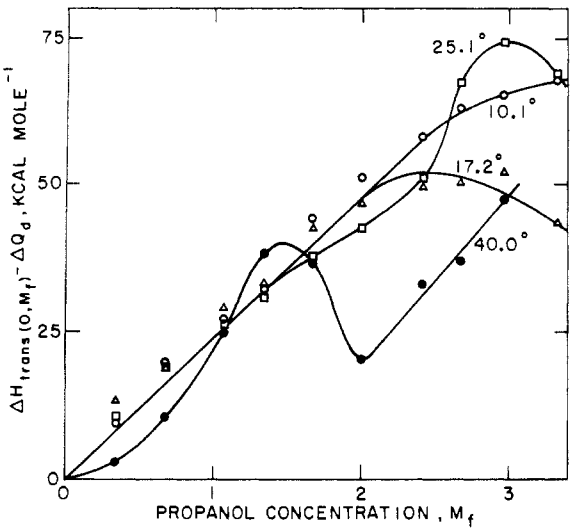

FIGURE 3: Result of deducting denaturational enthalpies, ΔQ_d, calculated from DSC data (Velicelebi & Sturtevant, 1979), from transfer enthalpies, $\Delta H_{trans(0,M_f)}$, displayed as a function of PrOH concentration.

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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Table I: Thermodynamics of Protein Association[a]

association process	ΔG°_u (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS°_u (cal K^{-1} mol^{-1})	ΔC_p° (cal K^{-1} mol^{-1})	ref[b]
trypsin (bovine) + inhibitor (soybean)	−14.6	8.6	78	−440	*c, d*
deoxyhemoglobin S gelation	−3.4	2.0	18	−200	*e, f*
lysozyme self-association (indefinite)	−3.9	−6.4	−8.3		*g*
glucagon trimerization	−12.1	−31	−64	−430	*h, i*
hemoglobin + haptoglobin	−11.5	−33	−73	−940	*j*
α-chymotrypsin dimerization	−7.1	−35	−95		*k, l*
S-peptide + S-protein (ribonuclease)	−13	−40	−90	−1100	*m, n*

[a] All thermodynamic parameters expressed per mole of complex formed except the indefinite association cases of hemoglobin S and lysozyme for which the mole refers to the monomeric protein reacted. Unitary entropy and free energy are given for processes of defined stoichiometry. Standard states are hypothetical 1 M protein, pH at which the reaction was measured. All pHs were close to 7 except for trypsin, pH 5, haptoglobin, pH 5.5, and glucagon, pH 10.5. All data for 25 °C except glucagon, $T = 30$ °C. [b] For each entry, the first reference is to calorimetric work and the second is to X-ray crystallographic structure determination. [c] Baugh & Trowbridge (1972). [d] Sweet et al. (1974). [e] Ross et al. (1977). [f] Wishner et al. (1975). [g] Banerjee et al. (1975). [h] Johnson et al. (1979). [i] Sasaki et al. (1975). [j] Lavialle et al. (1974). [k] Shiao & Sturtevant (1969). [l] Vandlen & Tulinsky (1973). [m] Hearn et al. (1971). [n] Wyckoff et al. (1970).

methodology and problems involved in such calculations have been critically reviewed by Némethy & Scheraga (1977).

In this paper we review the thermodynamics of protein association processes for the examples best characterized in terms of their chemistry and structure. From this survey we find that the thermodynamic parameters ΔG°, ΔH°, ΔS°, and ΔC_p° are predominantly of negative sign. This result poses severe difficulties for interpretations of protein association based upon the entropically driven hydrophobic effect. The aim of this paper is to attempt to account for the signs and magnitudes of these thermodynamic parameters for protein association reactions in terms of known molecular forces and the thermochemistry of small molecule interactions.

Results

In this paper we present thermodynamic parameters based only upon calorimetrically determined enthalpy changes. The reason for this criterion of data selection is that the measured heat, which is the net sum of all thermal effects, will more readily lead to a direct determination of the enthalpy change of the association process per se than will analysis of equilibrium data by the van't Hoff equation. This is especially true for processes in which several equilibria may be involved, as in several of the examples considered here.

In Table I we have assembled the thermodynamic parameters at 25 °C in order of (calorimetrically determined) increasing exothermic enthalpy change, for protein association complexes whose structures have been determined (in five of the seven cases) by X-ray crystallography. We note that (1) the values of ΔG° are all negative, favoring association, but no pattern in the magnitude of ΔG° is readily discernible; (2) the values of ΔH° and ΔS° range from positive to negative but in most cases these association processes are *enthalpically* controlled; (3) the values of ΔC_p° are large and negative in all instances; consequently the enthalpic contribution to ΔG° will become more predominant at higher temperatures; (4) the magnitude of ΔH° and ΔC_p° for protein–protein association are several-fold larger than those found for the protein–small molecule interactions, reflecting a multiplicity of interactions and large areas of subunit contacts in the former case.

