THE THERMODYNAMICS OF NUCLEOTIDE BINDING TO PROTEINS

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I. INTRODUCTION

During the last 10 years, the number of biological systems to which the methods of thermodynamics have been applied has increased dramatically.[1,2,3] As is frequently the case when a field undergoes rapid development, no methodical approach to the choice of systems for investigation has been used; rather, specific systems have been studied because of their intrinsic interest to the investigators or their topical appeal to the scientific community. Under these conditions, an overview of a specific area of interest can only be developed in retrospect.

The study of the binding of nucleotides and their analogs to proteins by microcalorimetric methods is currently in a state such that an overview is appropriate. Recent advances in microcalorimetric instrumentation and methods[4,5] have allowed many systems to be investigated in great detail. Of these, several have been examined by other physical methods, often allowing a correlation between thermodynamic and structural information. In this review, those nucleotide binding systems for which free energy, enthalpy, and entropy change data have been determined are examined in an effort to deduce plausible conclusions about the nature of the forces governing the interactions. To develop the appropriate context for the interpretation of these data, a brief review of solution thermodynamic theory is included with an emphasis on the determination of $\Delta G°$,$\Delta H°$, $\Delta S°$, and $\Delta C°_p$ for the binding process. We have relied heavily on the published work of several laboratories involved in these particular studies. The data included are the result of the application of reaction microcalorimetry for the determination of $\Delta H°$, with other methods, such as van't Hoff analysis, used only infrequently.

II. FUNDAMENTAL THERMODYNAMIC CONCEPTS

The general relations and theories of thermodynamics are extensively treated elsewhere,[6] but to provide the framework with which to understand the binding parameters discussed in this chapter, it is important to establish a uniform set of definitions and conventions. Our objective is to review the methods used to derive thermodynamic parameters from exerimental data, to indicate some of the advantages and disadvan-

tages of the methods, and to develop a conceptual approach to the use of calorimetry and thermochemical data.

In order to communicate data in a readily understandable form, a uniform set of units must be agreed upon. The thermodynamic standard state, i.e., all reactants and products at unit activity, is difficult to apply to biological systems. Thus, a standard state has been accepted through common usage of a specified pH and temperature, 1 atm pressure, and all reactants and products at unit molarity. Typically, the pH and temperature are 7.0 and 25°C respectively; however, quite a range of conditions is often used. To denote that the "biological" standard state differs from the thermodynamic one, a prime (′) is added as a superscript to the standard state thermodynamic quantity. In a practical sense, the adoption of the unit molarity scale means that all energy quantities are expressed on a "per mole" basis, while quantities of solutions are measured by volume.

The IUPAC convention of units and symbols calls for the use of the joule (J) as the unit of energy. However, most biochemists are more comfortable using the thermochemical calorie (cal), and this unit will be used throughout this article. To convert units, one uses the relation 1 cal = 4.184 J.

The thermodynamic parameters of most interest to the biochemist are those expressed by the relations:

$$\Delta G^{\circ\prime} = \Delta H^{\circ\prime} - T\Delta S^{\circ\prime} \quad (1)$$

$$\Delta G^{\circ\prime} = -RT\ln K' \quad (2)$$

$$\Delta C_p^{\circ\prime} = d(\Delta H^{\circ\prime})/dT \quad (3)$$

The application of these relations results from the need for very specific information. Typically, the first two quantities of a "thermodynamic" nature sought concerning the interaction of a small molecule and a protein are the stoichiometry, n, or moles of ligand bound per mole of protein, and the binding affinity or equilibrium constant K'_c, for the process $P + nL \rightleftharpoons PL_n$ for which

$$K'_c = \frac{[PL_n]}{[P]\,[L_{free}]} \quad (4)$$

(Note: many studies report the reverse reaction of Equation 4, i.e., the dissociation reaction.)

The determination of the stoichiometry is freqently approached using any of a variety of partitioning experiments such as equilibrium dialysis and gel chromatography. In any specific use of this approach, the experimental situation is arranged such that the concentration of bound and free ligand can be determined. This approach was recognized by Klotz[7] and by Scatchard[8] and for the particular case of identical, noninteracting sites, it can be recast into the familiar equation:

$$\frac{\bar{\nu}}{[L_{free}]} = K_C\,(n - \bar{\nu}) \quad (5)$$

where $\bar{\nu}$ is defined as the ratio of the moles of ligand bound per mole of protein. The detailed implications of this equation have been discussed extensively elsewhere.[9,10] We will consider the results of the application of this approach to obtain n and K'_c.

