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Biochemistry

Heat capacity and entropy changes in processes involving proteins

(thermodynamics/hydrophobic effect/vibrational modes/conformations)

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ABSTRACT **Six possible sources of the large heat capacity and entropy changes frequently observed for processes involving proteins are identified. Of these the conformational, hydrophobic, and vibrational effects seem likely to be of greatest importance. A method is proposed for estimating the magnitudes of the hydrophobic and vibrational contributions. Application of this method to several protein processes appears to achieve significant clarification of previously confusing and apparently contradictory data.**

In recent years direct calorimetric measurements have shown that many processes involving biopolymers, particularly proteins, take place with large changes in the apparent heat capacities of the reacting species. It now appears that these large values for ΔC_P constitute the rule rather than the exception. Many of the cases for which large values of ΔC_P have been observed are listed in Table 1. For comparisons between substances of widely different molecular weights, the changes in specific heats given in the third column of the table are of interest. The largest fractional change, amounting to 50%, is that for the unfolding of metmyoglobin.

Values of ΔS_u, the unitary entropy (1), in most cases at 25°, are also included in Table 1. It should be remembered that for a process with a large ΔC_P, ΔS_u is a strong function of temperature.

The purpose of this communication is to attempt to analyze the heat capacity and entropy data for a number of protein reactions in terms of the individual contributions arising from various sources, principally the hydrophobic effect and internal vibrational modes.

Sources of heat capacity and entropy changes

The Hydrophobic Effect. Edsall (27) was among the first to point out the effect of nonpolar groups in raising the apparent heat capacities of solutes in aqueous solutions. Since then the general nature of the hydrophobic effect (28–31) has been well established as resulting from the formation of cages of structured water of abnormally high heat capacity and low entropy around nonpolar groups.

Data are available in the literature which permit evaluation of ΔC_P for the transfer of many nonpolar substances or groups from nonpolar to aqueous medium, but in only 17 cases (Table 2) are entropy data, which are equally important in the present discussion, also available. For these cases, the ratio $\Delta S_u/\Delta C_P$ has a remarkably constant value of -0.263 ± 0.046 (standard deviation).

Electrostatic Charges. Edsall (27) also noted the effect of electrostatic charges in decreasing both the apparent heat capacities and entropies of charged solutes in aqueous solution. Numerous data indicate that the creation of a positive and negative pair of charges in aqueous solution leads to $\Delta S_u = -15$ to -30 cal K^{-1} mol^{-1} and $\Delta C_P = -20$ to -50 cal K^{-1} mol^{-1}. In view of the strong tendency of all charged groups to be immersed in the aqueous solvent rather than in the relatively low dielectric interior of a macromolecule, it seems likely that the degree of exposure of charged groups to the solvent does not change much in most macromolecular processes (37).

Hydrogen Bonds. The breaking of hydrogen bonds with increasing temperature is an important source of heat capacity, as in the case of water itself. It is thus to be expected that a change during a reaction in the net extent of hydrogen bonding will be reflected in a change in heat capacity. However, it seems likely that in most protein reactions the energy penalty for less than maximal hydrogen bonding will insure that there is no significant change in the extent of hydrogen bonding (37).

Conformational Entropy. An increase in the number of approximately iso-energetic conformations available to a molecule results in an increase in its entropy without any directly related change in heat capacity.

Intramolecular Vibrations. A protein has many "soft" internal degrees of freedom that are characterized by force constants weak enough to be significantly affected by chemical changes such as unfolding or ligand binding. We must therefore include changes in the number of easily excitable internal vibrational modes as a possible source of heat capacity changes. There is evidence (38–40) that a considerable fraction of the specific heat of solid proteins, usually close to 0.30 cal K^{-1} g^{-1}, is due to internal modes having fundamental frequencies less than 500 cm^{-1} and therefore readily perturbed by chemical reactions. This view is further supported by the following consideration: a typical protein contains about 16 atoms per 100 daltons, so that there are roughly 50 internal modes per 100 daltons. Because the heat capacity of solid proteins is approximately 30 cal K^{-1} per 100 daltons at 25° (41), the average internal mode has a fundamental frequency corresponding to approximately 800 cm^{-1}, or 4 times thermal energy, kT, at 25°. It may be noted that the heat capacity due to a vibrational mode having a fundamental frequency corresponding to 2.5 to 4.5 times kT is changed by 0.12–0.13 cal K^{-1} mol^{-1} by a 10% change in fundamental frequency.