In Table II we list some typical thermodynamic parameters (again exclusively calorimetrically determined) for the binding of coenzymes and other small molecules to proteins. The results presented for the binding of coenzymes and coenzyme analogues to rabbit muscle lactic dehydrogenase is representative of a much larger data base (Schmid et al., 1976; Subramanian & Ross, 1977, 1978; Hinz et al., 1978) comprised of these same ligands and six other dehydrogenases whose coenzyme binding sites have been shown by Rossman et al. (1975) to be closely identical by X-ray crystallography. The effect of variation in chemical structure upon the values of these thermodynamic parameters has been discussed by Subramanian & Ross (1978). The chymotrypsin data provide another example of the variation of thermodynamic parameters for inhibitor binding to a common site on the same protein. It may be seen from Table II that ΔG°, ΔH°, and ΔS° are of negative sign. Negative values of ΔC_p° characterize the coenzyme reactions (Hinz et al., 1978). Thus the thermodynamic parameters are again essentially all of negative sign.[1]

Table II: Thermodynamics of Protein–Ligand Association at 25 °C

ligand	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal K^{-1} mol^{-1})
Lactic Dehydrogenase[a]			
NAD^+	−3.8	−6.3	−8.3
NADH	−7.4	−6.9	+1.7
ADP-ribose	−4.6	−7.6	−10.0
iodosalicylic acid	−3.5	−21.7	−61.0
α-Chymotrypsin[b]			
proflavin	−6.0	−11	−18
indole	−4.3	−15	−37
N-acetyl-D-tryptophan	−3.3	−19	−53
hydrocinnamic acid[c]	−2.6	−26	−80

[a] From rabbit muscle, pH 7.6 (Subramanian & Ross, 1977, 1978). [b] pH 7.8 (Shiao & Sturtevant, 1969). [c] Canady & Laidler (1958).

[1] A referee has correctly pointed out that if ΔG° is evaluated without recognition of concomitant ancillary reactions (e.g., chloride ion displacement) and combined with calorimetrically determined values of ΔH° (for which these additional processes are recognized), then an undetermined error is introduced into the value of the thermodynamic parameters. The referee suggests that it is necessary to demonstrate agreement between calorimetrically determined values of ΔH° and those obtained by use of the van't Hoff equation before much confidence may be placed in the results presented in Tables I and II. In Table I, the ΔG°, ΔH°, and ΔS° for the self-associations of glucagon, lysozyme, and chymotrypsin are entirely self-consistent, all having been obtained from calorimetric heats of dilution. The van't Hoff and calorimetric enthalpy changes for the gelation of sickle cell hemoglobin are surprisingly in agreement. In Table II the thermodynamic parameters for the binding of NAD, ADP-ribose, and iodosalicylic acid have all been obtained from calorimetric data and thus all the parameters are inclusive of direct as well as indirect reactions. In summary, it does not appear that consideration of the van't Hoff vs. calorimetric technique will vitiate the major conclusion to be drawn from Tables I and II, which is that the values of the thermodynamic parameters are predominantly of negative sign. Finally, it should be mentioned that the van't Hoff equation is based upon a two-state model of equilibrium and will not be applicable to highly cooperative and/or multistate processes.

Table III: Thermodynamics of Self-Association of Purine Derivatives[a]

compound	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal K^{-1} mol^{-1})
purine	−0.4	−4.2	−13
6-methylpurine	−1	−6	−16
6-(dimethylamino)-purine[b]	−2.4	−9.1	−22.5
caffeine	−1.5	−3.4	−6
purine ribonucleoside	−0.4	−2.5	−7
adenosine	−0.9	−9.6	−29

[a] Thermodynamic parameters expressed per mole of monomer; water, 25 °C (Gill et al., 1967). [b] Marenchic & Sturtevant (1973).

Table IV: Expected Signs of Contributions to ΔH and ΔS

process	ΔH	ΔS	ref
hydrophobic association[a]	pos	pos	*b, c*
van der Waals	neg	neg	
H-bond formation in low dielectric medium	neg	neg	*d*
ionic (charge neutralization)	slight pos or neg	pos	*e*
protonation	neg	neg[f]	*g*

[a] By hydrophobic association, we are referring to the partial withdrawal of the nonpolar group from water and not to any further interaction between the nonpolar groups themselves. [b] Gill et al. (1976). [c] Gill et al. (1967). [d] Pimentel & McClellan (1971). [e] Ross & Shapiro (1974). [f] Standard state for H^+; unit activity at pH 7. [g] Shiao & Sturtevant (1976).

The polarizability of a ligand in binding to a protein contributes to large negative values for the thermodynamic parameters. This is illustrated by the binding of the inhibitor 5-iodosalicylic acid to dehydrogenases (Table II) and by the values for the binding of the first iodide ion to serum albumin ($\Delta H^\circ = -18$ kcal mol^{-1}, $\Delta S^\circ = -42$ cal K^{-1} mol^{-1}), which are strikingly more negative than those for the less polarizable chloride ion ($\Delta H^\circ = -8$ kcal mol^{-1}, $\Delta S^\circ = -12$ cal K^{-1} mol^{-1}) (Lovrien & Sturtevant, 1971).

