.25	0.02	62.7 ± 8.2
2.95	3.35	−52.28	−55.52	0.39	79.3 ± 8.2
3.35	3.68	−28.66	−30.00	1.00	81.5 ± 8.3

[a] Protein concentration 4.9–5.8 mg mL^{-1}; 17.20 °C. [b] In solution containing protein.

Table III: Enthalpies of Interaction of Aqueous Lysozyme with PrOH[a]

PrOH concn,[b] M_1	final PrOH concn, $M_f = {}^1/_2(M_1 + M_2)$	$\Delta q_{prot-alc}$ (mcal min^{-1})	$\Delta q_{alc\ diln}$ (mcal min^{-1})	$\Delta q_{prot\ diln}$ (mcal min^{-1})	$\Delta H_{trans(0,M_f)}$ [kcal (mol of protein)$^{-1}$]
0	0.33	−16.95	−18.50	−0.08	9.5 ± 0.9
0.33	0.67	−23.90	−25.48	−0.06	18.9 ± 1.7
0.67	1.07	−45.75	−46.93	−0.08	26.9 ± 1.8
1.07	1.34	−23.50	−24.08	−0.06	31.0 ± 2.0
1.34	1.74	−68.55	−69.55	−0.06	37.7 ± 2.7
1.74	2.00	−33.86	−34.91	−0.05	44.4 ± 2.8
2.00	2.40	−80.07	−84.42	−0.05	71.0 ± 3.2
2.40	2.67	−29.34	−35.07	0.71	101.1 ± 3.3
2.67	2.94	−23.76	−28.26	1.32	120.0 ± 3.4
2.95	3.35	−36.46	−37.63	0.16	125.5 ± 4.1

[a] Protein concentration 5.1–6.1 mg mL^{-1}; 25.06 °C. [b] In solution containing protein.

measured by a Radiometer pH meter standardized at pH 4.0 in aqueous solution. Densities were determined in a vibrating tube densimeter (Model 01D, Sodev, Inc.).

Isothermal calorimetric measurements were made with a sensitive flow calorimeter developed in collaboration with Beckman Instruments and described in detail elsewhere (Sturtevant, 1969; Sturtevant & Lyons, 1969; Velicelebi, 1978). The calorimeter was submerged in a water bath, the temperature of which was regulated within ±0.01 °C in the range 5–60 °C by using a Tronac precision temperature controller. Temperature measurements were made with a Hewlett-Packard quartz thermometer.

Measurements of the enthalpy of mixing of lysozyme solutions with PrOH were carried out over a range of final alcohol concentrations of 2.5–25% v/v at 10, 17, 25, and 40 °C. The calorimeter was calibrated at each temperature by reacting HCl with excess NaOH, using the heats of formation of H_2O given by Grenthe et al. (1970).

For each alcohol concentration at each temperature, three different enthalpy measurements were made: (1) the enthalpy of mixing protein in an alcohol solution of a certain concentration with an equal volume of a buffered alcohol solution of higher alcohol content ($\Delta q_{prot-alc}$); (2) the enthalpy of mixing the same lower and higher alcohol concentrations in the absence of protein ($\Delta q_{alc\ diln}$); (3) the enthalpy of dilution of the protein on addition of alcohol of the same composition ($\Delta q_{prot\ diln}$). The large heat of dilution of PrOH solutions precluded measuring directly the enthalpy of mixing aqueous lysozyme solutions with PrOH solutions at concentrations in excess of 0.67 M. The net enthalpy of transfer, $\Delta q_{trans(M_1,M_f)}$, of the protein from the initial alcohol concentration, M_1, to the final alcohol concentration, M_f, is given

$$\Delta q_{trans(M_1,M_f)} = \Delta q_{prot-alc} - \Delta q_{alc\ diln} - \Delta q_{prot\ diln} \quad (1)$$

It is convenient to express the observed heat effects in millicalories per minute normalized to the maximum available flow rate of 0.4351 mL min^{-1}. Actual flow rates were ${}^1/_5$–${}^1/_{20}$ this large. At these flow rates, heating due to viscous flow was negligible. Δq_{trans} is readily converted to a molar quantity by using the known protein concentration and molecular weight.