Measurements (41, 42) of the heat capacities and calculations of the third law entropies for four solid anhydrous "native" proteins gave the ratio $S°/C_P° = 1.047 \pm 0.003$ over the temperature range 260–310 K. I will assume in subsequent discussion that this relation is maintained, so far as internal vibrational modes are concerned, for proteins in aqueous solution over the temperature range 273–373 K. The fact that few if any water molecules are found in the interior of the hydrated protein molecules examined by the x-ray crystallographic method (43) supports this assumption.

Changes in Equilibria. A shift with temperature of an equilibrium between two or more states will appear experimentally as a contribution to the heat capacity. Careful chemical study may be required to determine in any individual case whether changes in such equilibria are of importance.

Table 3 gives a summary of the expected signs of the entropy and heat capacity changes arising from sources 1 through 5.

Table 1. Heat capacity and entropy changes in biochemical reactions at 25°*

System	ΔS_u, cal K^{-1} mol^{-1}	ΔC_P, cal K^{-1} mol^{-1}	Δc, cal K^{-1} g^{-1}	Refs.
Aldolase + hexitol-1,6-diphosphate	34	−410	−0.0073	2
GPDH + NAD^+ (pH 8.5)	−45	−750	−0.021	3
Lipase + colipase	13.6	−300	−0.027	4
Heart LDH + NAD^+	3.5	−84	−0.0024	5
Heart LDH + NADH	−2.8	−170	−0.0050	5
Heart LDH·NAD^+ + oxalate	8.0	−340	−0.0099	5
Heart LDH·NADH + oxamate	−20	−405	−0.012	5
Muscle LDH + NADH	9.0	−325	−0.0095	6
tRNA ligase + isoleucine	19.7	−430	−0.0042	7
Avidin + biotin	1.3	−240	−0.014	8
S-peptide + S-protein†	−90	−1100	−0.083	9
Hemoglobin + haptoglobin	−73	−940	−0.0063	10
Rabbit IgG, MOPC 315, MOPC 460 + Dnp-lysine	−7 to − 24	−200	−0.0027	11, 12
Bovine colostrum IgG_2 + Dnp-lysine	8.5	+140	+0.0019	13
α-Chymotrypsin unfolding (pH 7)	330	+3080	+0.122	14, 17
Chymotrypsinogen A unfolding (pH 5, 62°)	440	+3000	+0.129	15, 16
Cytochrome c unfolding (pH 7)	6.2	+1640	+0.132	17
Lysozyme unfolding (pH 7)	140	+1560	+0.111	18
Metmyoglobin unfolding (pH 11.5, 63°)	300	+2760	+0.154	16, 17
Ribonuclease unfolding (pH 2, 31°)	215	+1220	+0.089	16, 19
Poly(dA·dT), helix-coil‡	25	+23	+0.04	20
DNA (calf thymus and T_2), helix-coil§	21	+40	+0.06	21–23
$tRNA_1^{Val}$ unfolding¶	210	+1500	+0.061	24
Hemoglobin S gelation	—	−200	−0.0031	25
Flagellin polymerization	—	−3040	−0.076	26

* Except as noted, ΔC_P appears to be temperature independent in the vicinity of 25°. All values are average values per site in multisite cases. LDH, lactate dehydrogenase; Dnp, dinitrophenol.
† ΔC_P is a strong function of temperature in this case.
‡ Per base pair, pH 7.0, $\mu \approx 0.01$ M, 40°.
§ Per base pair, pH 7.0, $\mu \approx 0.05$ M, 77°.
¶ Average of five resolved steps, 52°–82°; pH 7.0, $\mu \approx 0.15$ M, 1 mM $MgCl_2$.

Method of analysis

In the present discussion I will neglect changes in electrostatic charges, hydrogen bonding, and temperature-dependent equilibria. I will make the following assumptions, which at present are admittedly based on scanty evidence.

1. ΔC_P and ΔS_u can be expressed as the sum of hydrophobic, vibrational and conformational contributions:

$$\Delta C_P = \Delta C_P(\text{hydro}) + \Delta C_P(\text{vib}) \quad [1]$$

$$\Delta S_u = \Delta S_u(\text{hydro}) + \Delta S_u(\text{vib}) + \Delta S_u(\text{conform}) \quad [2]$$

2. The empirical relations noted above,

$$\Delta S_u(\text{hydro}) = -0.26\ \Delta C_P(\text{hydro}) \quad (298\ \text{K}) \quad [3]$$

$$\Delta S_u(\text{vib}) = 1.05\ \Delta C_P(\text{vib}) \quad (273\text{–}373\ \text{K}) \quad [4]$$

hold generally for protein reactions in aqueous solution. In the application of Eq. 4 to reactions involving nonprotein species, the assumption is implicit either that such species also obey the equation, or that their contributions are sufficiently small not to invalidate the application.