In Table III we present thermodynamic parameters for the self-association of some purines and related molecules. These typically display negative values of ΔG°, ΔH°, and ΔS° and reflect, in part, the effect of van der Waals (stacking) interactions.

In Table IV we report the characteristic signs of the thermodynamic parameters associated with the various individual kinds of interactions that may take place in protein association processes. The conclusions reported in Table IV are based upon a large body of thermodynamic results for small molecule model systems and in the case of the last two entries also include ionic interactions with nucleic acids and proton dissociation of amino acids in proteins. Table IV contains the following important conclusions: (1) the only contributions to positive entropy and enthalpy changes arise from ionic and hydrophobic interactions (in the sense that the latter term is used in this paper; vide infra) and (2) the only sources of negative enthalpy and entropy changes arise from nonbonded (van der Waals) interactions and hydrogen-bond formation in low dielectric media and protonation accompanying association.

Discussion

The problem posed by the data presented under Results is to account for the signs and magnitudes of the thermodynamic parameters of protein association processes in terms of our knowledge of the thermochemical behavior of intermolecular interactions. Alternatively, the problem may be stated as a question: What information, if any, do the thermodynamic results for protein association processes contain regarding the intermolecular interactions taking place in those reactions? In the biochemical literature the role of the hydrophobic effect in such associations has been greatly emphasized. In our discussion, we shall first attempt to give a clear definition of the hydrophobic effect that will lead to a simple conceptual model of protein association which places the interpretation of the energetics in proper perspective. We will then examine the contributions of the various types of intermolecular interactions to the overall values of the thermodynamic parameters and point out examples in which thermodynamic evidence for specific interactions are supported by structural evidence from X-ray crystallography. Finally, we discuss the systematic effect of temperature upon the values of the observed thermodynamic parameters.

Several attempts (Chothia & Janin, 1975; Chothia et al., 1976; Janin & Chothia, 1976, 1978) have been made to account for the stability of protein association complexes in terms of hydrophobic interactions for many of the reactions cited in Tables I and II. These authors first compute the loss in translational and rotational entropies upon forming an association complex. The result is an unfavorable contribution of between 20 and 30 kcal mol^{-1} to the free energy of association at 27 °C. Janin and Chothia then estimate, from the known X-ray structure, the solvent-accessible surface areas of the reacting species that become buried upon association. The result of this hydrophobic portion of their calculation is a negative (favorable) contribution to the association free energy that outweighs the positive (unfavorable) contribution arising from the translational–rotational immobilization. Identical reasoning that the negative entropy change accompanying the loss of translational and rotational degrees of freedom would be offset by positive contributions to the entropy of association from hydrophobic and electrostatic effects had previously been presented by Steinberg & Scheraga (1963).

The theory of Janin & Chothia (Chothia & Janin, 1975; Chothia et al., 1976; Janin & Chothia, 1976, 1978) is logically incapable of predicting negative values of ΔH° and ΔS°, as are often observed (Tables I and II), while also obtaining the requisite negative values for ΔG°. Disregarding any possible uncertainties associated with the details of their calculations (evaluation of buried surface area, choice of energy conversion factors, etc.), we conclude that the theory of Chothia and co-workers (Chothia & Janin, 1975; Chothia et al., 1976; Janin & Chothia, 1976, 1978) is incomplete because van der Waals interactions and hydrogen bonds have been considered to be thermodynamically unimportant in these reactions. We will show below that these interactions assume an importance by the circumstances created by the hydrophobic effect.

The data presented in Tables I and II for protein association processes indicate that the criteria (Kauzmann, 1959) for hydrophobic interactions ($\Delta G^\circ < 0$, $\Delta H^\circ \sim 0$, $\Delta S^\circ > 0$, and $\Delta C_p^\circ < 0$) are generally satisfied for ΔG° and ΔC_p° but in most instances are *not* satisfied for the changes in enthalpy and entropy, ΔH° and ΔS°. This contradiction between negative values of ΔH° and ΔS° and the positive values expected for hydrophobic interaction has been noted previously for several protein association processes (Velick et al., 1971; Banerjee et al., 1975; Mills & Ackers, 1979). Thus, we draw the general conclusion, based upon the foregoing criteria for hydrophobic interaction, that *it is not possible to account for the stability of association complexes of proteins on the basis of hydrophobic interactions alone.* As we will discuss below,