Results

The heats of dilution of PrOH and lysozyme and the heats of transfer of the protein from one PrOH concentration to another at 10, 17, 25, and 40 °C are presented in Tables I–IV. In each case, the alcohol concentrations prepared as volume

Table IV: Enthalpies of Interaction of Aqueous Lysozyme with PrOH[a]

PrOH concn,[b] M_1	final PrOH concn, $M_f = {}^1/_2(M_1 + M_2)$	$\Delta q_{prot-alc}$ (mcal min^{-1})	$\Delta q_{alc\ diln}$ (mcal min^{-1})	$\Delta q_{prot\ diln}$ (mcal min^{-1})	$\Delta H_{trans(0,M_f)}$ [kcal (mol of protein)$^{-1}$]
0	0.33	−18.82	−19.57	0.00	4.5 ± 0.4
0.33	0.67	−24.33	−25.85	0.00	13.9 ± 0.5
0.67	1.06	−42.42	−46.51	0.23	36.8 ± 1.1
1.06	1.33	−17.98	−23.83	0.82	67.2 ± 1.3
1.33	1.72	−55.93	−62.74	1.15	101.7 ± 2.4
1.72	1.99	−26.28	−29.01	0.84	112.7 ± 2.4
1.99	2.38	−58.52	−59.48	0.25	117.0 ± 3.2
2.38	2.65	−20.53	−20.00	−0.04	113.8 ± 3.4
2.65	2.91	−15.16	−14.54	−0.11	110.8 ± 3.4

[a] Protein concentration 5.3–5.9 mg mL^{-1}; 40.00 °C. [b] In solution containing protein.

fractions were converted to molarities by using the appropriate density values (Velicelebi, 1978).

Apparent Molar Enthalpies of Propanol in 0.04 M Aqueous Glycine Buffer. The values obtained for $\Delta q_{alc\ diln}$ permit calculation of the relative apparent molar enthalpy, $\varphi_L = \varphi_H - \varphi_H^0$, of PrOH in solution in 0.04 M aqueous glycine buffer. The results for $\Delta q_{alc\ diln}$ at different alcohol compositions and temperatures are listed in Tables I–IV. Since the densities of PrOH–H_2O mixtures were found to be accurately linear in PrOH concentration, the final concentration of PrOH, M_f, was taken equal to the mean of the two initial concentrations, M_1 and M_2. The dilution process consists of the two steps

$$n_1 \text{ moles of PrOH } (M_1) \rightarrow n_1 \text{ moles of PrOH } (M_f): \ \Delta q_1 \quad (2)$$

$$n_2 \text{ moles of PrOH } (M_2) \rightarrow n_2 \text{ moles of PrOH } (M_f): \ \Delta q_2 \quad (3)$$

$$\Delta q_{alc\ diln} = \Delta q = \Delta q_1 + \Delta q_2 \quad (4)$$

Since

$$\Delta q_1 = n_1[\varphi_H(M_f) - \varphi_H(M_1)]$$
$$\Delta q_2 = n_2[\varphi_H(M_f) - \varphi_H(M_2)] \quad (5)$$

where $\varphi_H(M)$ is the apparent molar enthalpy of PrOH at concentration M, it follows that

$$\frac{\Delta q}{n_1 + n_2} = \varphi_H(M_f) - \frac{M_1}{M_1 + M_2}\varphi_H(M_1) - \frac{M_2}{M_1 + M_2}\varphi_H(M_2) \quad (6)$$