From these assumptions it readily follows that

$$\Delta C_P(\text{hydro}) = \frac{1.05\ \Delta C_P - [\Delta S_u - \Delta S_u(\text{conform})]}{1.31} \quad [5]$$

at 298 K. Calculations can be extended to other temperatures by means of the relation

$$\left(\frac{\partial \Delta S(\text{vib})}{\partial T}\right)_P = \frac{\Delta C_P(\text{vib})}{T} \quad [6]$$

which with Eq. 4 gives

$$\ln \frac{\Delta S_2(\text{vib})}{\Delta S_1(\text{vib})} = 0.95 \ln \frac{T_2}{T_1}. \quad [7]$$

In the protein reactions, for which heat capacity data are available, it has been found that ΔC_P is nearly independent of temperature. Calculations performed as outlined here lead to values of $\Delta C_P(\text{hydro})$ and $\Delta C_P(\text{vib})$ that also change slowly with temperature. It is interesting to note that this being the case, a factor of −0.23 in Eq. 4 in place of −0.26 would cause $\Delta S_u(\text{hydro})$ to vanish at approximately 373 K.

Glyceraldehyde-3-phosphate dehydrogenase + NAD^+

The thermodynamics of the binding of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase has been determined over a range of pH and temperature (3, 44). Data for the binding of four moles of NAD^+ at pH 6.5 (3) are given in Table 4. ΔS_u and ΔG_u are the standard unitary entropy and free energy changes, respectively. This system is characterized by a large decrease in apparent heat capacity, with the necessarily resultant enthalpy–entropy compensation giving an almost constant free energy change. If one attributes the heat capacity decrease to the hydrophobic effect, one is faced with the difficulty of understanding the large decreases in entropy at 25° and 40°. This difficulty is removed if the data are analyzed as

Table 2. Thermodynamic data for the transfer of groups and substances from various nonaqueous media to water at 25°

Substance	cal K^{-1} mol^{-1} $-\Delta S°$*	cal K^{-1} mol^{-1} ΔC_P†	$-\frac{\Delta S°}{\Delta C_P}$
C_2H_6	19.5	61	0.320
C_2H_5OH	10.7	36	0.297
1-C_3H_7OH	13.4	49	0.273
C_4H_{10}	23	63	0.365
1-C_4H_9OH	15.6	62	0.252
1-C_3H_7COOH	7.7‡	35	0.220
C_5H_{12}	24.5§	95.6§	0.257
1-$C_5H_{11}OH$	17.1	74	0.231
cyclo-C_6H_{12}	22.6§	86.0§	0.263
C_6H_{14}	26.1§	105.2§	0.248
C_6H_6	13	57	0.228
	13.8§	53.8§	0.257
$C_6H_5CH_3$	16	66	0.242
	16.9§	62.9§	0.269
$C_6H_5CH_2CH_3$	19	77	0.247
	19.4§	76.0§	0.255
$C_6H_5CH_2CH_2CH_3$	21.2§	93.5§	0.227
Alanyl side chain	4.4¶	26¶	0.169
Valyl side chain	10.8¶	30¶	0.360
Norleucyl side chain	11.5¶	40¶	0.287
	Mean		0.263
	Standard deviation		± 0.046

* Ref. 30, pages 18 and 20, unless otherwise noted.
† These values are obtained from the data given by Nichols *et al.* (32) for solutes in aqueous solutions and the specific heats of the pure materials listed in the *Handbook of Physics and Chemistry*, 44th edition, 1963, unless otherwise noted.
‡ Ref. 33.
§ Ref. 34.
¶ Refs. 35 and 36. These data are based on solubility measurements only, so that the values for ΔC_P are less reliable than the other values in the table, which are based on calorimetric measurements. The data for the leucyl side chain derived from the data in these references have been omitted from the table.

outlined above, with the assumption that $\Delta S_u(\text{conform}) = 0$, to give the results in Table 5. According to this treatment, the change in sign of ΔS_u is the result of a delicate balance between a hydrophobic contribution, decreasing in magnitude as expected with increasing temperature, and a vibrational contribution that increases in magnitude with increasing temperature. It appears that, so far as entropy is concerned, the hydrophobic and vibrational effects are of roughly equal importance.