Graphical use of Equation 5 or any of several transformations[8,9] of it provide information necessary to determine n and K'_c. Data of high precision are required for all but the simplest cases. Once these parameters are defined, the application of Equation 2 provides an estimate of the total free-energy change for the process of interest. Sev-

eral other methods are available for determining n and K'_c. One which is quite useful involves curve fitting to a plot of a signal proportional to the extent of binding as a function on the mole ratio of ligand to protein. Binding parameters can frequently be extracted from such an analysis. In addition, a graph of this type based on assessed parameters can also be interpreted in terms of the feasibility of a particular experimental design, and the upper and lower limits of derived parameters can be determined for the magnitude of the signal.

For the equilibrium expressed by Equation 4, assuming n = 1,

$$K'_C = [PL]/[P][L] \quad (6)$$

$$= [PL]/([P_t] - [PL])([L_t] - [PL]) \quad (7)$$

where $[P_t]$ is the total protein concentration. Solving this equation for the equilibrium concentration of product, [PL] provides the information necessary for Figure 1. From the calculated value of [PL] the extent of reaction is calculated from the ratio $[PL]/[P_t]$ and the titration variable is $[L_t]/[P_t]$. The factor determining the feasibility of the reaction is the product $K'_c[P_t]$. Thus, the feasibility of a given experiment is proportional to both the equilibrium constant and the concentration of protein. Since the equilibrium constant is usually not amenable to variation without grossly changing solution conditions, the term $K'_c[P_t]$ can then only be conveniently changed by varying the protein concentration.

An experimentally determined graph similar to Figure 1 will provide information about n and K, under appropriate conditions. If $K'_c[P_t]$ is sufficiently large, the intersection of tangents drawn at $[L_t]/[P_t] \rightarrow 0$ and $[L_t]/[P_t] \rightarrow \infty$ will give n directly on the mole ratio axis. The curvature is related to both the equilibrium constant and $[P_t]$. Thus, an appropriate linearization of Equation 7 will define a procedure for the determination of the equilibrium constant. The detailed procedures for extracting the equilibrium constant from data which define a curve such as shown in Figure 1 have been treated extensively by Biltonen and Langerman.[4] The procedures are based in part on expressing $\bar{\nu}$ as the ratio of the observed signal, λ, to the maximum value the signal may have when the protein is fully saturated with the ligand, λ_{max}:

$$\nu = \lambda/\lambda_{max} \quad (8)$$

Use of this relation (or any procedure based upon it) requires an assumption which should be understood whenever any but the simplest (n = 1) system is being studied. The direct application of Equation 8 implies that the change in λ upon binding each mole of ligand is independent of $\bar{\nu}$. Failure to recognize this limitation can directly contribute to a misinterpretation of the data.

When $K'_c[P_t]$ is large, it is not possible to deduce a value of K, due to insufficient curvature. The ideal approach is to adjust the protein concentration to a very high value to determine n and if necessary, to a lower value to determine K'_c.

These general approaches have proved quite useful when the limitations of each approach are respected. Complicated binding processes require data of very high precision. Once K'_c and n are determined, it is then possible to try to determine the enthalpy change for bindng. There are two general approaches: application of the van't Hoff equation to temperature variation of the equilibrium constant data or direct calorimetric measurement.