If it is assumed that φ_H is a linear function of PrOH concentration, it follows that

$$\varphi_H(M_f) = {}^1/_2[\varphi_H(M_1) + \varphi_H(M_2)]$$

and

$$\frac{d\varphi_H}{dM} = \frac{d\varphi_L}{dM} = \frac{\varphi_H(M_2) - \varphi_H(M_1)}{M_2 - M_1} = -\frac{2\Delta q(M_1 + M_2)}{(n_1 + n_2)(M_2 - M_1)^2} \quad (7)$$

φ_L may thus be obtained as a function of M by plotting $d\varphi_L/dM$ vs. M_f and graphically integrating. The resulting values of φ_L are plotted as a function of alcohol concentration in Figure 1.

Enthalpies of Transfer of Lysozyme. The enthalpy of transfer, $\Delta H_{trans(M_1,M_f)}$, of lysozyme from one PrOH concentration, M_1, to another, M_f, was determined over the PrOH concentration range 0–3 M and at 10, 17, 25, and 40 °C. The results are tabulated in Tables I–IV. For evaluation of the molar enthalpy of transfer of the protein from water to the final alcohol concentration, $\Delta H_{trans(0,M_f)}$, the values of

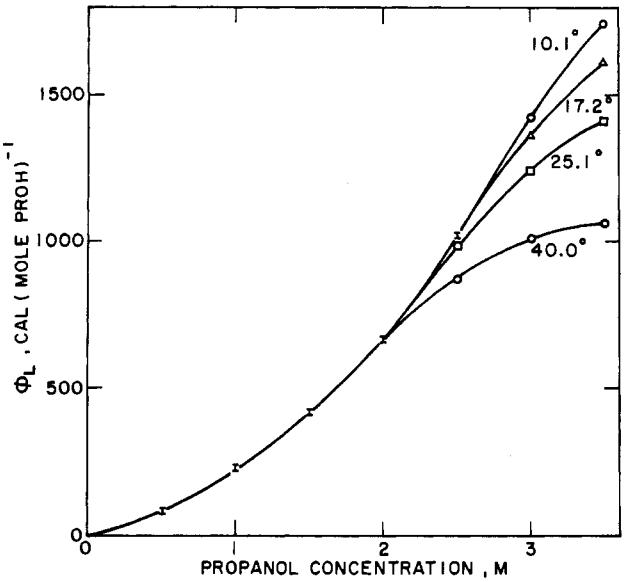

FIGURE 1: Variation of φ_L, relative apparent molar enthalpy of PrOH in 0.04 M aqueous glycine buffer, with PrOH concentration at 10.13 (○), 17.20 (△), 25.06 (□), and 40.00 (●) °C.

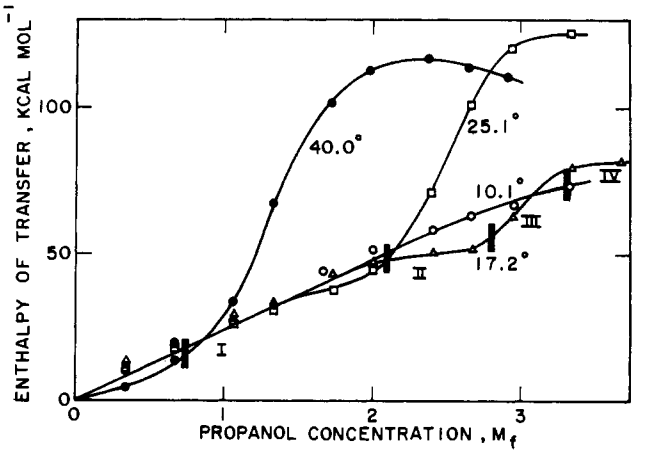

FIGURE 2: Variation of enthalpy of transfer of lysozyme from H_2O to M_f molar PrOH, $\Delta H_{trans(0,M_f)}$, as a function PrOH concentration at 10.13, 17.20, 25.06, and 40.00 °C. A broad vertical line through each curve indicates the alcohol concentration at which, according to DSC data (Velicelebi & Sturtevant, 1979), the protein is 5% denatured.