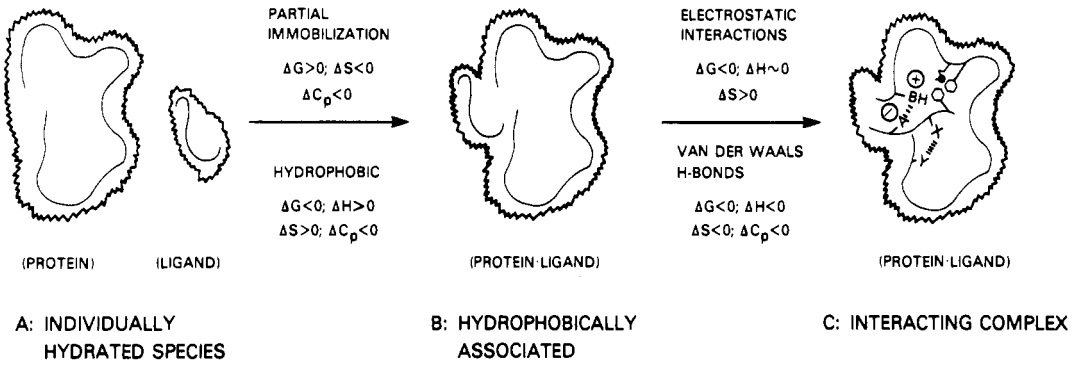

FIGURE 1: Schematic drawing of protein association process. In this hypothetical thermodynamic model, the protein is shown reacting with another species (labeled ligand) which may be either the same protein or another protein, peptide, or ligand molecule. The fuzzy outline surrounding reaction species denotes domain of water that is "more ordered" than bulk solvent. Protein association is visualized as occurring in two steps, A → B consisting of hydrophobic association and partial immobilization and B → C representing all other intermolecular interactions. The signs of the thermodynamic parameters are shown for each step.

we do not minimize the importance of hydrophobic contributions in the association process but emphasize that hydrophobic interactions *by themselves* are incapable of accounting for many of the observed thermodynamic parameters.

Examination of the thermodynamics of protein associations in terms of $\Delta G°$ alone as made by Chothia and co-workers (Chothia & Janin, 1975; Chothia et al., 1976; Janin & Chothia, 1976, 1978) overlooks the interplay of the contributions from $\Delta H°$ and $\Delta S°$. From this perspective, the nonconformity of $\Delta H°$ and $\Delta S°$ with the criteria for hydrophobic interaction renders the agreement in $\Delta G°$ ($\Delta G° < 0$) only superficial. Only for the first two entries in Table I are the signs of all of the thermodynamic parameters in accord with the criteria for hydrophobic interactions. *These examples, consistent with hydrophobically driven reactions, appear to be the exception rather than the rule for the majority of associations involving proteins reported in Tables I and II.* This assessment of the thermodynamic data and our own extensive experience with the thermodynamics of coenzyme binding to dehydrogenases (Subramanian & Ross, 1977, 1978) motivated us to search for other forces responsible for the association reactions. Specifically and most importantly the sources of the negative values observed for $\Delta H°$ and $\Delta S°$ require delineation.

Conceptual Model of Protein Association. We interpret the thermodynamics of protein association processes in terms of the following conceptual model. Protein associations may be envisioned as proceeding from *hypothetical thermodynamic states* A to B and then to C, as schematized in Figure 1. In this scheme, no consideration is given to kinetics. State A represents isolated hydrated species which in state B partially interact so that there is a mutual penetration of their hydration layers to form what we refer to as a "hydrophobically associated species". This hydrophobic association is a result of the tendency of water to form a more ordered structure in the vicinity on nonpolar hydrocarbon groups (Frank & Evans, 1945). Our description of protein association as a hydrophobic process merely means that hydrophobic amino acid side chains which were previously accessible to solvent in the isolated subunits become buried upon complex formation and produce an increase in the number of "destructured" water molecules. The hydrophobic free energy calculated by Chothia & Janin (1975) qualitatively corresponds to our process A → B. We estimate that the signs for the accompanying thermodynamic parameters shown for A → B in Figure 1 will be $\Delta G°$ negative, $\Delta H°$ positive, $\Delta S°$ and $\Delta V°$ positive, and $\Delta C_p°$ negative (Table IV). The hydrophobic interaction pictorially described by Némethy & Scheraga (1962) occurs when two hydrophobic groups (e.g., the side chains of the amino acids phenylalanine and leucine) come into proximity, cause a partial disordering of the more highly organized water molecules that were formerly surrounding these groups, and engage in van der Waals interactions as well. The hydrophobic interaction in proteins is thus similar to a partially nonaqueous environment (Kauzmann, 1959; Némethy & Scheraga, 1962).

In the second step of protein association, depicted in Figure 1 as B → C, the hydrophobically associated species B participate in further interactions as depicted in state C, the fully interacting association complex. In essence the process B → C is a description of the intermolecular interactions between the protein and the ligand species, and solvent molecules are not considered to be involved to any appreciable extent. The intermolecular interactions involved include ionic interactions, hydrogen bonds, and what we shall refer to in this paper as van der Waals interactions (dipole–dipole and dipole–induced dipole forces and the London dispersion effect). The thermodynamic characteristics of these interactions as known from work with small molecules given in Table IV indicate that the van der Waals and hydrogen-bonding interactions are associated with negative signs for ΔH and ΔS and thus along with protonation (which may be incidental to the association process) are the most likely (if not the only) sources capable of accounting for the negative values of $\Delta H°$ and $\Delta S°$ so often observed in protein associations. The interactions that occur in B → C in Figure 1 and their associated thermodynamic parameters have been neglected in the descriptions of protein association by their implicit inclusion under hydrophobic interactions, and thereby their importance has been ignored (Chothia & Janin, 1975). To summarize our view: proteins are involved in hydrophobic interaction as in A → B but the other types of interactions represented by B → C in Figure 1 play a crucial if not predominant role in determining the thermodynamics of protein association.