The van't Hoff equation

$$d(\ln K)/dT = \Delta H^{\circ\prime}/RT^2 \quad (9)$$

or

$$d(\ln K)/d(1/T) = -(\Delta H^{\circ\prime}/R) \quad (10)$$

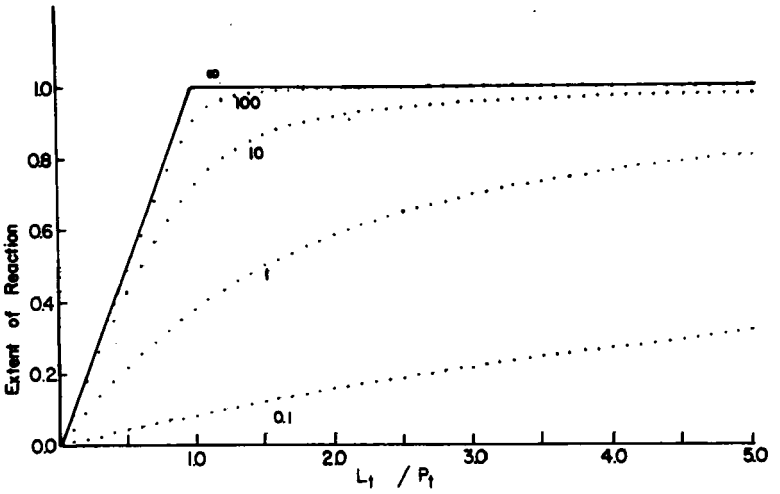

FIGURE 1. Calculated curves showing the dependence of the extent of reaction as a function of the mole ratio of reactants on the product K'_C [P_t]. The infinite energy isotherm (∞) indicates the values of the product for which no curvature in the isotherm is expressed.

indicates that the enthalpy change may be calculated from the slope of a graph of lnK versus the reciprocal absolute temperature. Experimentally, this necessitates that a series of binding experiments be performed at various temperatures and the first derivative of the equilibrim constant with respect to the inverse of temperature be calculated.

Several difficulties are inherent in this approach. In general, the equilibrium constant at each temperature is derived by a curve fitting procedure. The result of either is then subject to a differentiation with respect to temperature. Errors propagate badly by this treatment. Thus, data of extremely high precision are required. The derivation of Equation 9 requires that K'_C be the true thermodynamic equilibrium constant for binding. Frequently, this is not the case for protein-ligand binding reactions. In addition, if a partition method is being employed to generate the primary data, the temperature variation of the equilibrium constant will contain a term reflecting the temperature dependence of the ligand partition coefficient between the solution and stationary phases.[11] A third consideration is the frequently made assumption that the enthalpy change itself is invariant over the temperature interval examined. The usual argument in favor of this assumption is that the van't Hoff graphs are frequently linear. However, since $\Delta C_P°'$ is frequently non-zero for protein-ligand binding reactions, the enthalpy change derived using Equation 10 represents an average value over the experimental range.

Direct calorimetric measurement of the enthalpy change is now within the ability of many biochemistry laboratories. During a calorimetric experiment, the energy change occurring concomitant with Reaction 4 is measured directly. Recent developments in instrumentation, methods, and theories, have made this approach quite attractive. It is useful to note that $\bar{\nu}$ (for simple interactions) may be readily expressed as the ratio of the observed molar heat of reaction, Q, to the molar enthalpy change:

$$\bar{\nu} = Q/\Delta H°' \qquad (11)$$

This immediately opens up all of the approaches discussed above for the determination of thermodynamic information. Thus, from one properly designed thermal titration experiment, it is possible to determine n, K'_C, $\Delta G°'$, $\Delta H°'$, ad $\Delta S°'$. Such an experiment