$\Delta H_{trans(M_1,M_f)}$ were added stepwise. The molar enthalpies of transfer from water are also listed in Tables I–IV and plotted in Figure 2.

Discussion

The relative apparent molar enthalpy, φ_L, of PrOH is shown in Figure 1 as a function of temperature and alcohol concentration. It is seen that within experimental uncertainty φ_L

Scheme I

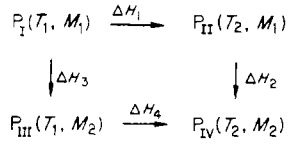

Table V: Coefficients B and D in Equation 8 as Functions of PrOH Concentration

	PrOH concn (M)					
	0	0.67	1.34	2.00	2.67	3.34
B (cal K^{-2} mol^{-1})	17	34	22	52	82	86
D (cal K^{-2} mol^{-1})	1.3	−4.9	14	35	58	65

is independent of temperature up to 2 M. Above this concentration φ_L decreases with increasing temperature at constant PrOH concentration. The general shape of the curve for φ_L at 25 °C is similar to that deduced by Dimmling & Lange (1951) from integral heats of dilution, but our values are ~20% higher. This difference is presumably at least in part due to the presence of 0.04 M glycine in our solutions.

The transfer enthalpies, $\Delta H_{trans(0,M_1)}$, given in Tables I–IV and Figure 2 are subject to considerable uncertainty since their derivation from the experimental data involves the evaluation of small differences between large quantities. The error ranges given in the last column of each of the tables are based on the standard errors for each of the series of experiments listed in columns 3–5 of the tables. The actual uncertainties are thought to be no larger than 1.5 times the listed error ranges. The reliability of the data is supported by the fact that repetition of the experiments at 25 °C after a lapse of 2 years gave results falling well within the previously assigned uncertainty limits.

The transfer enthalpies can be used in conjunction with the unfolding enthalpies determined by DSC (Velicelebi & Sturtevant, 1979) to compute the enthalpy changes involved in going between two different states of lysozyme by two different pathways, as shown in Scheme I. If the states of the protein, P_I–P_{IV}, are thermodynamically defined states, then the enthalpy is a state function and we must have $\Delta H_1 + \Delta H_2 = \Delta H_3 + \Delta H_4$. However, as discussed below, it is necessary to include heat capacity contributions as well as unfolding contributions in ΔH_1 and ΔH_4. As pointed out earlier (Velicelebi & Sturtevant, 1979), the unfolding transitions observed in the DSC experiments can be regarded as being closely two state in character since the ratio $\Delta H_{vH}/\Delta H_{cal}$ is approximately unity. It was observed that the apparent heat capacities of both the native (N) and unfolded (D) states of the protein are linear functions of the temperature within experimental uncertainty:

$$C_N = A + B(T - T_d) \qquad C_d = C + D(T - T_d) \quad (8)$$

where T_d is the temperature at which half of the unfolding enthalpy has been absorbed. The average apparent heat capacity is then

$$C_{av} = A + \alpha(C - A) + [B + \alpha(D - B)](T - T_d) \quad (9)$$

where α is the extent of the unfolding transition ($\alpha = 0.5$ at T_d) and the enthalpy at temperature T is

$$\Delta H_T = \Delta H_{cal} + (C - A)(T - T_d) + \tfrac{1}{2}(D - B)(T - T_d)^2 \quad (10)$$

Integration of the van't Hoff equation, assuming the van't Hoff enthalpy, ΔH_{vH}, to be governed by the same heat capacity change as the calorimetric enthalpy, ΔH_{cal}, and noting that the equilibrium constant for the unfolding is given by $\alpha/(1 - \alpha)$ leads to the result

$$R \ln \frac{\alpha}{1-\alpha} = \left[\frac{\Delta H_{vH}}{T_d} - (C - A) + \tfrac{1}{2}(D - B) \times (T + T_d) \right] \frac{T - T_d}{T} + [(C - A) - (D - B)T_d] \ln \frac{T}{T_d} \quad (11)$$