The thermodynamic parameters associated with the process A → B describe only the reorganization of the solvent structure around the protein and the ligand species toward a greater disorder of the solvent compared to the isolated individually hydrated species. This favorable entropy-driven association applies to totally nonpolar molecules as well as to the nonpolar segments of amphipathic molecules. The hydrophobic step A → B, however, is *necessary but not sufficient* to explain the experimental thermodynamic parameters observed in a vast majority of protein-association reactions given by some representative examples in Tables I and II. Studies with small molecules (Table III) have shown that associations between analogues of nucleic acid bases are characterized by negative

$\Delta H°$ and $\Delta S°$. The intramolecular folding interaction of the nicotinamide and adenine halves of NAD^+ (Sovago & Martin, 1979), a process akin to the phenylalanine–phenylalanine interaction (Némethy & Scheraga, 1962), is accompanied by $\Delta H° = -3$ kcal mol^{-1} and $\Delta S° = -11$ cal K^{-1} mol^{-1}, values which are opposite in sign predicted for a hydrophobic interaction. To this extent, step B → C resolves this apparent discrepancy by recognizing the stacking and other van der Waals interactions along with H bonds in describing the association process. The net $\Delta G°$ for the complete association process is essentially determined by the relative magnitudes of positive ΔS(A → B) and negative ΔH(B → C). The ΔS(B → C) is of the same sign as ΔH(B → C) and thus partially offsets the contribution of ΔH(B → C) toward the net $\Delta G°$. The ΔH(A → B) contribution is insignificant.

Partial immobilization of the protein and the ligand occurs in the step A → B which would contribute negatively to ΔS, and the immobilization is more extensive in the step B → C. To this extent, the positive ΔS due to the hydrophobic association would be partially offset by the negative ΔS due to the loss of translation and rotation (if any) in the hydrophobically associated species. Thus it can be seen that the net $\Delta G°$ is, to a large extent, due to a negative ΔH in the step B → C.

Both of the processes described above as A → B and B → C have been considered in a recent approach to the hydration and short-range interactions of peptides by Hodes et al. (1979). In this paper, we reiterate the importance of van der Waals and hydrogen-bond interactions (but not neglect the hydration aspects) in order to account for the thermodynamic parameters in Tables I and II. This will now be discussed.

Summary of Interactions Contributing to Negative Values of $\Delta H°$ and $\Delta S°$: (a) van der Waals Interactions. A dramatic effect upon the thermodynamic parameters of coenzyme binding is produced by the presence of a highly polarizable iodine atom (Table II). Induced dipole and dispersion interactions depend upon polarizability. This result suggests that sulfur atoms in proteins such as the one in methionine, with its large electron donor capability, when interacting with the delocalized charge cloud of an aromatic group (e.g., Met-13 with Phe-120 in ribonuclease), would result in negative values of $\Delta H°$ and $\Delta S°$ as in the interaction between S-peptide and ribonuclease S-protein. Morgan et al. (1978) have reported interactions of alternating chains of sulfur and π electrons to be a feature of globular protein structure.

The thermodynamics for the self-associating aromatic ring systems incapable of forming intermolecular hydrogen bonds or other ionic bonds in the associated state, as in the case of the dimerization of caffeine (Table III), typically display negative values of $\Delta H°$ and $\Delta S°$. In these cases the source of the dimer or multimer stability should reside in the optimization of van der Waals contacts between stacked molecules. For the other purine derivatives shown in Table III, such as purine ribonucleoside and adenosine, van der Waals interactions would be only one of the factors governing the mechanism of association. Interactions other than stacking do not contribute significantly to the stability ($\Delta G°$) of the associated species but are manifested in additional entropy-compensated enthalpic changes. The negative $\Delta H°$ and $\Delta S°$ parameters of self-association increase in magnitude with increasing polarizability of the π electron charge cloud as observed for 6-(dimethylamino)purine > 6-(methylamino)purine > purine (Marenchic & Sturtevant, 1973).

The interactions involving delocalized electrons of aromatic ring systems make significant contributions of negative sign to both $\Delta H°$ and $\Delta S°$. Similar nonbonded interactions (stacking) in aromatic hydrocarbons dominated by negative enthalpy changes have been reported in recent thermodynamic transfer studies of several aromatic hydrocarbons by Amidon & Anik (1980). In the low dielectric interior of the protein such interactions are very likely further enhanced, resulting in even more negative values of $\Delta H°$ and $\Delta S°$ than obtained for the model systems in water. In support of van der Waals interactions in protein complexes, we cite the following examples from X-ray crystallographic results.

(1) It is known that the active sites of dehydrogenases contain both aromatic and aliphatic nonpolar side chains which interact wiith the adenine of the coenzymes NAD(H) (Rossman et al., 1975). In the binding of NAD^+ to glyceraldehyde-3-phosphate dehydrogenase the adenine ring of the coenzyme is nestled between Phe-99 and Phe-34, with Phe-8 situated close by [Figure 10 of Rossman et al. (1975)].