From Suurkuusk's protein specific heat data (45), it can be concluded that per mole of water lost from hydration at 25° there is, on average, a decrease in heat capacity of 5.6 cal K^{-1} mol^{-1}. If it is assumed that this water has the same heat capacity as "hydrophobic" water, then the value for ΔC_P(hydro) at 25° in Table 5 corresponds to a loss of roughly 280 moles of water per mole of protein of 144,000 molecular weight. A somewhat larger figure was obtained by Sloan and Velick (46) from buoyant density measurements, and by Durchslag *et al.* (47) from low angle x-ray scattering, on the assumption that the volume decrease deduced from the measurements is due entirely to the loss of water of hydration. I would suggest that part of the volume decrease is to be ascribed to the conversion of a few hundred soft internal modes to stiffer modes, as reflected in ΔC_P(vib) and ΔS_u(vib), with resultant tightening of the structure of the protein. The magnitude of ΔC_P(vib) does not seem unduly large when it is considered that it is only one to two percent of that part of the heat capacity of the protein which may reasonably be assigned to soft internal modes.

Table 3. Expected signs of ΔC_P and ΔS_u in protein reactions

For an increase in	ΔC_P	ΔS_u
Exposure of nonpolar groups	+	−
Exposure of electrostatic charges	−	−
Hydrogen bonds	+	−
Conformations (isoenergetic)	0	+
Soft internal modes	+	+

Table 4. Thermodynamic data for the binding of four molecules of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase at pH 6.5

Temp., °C	ΔH, kcal mol^{-1}	ΔG_u, kcal mol^{-1}	ΔS_u, cal K^{-1} mol^{-1}	ΔC_P, cal K^{-1} mol^{-1}
5	−14.5	−43.2	103	−2100
25	−56.4	−43.8	−42	
40	−84.8	−42.6	−138	−1900

Data from ref. 3.

L-Isoleucine tRNA ligase + various substrates

Hinz *et al.* (7) have studied the binding of six different ligands to the L-isoleucine tRNA ligase of *Escherichia coli* MRE 600. The unitary free energies of binding, together with hydrophobic and vibrational contributions to ΔC_P and ΔS_u, are listed in Table 6. Because all the substrates except the first one have identical enthalpies of binding, −3.7 kcal mol^{-1} at 25°, the specificity of the enzyme can be said to reside mainly in the entropy of binding, but this again appears to result from approximately balanced hydrophobic and vibrational contributions, the one favorable and the other unfavorable.

Unfolding of lysozyme at pH 7

Extensive thermodynamic data for the unfolding of lysozyme have been reported by Pfeil and Privalov (18). The values for pH 7 at several temperatures are given in Table 7. In this case, in contrast to the protein–ligand reactions considered above, the contribution to the entropy change resulting from the enormous increase in the number of approximately isoenergetic conformations available to the protein must be included. It is assumed that there is no significant conformational contribution to the enthalpy change, or to the heat capacity change as distinct from the vibrational contribution. Because it is not possible to compute directly ΔS(conform), the conformational part of the entropy, I have assumed two different values for it in arriving at the hydrophobic and vibrational quantities listed in Table 8. ΔC_P(hydro) and ΔS(hydro) are not much affected by a

Table 5. Analysis of the data in Table 4 in terms of hydrophobic and vibrational contributions

Temp., °C	ΔC_P(hydro)	ΔC_P(vib)	ΔS_u(hydro)	ΔS_u(vib)
5	−1820	−390	515	−410
25	−1560	−430	405	−450
40	−1360	−460	345	−485

All quantities in cal K^{-1} mol^{-1}.

Table 6. Binding of various substrates to the L-isoleucine tRNA ligase of *E. coli* MRE 600 at 25°, pH 7.5

Substrate	ΔG_u, kcal mol^{-1}	ΔC_P(hydro), cal K^{-1} mol^{-1}	ΔC_P(vib), cal K^{-1} mol^{-1}	ΔS_u(hydro), cal K^{-1} mol^{-1}	ΔS_u(vib), cal K^{-1} mol^{-1}
L-Isoleucinol	−5.2	−307	−56	78	−58
L-Leucine	−5.9	−388	−89	101	−93
L-Norvaline	−6.5	−305	−67	79	−70
L-Valine	−6.8	−392	−87	102	−92
L-2-Amino-3,4 dimethyl-pentanoic acid	−8.2	−354	−74	92	−77
L-Isoleucine	−9.6	−357	−70	93	−73

Experimental data from ref. 7.

change in ΔS(conform), whereas the vibrational quantities are decreased by an increase in ΔS(conform). Nevertheless, it appears that the vibrational effect, which is a destabilizing effect here, as expected, must be considered in any discussion of the stability of the native structure.