The heat absorbed in a short temperature interval is given by

$$\delta Q = \Delta H_T \, \delta\alpha + C_{av} \delta T = \delta Q_d + \delta Q_c \quad (12)$$

and integration over a longer temperature interval is accomplished by summation of δQ's. A value of $\delta T = 0.2$ °C gives accurate results with the fairly broad transitions observed with lysozyme. The values of T_d, ΔH_{cal}, ΔH_{vH}, and $\Delta C_p^d = C - A$ have been given previously (Velicelebi & Sturtevant, 1979) for several PrOH concentrations. In addition, the coefficients B and D in eq 8 are needed for the integrations; these are listed in Table V. Integrals from 0 °C to the various temperatures at which heats of transfer were evaluated are listed in Table VI.

In obtaining the enthalpy values in Table VI, we set the apparent specific heat of the protein, C_N, equal to 0 at 0 °C *at each PrOH concentration.* In Scheme I, however, we must use a single reference value for C_N, for convenience setting it equal to 0 at T_1, M_1. This introduces an additional contribution into ΔH_4, since for concentrations of PrOH above 1.33 M there is a substantial increase in C_N in going from T_1, M_1 to T_1, M_2. This increase in C_N of course adds nothing to the isothermal ΔH_3, but it does mean that in the heating step to T_2, M_2, extra enthalpy must be absorbed in order to carry this increased heat capacity through the temperature range T_1 to T_2. Thus, if values taken directly from Table VI are used in connection with Scheme I, it is found that $\Delta H_1 + \Delta H_2 \neq \Delta H_3 + \Delta H_4$ for $M_2 > 1.33$.

If a series of cycles with constant T_1, M_1, and M_2 is computed, the quantity

$$C_{III} - C_I = (\Delta H_3 + \Delta H_4 - \Delta H_1 - \Delta H_2)/(T_2 - T_1) \quad (13)$$

should be constant and equal to the increase in apparent heat

Table VI: Denaturational and Heat Capacity Contributions to Enthalpy Change on Heating Lysozyme in PrOH–H_2O Mixtures from 0 °C to the Indicated Temperatures

PrOH concn (M)	10.13 °C		17.20 °C		25.06 °C		40.00 °C	
	δQ_d[a]	δQ_c[a]	δQ_d[a]	δQ_c[a]	δQ_d[a]	δQ_c[a]	δQ_d[a]	δQ_c[a]
0	0	0.87	0	2.53	0	5.35	0.57	13.7
0.67	0	0.88	0	2.52	0.01	5.34	3.27	13.8
1.33	0	1.13	0	3.25	0.06	6.91	28.87	18.78
2.00	0	2.67	0.05	7.69	1.61	16.37	92.31	54.67
2.67	0.07	4.22	1.59	12.21	33.54	25.23	76.77	78.14
3.34	5.20	4.84	35.72	15.89	56.93	37.07	58.48	90.42

[a] In kilocalories per mole.

Table VII: Computation of Cycles According to Scheme I, with T_1 = 10.13 °C, M_1 = 0, and Evaluation of $c_{III} - c_I$ at 10.13 °C