(2) In the trimerization of glucagon, one structure involves close interactions between Trp-25 and Phe-22 of one molecule with Phe-6, Tyr-10, and Tyr-13 of another [Figures 3 and 4 of Sasaki et al. (1975)]. In another possible structure, contacts between Phe-22, Val-23, Leu-26, and Met-27 of three identical molecules are noted at the interfaces of the trimer [Figure 5 of Sasaki et al. (1975)].

(3) In the chymotrypsin dimer there is an extremely close contact between the two Phe-39 residues which are stated to be nearly parallel to each other (Vandlen & Tulinsky, 1973).

(4) In the association of the S-peptide with the S-protein of ribonuclease, extensive van der Waals interactions occur among the aromatic rings of Phe-8, His-12, and Phe-120 and also the sulfur atom of Met-13. These groups are surrounded by the aliphatic side chains of Leu-51, Val-47, and Val-54 (Wyckoff et al., 1970).

(5) In the description of the structure of dihydrofolate reductase (*L. casei*), Matthews et al. (1978) describe a "stacking interaction" in which "the side chain of Phe-49 is nearly parallel to and in van der Waals contact with the benzene ring in the inhibitor", methotrexate.

(b) Hydrogen Bonds. It is generally accepted that there is little net energetic difference when an amino acid forms a hydrogen bond with another group in an aqueous environment. However, an important source of negative contribution to $\Delta H°$ and $\Delta S°$ will arise if a hydrogen bond is formed in an environment of low dielectric constant (Pimentel & McClellan, 1971). Typical values for such processes would be considerable enthalpy and entropy changes of $\Delta H = -5$ kcal mol^{-1} and $\Delta S = -10$ to -20 cal K^{-1} mol^{-1}, but only a marginal contribution to the overall stability, $\Delta G = -1$ kcal mol^{-1}, would be made (Pimental & McClellan, 1971). The same conclusion was reached by Némethy et al. (1963) in their assignment of thermodynamic parameters for hydrogen-bond formation. Thus several hydrogen bonds formed in a low dielectric environment such as parts of the contact areas between proteins which are inaccessible to water or the ligand binding sites in the interior of an enzyme could collectively make substantial negative contributions to $\Delta H°$ and $\Delta S°$. As probable examples of this, we note that several hydrogen bonds are formed at the interface between the subunits of the α-chymotrypsin dimer (Vandlen & Tulinsky, 1973) and between 7 and 10 new hydrogen bonds are formed upon reaction of the S-peptide with the S-protein of ribonuclease in the buried region from which solvent is excluded (Wyckoff et al., 1970; Hearn et al., 1971).

Interactions Contributing to Positive Values of $\Delta H°$ and $\Delta S°$. (a) Ionic Interactions and Salt Bridges. Interactions between ionic species in aqueous solution are characterized by extremely small enthalpy changes (of either sign) and

positive entropy changes (Table IV). The extent of such favorable contribution to the free energy from positive entropy changes in protein association processes will depend upon the details of specific associations. We believe that the crystallographically demonstrated salt bridge between Lys-15 of bovine pancreatic trypsin inhibitor and Asp-189 of trypsin (Rühlmann et al., 1973) may contribute significantly to the thermodynamics of that reaction. Such ionic effects are responsible for the interactions between DNA and basic amino acid species (Ross & Shapiro, 1974) as in DNA–histone binding and in the entropically driven polymerization of tobacco mosaic virus (Ansevin & Lauffer, 1969).

(*b*) *Role of Protons.* Protein association processes are often accompanied by the release (and, less frequently, uptake) of protons (Allewell et al., 1979; Subramanian & Ross, 1979). In the binding of organic phosphates to hemoglobin, Noll et al. (1979) have shown that the major driving force for this reaction is the protonation of an imidazolium nitrogen of histidine and/or an α-amino nitrogen atom. The release of the Bohr protons estimated at $\Delta H = 11$ kcal (mol proton)$^{-1}$ (Mills et al., 1979) accompanying the binding of oxygen to hemoglobin makes a *major* unfavorable contribution to the energetics of that vital process. Such proton effects, accompanying the association process, will not significantly alter the magnitude of any of the results we have presented in Tables I and II except perhaps for the association of trypsin with trypsin inhibitor in which case 1.8 protons are released (Laskowski et al., 1971). However, the identity of the groups dissociating protons is not known, so it is not possible to make a quantitative estimate of the energetics of proton-transfer contributions in this example.[2] While the energetics of proton release (or uptake) incidental in protein association processes, in general, may make only minor contributions to the overall energetics, it is to be recognized that in specific cases proton transfer could control the overall energetics (Subramanian & Ross, 1979).