Vibrational contributions to enthalpy and free energy changes

The available heat capacity data for anhydrous proteins (42) suggest that the vibrational part of the enthalpy function can be approximated to within 1–2% in the temperature range 200–310 K by the equation

$$\left(\frac{H^\circ - H_0^\circ}{T}\right)(\text{vib}) = \frac{C_P^\circ(\text{vib})}{1.9}. \qquad [8]$$

Aside from zero point energies, the vibrational contributions to ΔH and ΔG_u are then

$$\Delta H(\text{vib}) = 0.53\ T\ \Delta C_P(\text{vib});$$
$$\Delta G_u(\text{vib}) = -0.52\ T\ \Delta C_P(\text{vib}) \qquad [9]$$

Several values calculated in this way are listed in Table 9. It appears that although the vibrational effect may make the major contribution to the enthalpy change in a protein reaction at 298 K, there must be large contributions of opposite sign to the free energy change. For example, Chothia (48), using x-ray crystallographic data and the hydrophobicity values for amino acid sidechains proposed by Nozaki and Tanford (49), estimated that the hydrophobic contribution to the free energy change at 298 K in converting lysozyme from its native conformation to a fully extended chain with *trans* sidechains is 340 kcal mol^{-1}. Although the hydrophobic contribution in the less complete unfolding accomplished by heating the protein is certainly much smaller than this limiting figure, it is probably significantly larger in magnitude than ΔG_u(vib).

Because $|\Delta C_P(\text{vib})| < |\Delta C_P|$ the variation of ΔH(vib) with temperature is much smaller than that of ΔH, and the above comments cannot be blindly taken over to other temperatures.

Table 7. Thermodynamic data for the unfolding of lysozyme at pH 7

Temp., °C	ΔH, kcal mol^{-1}	ΔG, kcal mol^{-1}	ΔS, cal K^{-1} mol^{-1}	ΔC_P, cal K^{-1} mol^{-1}
10	32.8	16.1	59.1	1560
25	56.4	14.5	140	1560
60	112	6.5	315	1560
100	175	−9.9	494	1560

Discussion

It has often been emphasized that the stability of proteins and other biopolymers in their native conformations is a marginal situation resulting from the delicate balance of large opposing effects. The analysis proposed here suggests that other important properties of proteins are likewise the result of a balance between at least two large counteracting effects.

Although some clarification of the nature of protein reactions has perhaps been obtained by the present treatment, it is unfortunately still not possible to identify the specific regions in a protein which give rise to the effects cited, much less to predict the magnitudes of the effects.

Table 8. Conformational, hydrophobic, and vibrational contributions to the thermodynamics of unfolding of lysozyme at pH 7

Temp., °C	ΔC_P(hydro)	ΔC_P(vib)	ΔS(hydro)	ΔS(vib)
ΔS(conform) = 200; 2.2 new conformations per residue				
10	1300	260	−410	270
25	1290	270	−335	280
60	1260	300	−200	310
100	1230	330	−60	350
ΔS(conform) = 400; 4.8 new conformations per residue				
10	1450	110	−460	120
25	1440	120	−375	120
60	1430	130	−220	140
100	1410	150	−60	150

All quantities in cal K^{-1} mol^{-1}.

Table 9. Vibrational contributions to enthalpy and free energy changes at 25°

Data from	ΔH	ΔH(vib)	ΔG_u	ΔG_u(vib)
Tables 4 and 5	−56.4	−68	−43.8	67
Table 6	−3.65	−11.1	−9.55	10.9
Table 8	56.4	43*	74*†	−42*
		19‡	134†‡	−19‡

All quantities in kcal mol^{-1}.
* ΔS(conform) = 200.
† Values for $\Delta G_u - \Delta G$(conform).
‡ ΔS(conform) = 400.

The present treatment removes some of the difficulties that have arisen in attempts to interpret the thermodynamics of protein reactions primarily in terms of the hydrophobic effect. It has been noted by several authors (48–53) that similar difficulties arise in connection with the volume changes in protein reactions, which are usually much smaller in magnitude than expected on the basis of the hydrophobic effect alone. It seems likely that these difficulties might be alleviated to some extent if it were possible to bring into consideration volume changes resulting from changes in the number of easily excitable internal modes. The increases in ΔC_P(vib) and ΔS(vib) accompanying protein unfolding (Table 8) indicate an increase in the excitability of internal modes which in turn should lead to a positive contribution to the volume change working against the expected negative hydrophobic contribution.

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