T_2 (°C)	final PrOH concn, M_f	$\Delta H_1 + \Delta H_2$ (kcal mol^{-1})	$\Delta H_3 + \Delta H_4$ (kcal mol^{-1})	$c_{III} - c_I$ (cal K^{-1} g^{-1})
17.20	0.67	19.3	20.6	0.013
25.06	0.67	20.8	23.5	0.012
40.00	0.67	27.1	35.5	0.019
17.20	1.33	33.5	32.9	−0.006
25.06	1.33	32.9	34.9	0.009
40.00	1.33	75.4	72.2	−0.007
17.20	2.00	47.3	52.6	0.052
25.06	2.00	46.3	58.8	0.058
40.00	2.00	120.9	172.5	0.120
17.20	2.67	52.2	66.8	0.143
25.06	2.67	103.0	107.3	0.020
40.00	2.67	122.0	189.0	0.155
17.20	3.34	79.7	113.9	0.335

capacity in going from state I to state III. A series of cycles with T_1 = 10.13 °C and M_1 = 0 is given in Table VII. It is seen that for each value of M_2 the three calculated values of $c_{III} - c_I$, expressed in terms of specific heats, are reasonably constant except for those corresponding to T_2 = 40.00 °C, M_2 = 2.00 and T_2 = 25.06 °C, M_2 = 2.67. It is difficult to accept that the values for ΔH_{trans} involved in these two cases are in error by as much as 28 kcal mol^{-1}, one being too small and the other too large, but we can propose no other explanation.

It appears that addition of PrOH has very little effect on the apparent specific heat of the protein at 10.13 °C, which is ~0.31 cal K^{-1} g^{-1} in water, at concentrations of 0.67 and 1.33 M, but that significant increases are caused by 2.00–3.34 M PrOH. At the latter concentration the apparent specific heat is more than *doubled*.

It has been suggested by one of the reviewers of this paper that a major part of these large changes in apparent specific heat could arise from the effect of temperature on the binding of PrOH to the protein. If we take as a highly simplified model a set of n equivalent binding "sites" for PrOH, we will have the equilibria:

$$P + A \rightleftarrows PA \qquad K = PA/P{\cdot}A;\ PA = KA{\cdot}P \quad (14)$$

$$PA + A \rightleftarrows PA_2 \qquad K = PA_2/PA{\cdot}A;\ PA_2 = (KA)^2P \quad (15)$$

$$PA_{n-1} + A \rightleftarrows PA_n \qquad K = PA_n/PA_{n-1}{\cdot}A;\ PA_n = (KA)^nP \quad (16)$$

In these equations A, P, PA, etc. stand for the species PrOH, lysozyme, a 1:1 complex of PrOH and lysozyme, etc. and also for their concentrations (italic). Setting $X = KA$, protein conservation gives

$$P_0 = P + PA + PA_2 + \ldots + PA_n = P(1 + X + X^2 + \ldots + X^n) \quad (17)$$

For $n \geq 100$ and $X \leq 0.95$ we can with sufficient accuracy write

$$P_0 = \frac{P}{(1 - X)} \quad (18)$$

Since the alcohol concentration is very much larger than that of the protein, we may set $X = KA_0$ and obtain by differentiation and the van't Hoff equation

$$\frac{dX}{dT} = X\frac{\Delta H}{RT^2} \quad (19)$$

where ΔH is the enthalpy change in each step of the reaction. The total heat change, Q, is

$$Q = \Delta H{\cdot}PA + 2\Delta H{\cdot}PA_2 + \ldots + n\Delta H{\cdot}PA_n = XP\Delta H(1 + 2X + \ldots + nX^{n-1}) \quad (20)$$

For $n \geq 100$ and $X \leq 0.95$, this becomes with sufficient accuracy

$$Q = \frac{X}{1 - X}P_0\Delta H \text{ cal (mol of protein)}^{-1} \quad (21)$$

The excess apparent heat capacity due to increased binding of PrOH to the protein resulting from an increase in temperature is then

$$C_{ex} = \frac{1}{P_0}\frac{dQ}{dT} = \frac{X}{(1 - X)^2}\frac{\Delta H^2}{RT^2} \quad (22)$$

If we assume n = 100 and ΔH = 1250 cal (mol of sites)$^{-1}$, then at 10.13 °C a value of X = 0.935 gives C_{ex} = 2150 cal K^{-1} mol^{-1} or c_{ex} = 0.15 cal K^{-1} g^{-1}, the value found at 2.67 M PrOH. This contribution to the apparent heat capacity of the protein must also be present in the DSC experiments reported earlier (Velicelebi & Sturtevant, 1979), further complicating the interpretation of those results.