Temperature Dependence of Thermodynamic Parameters and Heat Capacity Changes. We now consider the effect of temperature on the relative contributions of $\Delta H°$ and $\Delta S°$ to the $\Delta G°$ for protein association reactions. By virtue of the predominantly negative heat capacity changes, the $\Delta H°$ will become more negative at higher temperatures, thereby gaining greater control of $\Delta G°$. But at lower temperatures (between 0 and 25 °C) there are several cases known where both $\Delta H°$ and $\Delta S°$ contribute favorably to the $\Delta G°$ together, as in the binding of NAD^+ to glyceraldehye-3-phosphate dehydrogenase (Niekamp et al., 1977), and in some cases, especially where negative $\Delta C_p°$ values are large, the $\Delta G°$ is totally under entropic control. Two (noncalorimetric) examples of reactions entropically driven at low temperature and enthalpically driven at high temperature are the self-association of carboxymethylated high-density serum lipoprotein (Osborne et al., 1976) and the binding of insulin to its receptor (Waelbroeck et al., 1979). This development of entropic control of $\Delta G°$ (mainly at low temperatures) is a consequence of the hydrophobic interaction dominating the association process at low temperatures. The enhancement of solvent structure by the dissolution of nonpolar groups in water is greatest at low temperatures, and the tendency for hydrophobic association is increased because of this. Thus the large positive ΔS of such hydrophobic association can more than compensate the negative ΔS due to van der Waals and other interactions. But at higher temperatures as the solvent structure is randomized, the positive contribution to $\Delta S°$ by hydrophobic association is diminished while the enthalpy term dominates the stability of the complex. Thus it must be borne in mind that the magnitude of the heat capacity changes (which in turn reflect a hydrophobic index) could determine the enthalpic or entropic control of the free energy change. However, the main argument of this paper is to explain the sources of the large and negative $\Delta H°$ and $\Delta S°$ contributions to $\Delta G°$ that are observed while not neglecting the importance of the hydrophobic interactions.

Sturtevant (1977) has discussed the important and admittedly complex parameter $\Delta C_p°$ in his semiempirical analysis of the $\Delta C_p°$ and $\Delta S°$ of protein association reactions. He emphasized two major contributions to $\Delta C_p°$ and $\Delta S°$, one arising from the hydrophobic effect and the other from the changes in low-frequency (0–500 cm^{-1}) vibrational modes resulting from the "tightening" of the protein structure upon association. According to Sturtevant (1977), the contributions to $\Delta C_p°$ from the hydrophobic effect and changes in soft vibrational modes are both negative, producing the observed large negative values of $\Delta C_p°$. As for the entropy, the hydrophobic contribution is positive while the vibrational contribution is negative. With increase of temperature the vibrational contribution becomes more negative but the positive hydrophobic contribution is diminished, thus making the $\Delta S°$ more negative (or less positive) at higher temperatures. Sturtevant's analysis (1977) is thus able to reconcile the apparent conflict between negative $\Delta C_p°$ and negative $\Delta S°$ being grossly ascribed to hydrophobic interactions.

Our emphasis is on the thermochemical aspects of the complex formation while Sturtevant (1977) has addressed the origins of the $\Delta C_p°$ parameter. To this extent the two approaches are to be considered parallel, and the conclusions reached concerning the large negative heat capacity changes and negative entropy changes at higher temperatures are quite compatible. Both approaches stress the importance of the diminishing solvent structure at higher temperatures resulting in the elimination of positive $\Delta S°$ due to hydrophobic interaction. It is this general property of water that promotes enthalpic domination of protein association processes at higher temperatures. The observation that the negative enthalpy and entropy changes of protein association reactions primarily arise from hydrogen-bonding and van der Waals interactions has been the main burden of this paper.

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References

Allewell, N. M., Hoffman, G. E., Zaug, A., & Lennick, M. (1979) *Biochemistry 18*, 3008–3015.

Amidon, G. L., & Anik, S. T. (1980) *J. Phys. Chem. 84*, 970–974.

Ansevin, A. T., & Lauffer, M. A. (1959) *Nature (London) 183*, 1601–1602.

[2] The reaction heat for the soybean trypsin inhibitor–trypsin interaction as a function of pH has been reported recently (Yung & Trowbridge, 1980). At pH values greater than 5, the measured ΔH becomes progressively less endothermic, and above pH 6, the enthalpy change becomes exothermic. This result reinforces our analysis that the intrinsic association process in the trypsin–trypsin inhibitor reaction is characterized by a negative enthalpy change and that proton release masks this process in making the overall reaction endothermic (top entry, Table I).