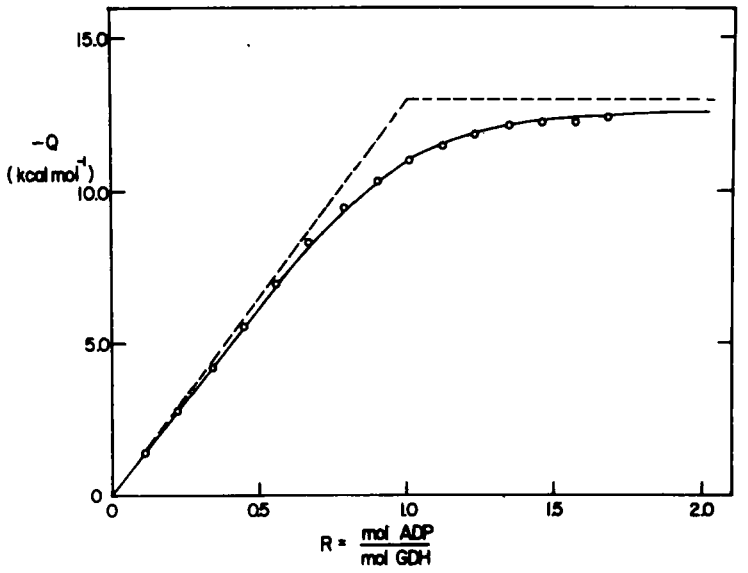

FIGURE 2. Isotherm for the binding of ADP to L-glutamate dehydrogenase in 0.1 *M* potassium phosphate, pH 7.6, 25°C, obtained using the Tronac Titration Calorimeter. The broken and solid lines represent, respectively, the infinite binding energy isotherm and the best theoretical fit for single-site biding. (From Beaudette, N.V. and Langerman, N., *Anal. Biochem.*, 90, 693, 1978. With permission.)

is illustrated in Figure 2 for the binding of ADP to glutamate dehydrogenase. It is important to realize that no assumptions or approximations are involved in the determination of $\Delta H^{\circ\prime}$ or n. K still contains approximations discussed elsewhere (Reference 4). Calorimetric studies of the interaction of a small molecule with a protein must be carefully performed, since the instrument responds to *all* processes which generate heat within it. Thus, if the binding reaction occurs with a net movement of protons to or from the buffer, this will produce a heat effect. This observation can be put to good use. The observed heat, $\Delta H^{\circ\prime}_{obs}$, is defined by the relation

$$\Delta H^{\circ\prime}_{obs} = \Delta H^{\circ\prime}_{rxn} + n_H \Delta^{\circ\prime}_{ion} \qquad (12)$$

where $H^{\circ\prime}_{rxn}$ refers to the actual heat of the reaction under the conditions of the experiment, n_H is the number of moles of protons bound (released) by the buffer species, and $\Delta H^{\circ\prime}_{ion}$ is the enthalpy change for the buffer reaction:

$$H^+ + B \rightleftarrows HB^+$$

The parameter desired is the molar enthalpy change for the reaction, $\Delta H^{\circ\prime}_{rxn}$. By performing the experiment in two or more buffers of differet heat of ionization, at constant pH, ΔH_{rxn} and n_H can be determined. This very useful technique will be illustrated below by the work of Beaudette et al.[14,15]

The temperature coefficient of the enthalpy change is the heat capacity change:

$$\Delta C^{\circ\prime}_p = d(\Delta H^{\circ\prime})/dT \qquad (13)$$

The subscript indicates that these parameters are determined at constant (usually atmospheric) pressure. $\Delta C^{\circ\prime}_p$ is readily determined from the slope of a plot of $\Delta H^{\circ\prime}$

versus temperature. No assumptions are involved in this determination and the values of $\Delta C°'$ are typically non-zero for reactions of the type of interest in this article.

The final parameter of interest, $\Delta S°'$, is calculated in a straightforward manner from enthalpy and free-energy change data by the application of Equation 1. While no assumptions are involved in this calculation, the interpretation of entropy data is probably the most controversial of any of the thermodynamic parameters.

A large numbr of experimental observations,[16,17] indicate that for a series of related reactions, $\Delta H°'$ and $\Delta S°'$ are altered in such a manner as to maintain the free-energy change relatively constant. This behavior is referred to as compensation.[18] While several investigators[17,18] have attempted to treat the theoretical basis of this phenomenon, no generally accepted theory has yet emerged. It is quite interesting that when $\Delta H°'$ is plotted as a function of $\Delta S°'$ for an homologous series of reactions, the slope, which has units of temperature, is about 298 K. Some attempts have been made to use this behavior in interpreting data for the interaction of nucleotides with proteins. However until a unified and generally accepted theory emerges, the interpretation of compensation in specific cases remains speculative.

III. STABILIZING FORCES

The wealth of information available (see for example, Reference 19) from X-ray analysis of protein molecules has made it abundantly clear that the free energy stabilizing the protein-ligand complex is the result of a large number of weak interactions. These weak interactions are all noncovalent and result from a combination of direct and indirect forces. Némethy[20] has examined the mathematical expression of some of the specific interactions in detail. He has pointed out that a complete description of the binding of a ligand to a protein requires detailed knowledge of the conformation of both species. However, a set of macroscopic phenomenological constants, such as $\Delta G°'$, $\Delta H°'$, $\Delta S°'$, and $\Delta C_p°'$ are of themselves sufficient to describe all but the detailed molecular basis of the interaction.