It is quite likely that some of the increase in apparent specific heat caused by the addition of PrOH is due to an increase in the number of excitable internal degrees of freedom (Sturtevant, 1977). There is, unfortunately, no way of estimating the importance of this effect in the present case.

Inspection of Figure 2 shows that the dependence of $\Delta H_{trans(0,M)}$ on M_f has four distinguishable regions which are most clearly evident in the curve for 17.2 °C. In region I the enthalpy increases linearly with PrOH concentration with a slope of 23.8 ± 2.2 kcal (mol of protein)$^{-1}$ (M PrOH)$^{-1}$ independent of temperature. This slope can be taken as a measure of the enthalpy of binding of PrOH to the native protein. No information concerning the stoichiometry of this binding is available. An inflection region, II, is followed by another approximately linear region, III, with a slope higher than that of region I. Finally, in region IV there is a transition to decreasing enthalpies. At 10 °C the curve has just barely entered region II at the highest PrOH concentration studied, while at 40 °C at the lowest PrOH concentration the curve is already leaving region II and entering region III. The broad vertical lines in Figure 2 indicate for each temperature the alcohol concentration at which DSC data (Velicelebi & Sturtevant, 1979) show the protein to be 5% denatured. At 17, 25, and 40 °C the end of region II corresponds well with the onset of denaturation. This suggests that region II may indicate a saturation of the binding of PrOH to the native protein.

The values for ΔH_{trans} include contributions arising from unfolding of the protein. Similarly, the apparent heats of unfolding determined by DSC (Velicelebi & Sturtevant, 1979) undoubtedly are affected by changes in the binding of PrOH. There appears to be no way to separate these two effects. Denaturational contributions, ΔQ_d, including unknown binding enthalpies, can be estimated from the DSC data as described above, and deduction of these contributions gives the curves plotted in Figure 3. The irregularities in the 25 and 40 °C curves arise from the same points as those which gave anomalous results in Table VII.

The only regularity which can be discerned in Figure 3 is that at 10–25 °C and 0–2.4 M PrOH, $\Delta H_{trans(0,M)} - \Delta Q_d$ has the same slope, 23.8 ± 2.2 kcal (mol of protein)$^{-1}$ (M PrOH)$^{-1}$, as occurs in Figure 2. At 25 °C and 2.4 M PrOH the protein is ~$^1/_4$ denatured. It appears that at 25 °C and 3 M PrOH,

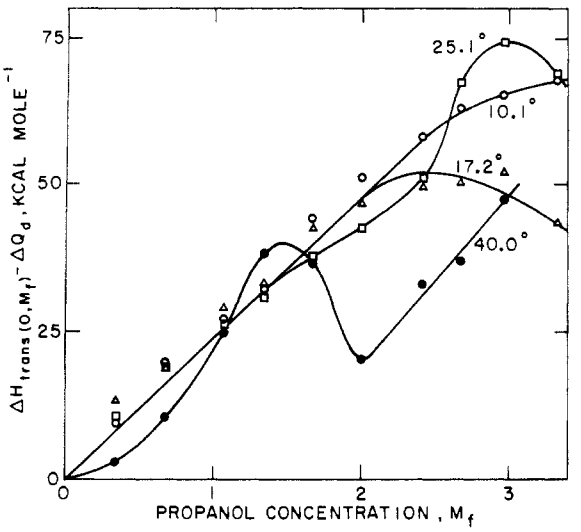

FIGURE 3: Result of deducting denaturational enthalpies, ΔQ_d, calculated from DSC data (Velicelebi & Sturtevant, 1979), from transfer enthalpies, $\Delta H_{trans(0,M_f)}$, displayed as a function of PrOH concentration.

where the protein is $\sim^2/_3$ denatured, the course of the binding enthalpy is not very different from that observed with the native protein.