Banerjee, S. K., Pogolotti, A., Jr., & Rupley, J. A. (1975) *J. Biol. Chem. 250*, 8260–8266.
Baugh, R. J., & Trowbridge, C. G. (1972) *J. Biol. Chem. 247*, 7498–7501.
Canaday, W. J., & Laidler, K. J. (1958) *Can. J. Chem. 36*, 1289–1296.
Chothia, C., & Janin, J. (1975) *Nature (London) 256*, 705–708.
Chothia, C., Wodak, S., & Janin, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3793–3797.
Frank, H. S., & Evans, M. W. (1945) *J. Chem. Phys. 13*, 507–532.
Gelin, B. R., & Karplus, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2002–2006.
Gill, S. J., Downing, M., & Sheats, G. F. (1967) *Biochemistry 6*, 272–276.
Gill, S. J., Nichols, N. F., & Wadso, I. (1976) *J. Chem. Thermodyn. 8*, 445–452.
Hearn, R. P., Richards, F. M., Sturtevant, J. M., & Watt, G. D. (1971) *Biochemistry 10*, 806–817.
Hinz, H. J., Steininger, G., Schmid, F., & Jaenicke, R. (1978) *FEBS Lett. 87*, 83–86.
Hodes, Z. I., Némethy, G., & Scheraga, H. A. (1979) *Biopolymers 18*, 1565–1610.
Janin, J., & Chothia, C. (1976) *J. Mol. Biol. 100*, 197–211.
Janin, J., & Chothia, C. (1978) *Biochemistry 17*, 2943–2948.
Johnson, R. E., Hruby, V. J., & Rupley, J. A. (1979) *Biochemistry 18*, 1176–1179.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.
Laskowski, M., Jr., et al. (1971) in *Proceedings of the International Conference on Proteinase Inhibitors* (Fritz, H., & Tschesche, H., Eds.) pp 117–134, W. de Gruyter, Berlin.
Lavialle, F., Rogard, M., & Alfsen, A. (1974) *Biochemistry 13*, 2231–2234.
Levitt, M., & Warshel, A. (1975) *Nature (London) 253*, 694–698.
Lovrien, R., & Sturtevant, J. M. (1971) *Biochemistry 10*, 3811–3815.
Marenchic, M. G., & Sturtevant, J. M. (1973) *J. Phys. Chem. 77*, 544–548.
Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisluik, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946–6954.
Mills, F. C., & Ackers, G. K. (1979) *J. Biol. Chem. 254*, 2881–2887.
Mills, F. C., Ackers, G. K., Gaud, H. T., & Gill, S. J. (1979) *J. Biol. Chem. 254*, 2875–2880.
Morgan, R. S., Tatsch, C. E., Gushard, R. H., McAdon, J. M., & Warme, P. K. (1978) *J. Pept. Prot. Res. 11*, 209–217.
Némethy, G., & Scheraga, H. A. (1962) *J. Phys. Chem. 66*, 1773–1789.
Némethy, G., & Scheraga, H. A. (1977) *Q. Rev. Biophys. 10*, 239–352.
Némethy, G., Steinberg, I. Z., & Scheraga, H. A. (1963) *Biopolymers 1*, 43–69.
Niekamp, C. W., Sturtevant, J. M., & Velick, S. F. (1977) *Biochemistry 16*, 436–445.
Noll, L. A., Gaud, H. T., Gill, S. J., Gersonde, K., & Barisas, B. G. (1979) *Biochem. Biophys. Res. Commun. 88*, 1288–1293.
Osborne, J. C., Jr., Palumbo, G., Brewer, H. B., Jr., & Edelhoch, H. (1976) *Biochemistry 15*, 317–320.
Pimentel, G. C., & McClellan, A. L. (1971) *Annu. Rev. Phys. Chem. 22*, 347–385.
Ross, P. D., & Shapiro, J. T. (1974) *Biopolymers 13*, 415–416.
Ross, P. D., Hofrichter, J., & Eaton, W. A. (1977) *J. Mol. Biol. 115*, 111–134.
Rossman, M. G., Liljas, A., Branden, C. I., & Banaszak, L. J. (1975) *Enzymes, 3rd Ed. 11*, 61–102.
Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., & Huber, R. (1973) *J. Mol. Biol. 77*, 417–436.
Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., & Blundell, T. (1975) *Nature (London) 257*, 751–757.
Schmid, F., Hinz, H. J., & Jaenicke, R. (1976) *Biochemistry 15*, 3052–3059.
Shiao, D. D. F., & Sturtevant, J. M. (1969) *Biochemistry 8*, 4910–4917.
Shiao, D. D. F., & Sturtevant, J. M. (1976) *Biopolymers 15*, 1201–1211.
Sovago, I., & Martin, R. B. (1979) *FEBS Lett. 106*, 132–134.
Steinberg, I. Z., & Scheraga, H. A. (1963) *J. Biol. Chem. 238*, 172–181.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Subramanian, S., & Ross, P. D. (1977) *Biochem. Biophys. Res. Commun. 78*, 461–466.
Subramanian, S., & Ross, P. D. (1978) *Biochemistry 17*, 2193–2197.
Subramanian, S., & Ross, P. D. (1979) *J. Biol. Chem. 254*, 7827–7830.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4212–4228.
Vandlen, R. L., & Tulinsky, A. (1973) *Biochemistry 12*, 4193–4200.
Velick, S. F., Baggott, J. P., & Sturtevant, J. M. (1971) *Biochemistry 10*, 779–786.
Waelbroeck, M., Van Obberghen, E., & DeMeyts, P. (1979) *J. Biol. Chem. 254*, 7736–7740.
Wishner, B. C., Ward, K. B., Lattman, E. E., & Love, W. E. (1975) *J. Mol. Biol. 98*, 179–194.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305–328.
Yung, B. Y. K., & Trowbridge, C. G. (1980) *J. Biol. Chem. 255*, 9274–9730.