It is appropriate to compare our isothermal calorimetric results for the interaction of lysozyme with propanol with those of Pfeil & Privalov (1976b) for its interaction with Gdn·HCl. Their curves of enthalpy vs. Gdn·HCl concentration displayed three distinguishable regions: exothermic effects with linear concentration dependence at low concentrations, followed by a sigmoidal curvature corresponding to endothermic effects, and finally another linear region with a large slope up to saturation. Privalov and Pfeil attributed the linear portions of the curves to binding of Gdn·HCl to the native and denatured protein and the sigmoidal region to denaturation. It is evident that our results are not interpretable in this relatively simple manner. Privalov and Pfeil found that all their enthalpies of denaturation, whether observed by DSC in the absence or presence of Gdn·HCl or with change of pH or observed isothermally by the addition of Gdn·HCl, when plotted against temperature fell on the same straight line of slope equal to the value of ΔC_p^d observed in the DSC experiments. Again, it is evident that neither our isothermal data reported here nor our earlier DSC data (Velicelebi & Sturtevant, 1979) follow this simple and significant behavior. It must be concluded that Gdn·HCl and PrOH alter the stability of lysozyme by entirely different mechanisms.

References

Bjurulf, C., & Wadsö, I. (1972) *Eur. J. Biochem. 31*, 95.
Dimmling, V. W., & Lange, E. (1951) *Z. Elektrochem. 55*, 322.
Grenthe, I., Ots, H., & Ginstrup, O. (1970) *Acta Chem. Scand. 24*, 1067.
Pfeil, W., & Privalov, P. L. (1976a) *Biophys. Chem. 4*, 23.
Pfeil, W., & Privalov, P. L. (1976b) *Biophys. Chem. 4*, 33.
Sturtevant, J. M. (1969) *Fractions No. 1*, 1-7.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236.
Sturtevant, J. M., & Lyons, P. A. (1969) *J. Chem. Thermodyn. 1*, 201.
Velicelebi, G. (1978) Ph.D. Thesis, Yale University, New Haven, CT.
Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180.

Thermodynamics of Protein Association Reactions: Forces Contributing to Stability†

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ABSTRACT: Reviewing the thermodynamic parameters characterizing self-association and ligand binding of proteins at 25 °C, we find $\Delta G°$, $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ are often all of negative sign. It is thus not possible to account for the stability of association complexes of proteins on the basis of hydrophobic interactions alone. We present a conceptual model of protein association consisting of two steps: the mutual penetration of hydration layers causing disordering of the solvent followed by further short-range interactions. The net $\Delta G°$ for the complete association process is primarily determined by the positive entropy change accompanying the first step and the negative enthalpy change of the second step. On the basis of the thermochemical behavior of small molecule interactions, we conclude that the strengthening of hydrogen bonds in the low dielectric macromolecular interior and van der Waals' interactions introduced as a consequence of the hydrophobic effect are the most important factors contributing to the observed negative values of $\Delta H°$ and $\Delta S°$ and hence to the stability of protein association complexes. The X-ray crystallographic structures of these complexes are consonant with this analysis. The tendency for protein association reactions to become entropy dominated and/or entropy–enthalpy assisted at low temperatures and enthalpy dominated at high temperatures (a consequence of the typically negative values of $\Delta C_p°$) arises from the diminution of the hydrophobic effect with increasing temperature which is a general property of the solvent, water.

In the past decade, a complete thermodynamic description of the self-association of many proteins and their interactions with small molecular substrates has become available. Concomitantly, X-ray crystallography has provided a detailed picture of some of these associations, and this has stimulated a number of theoretical studies (Levitt & Warshel, 1975; Gelin & Karplus, 1975; Chothia & Janin, 1975), based upon energetic considerations, to account for these structures. The

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