It is instructive to examine the specific forces contributing to the stability of the protein-ligand complex. Results of a large number of model studies allow some generalizations to be made about the sign and magnitude of the thermodynamic functions for these forces in a variety of situations. The forces normally considered are van der Waals, hydrogen-bonding, ionic (electrostatic), and hydrophobic.

The generalized van der Waals forces produce weak short-range bonds. These are nonspecific and do not require orientation of the contributing atomic pair. Because of the weak nature of these forces, they are significant only in situations in which the contributing atomic pairs are in very close proximity. Van der Waals energies fall in the range of 0 to -0.6 kcal mol^{-1}; many of them are required to make a significant contribution to the total free energy of stabilization of the PL complex. They are especially significant in defining the spatial constraints imposed upon the complex, rather than as contributors to the stabilization of the complex. Some forms of Van der Waals interactions, such as dipole-dipole forces, are highly oriented and of moderate strength. However it is difficult to study them without extensive extrathermodynamic information and, they are thus beyond the scope of this review.

The physical chemistry of the hydrogen bond has been reviewed in detail by Pimental and McClellan.[21] It is specific and directed and its strength depends upon the electronegativity of the atoms involved and the protic nature of the solvent. In aprotic conditions, a strong hydrogen bond can contribute 4 to 6 kcal mol^{-1} to the stabilizing free energy. In aqueous situations, however, this stabilizing force is markedly reduced by competition with water. The stabilizing free energy is usually thought to be in the range

of 0 to −3 kcal mol^{-1}, with Klotz[22] arguing for the lower portion of the range. Alvarez and Biltonen,[57] however, on the basis of heated solution measurements of thymine into water and ethanol, argue that the hydrogen bond plays a significant role in protein-solution interactions.

Theoretical treatment[23] of ionic interactions typically begins with an analysis of the potential energy between two point charges separated in a vacuum and examines the effect as they are allowed to approach each other, in the absence and in the presence of a solvating medium. Results of this type of analysis and appropriate experiments reveal that ions in solution are surrounded by compressed, oriented solvation shells arising from the strong electric fields in the vicinity of the charges. As oppositely charged ions approach each other, the electric fields are concentrated in the region between them and a rather strong, highly oriented interaction occurs. Because of the dependence of ionic interactions on the ionic strength of the medium, this is frequently a well-studied interaction.

The tendency for nonpolar groups or molecules to associate in aqueous media with each other and thus minimize their contact with the water molecules is referred to as a hydrophobic bond. This bond is the least understood of all forms of noncovalent bonding which occur between a protein and a ligand. The driving force is apparently the minimization of the surface free energy of the solvation shell about the hydrophobic moieties. This suggests that the stabilization energy should be expressed primarily by an entropic contribution.[24,26] The most frequently cited data for the magnitude of the hydrophobic bond energy is from the work of Nozaki and Tanford[24] for the transfer of various amino acids from ethanol to water. Typical values range from −0.5 kcal mol^{-1} for alanine to −3.5 kcal mol^{-1} for tryptophan side chains. There is little doubt that the tendency of nonpolar groups to move to areas of low polarity also plays a significant role in the stabilization of the protein-ligand complex. The lack of a generally accepted description of the interaction, however, makes the assessment of the magnitude of the contribution difficult.

No mention has yet been made of the parts of the protein removed from the actual binding domain of the ligand. Because of the flexibility of the polypeptide chain, the large network of cooperative interactions stabilizing the protein conformation and, in oligomeric species, the possibility of facile changes in the quaternary structure, this remainder of the protein cannot be ignored. Again, however, only detailed spatial analysis can specify its exact contributon to the stability of the PL complex. Némethy[20] and Weber[25] have each recently examined the implications of chain conformation and/or multiligand binding resulting in cooperativity on the energetics of binding. In these complex systems, the determination of the thermodynamic equilibrium constant is difficult, thus the application of the van't Hoff relation to determine $\Delta H^{\circ\prime}$ is quite complicated. Indeed in complex, highly cooperative systems such as the binding of oxygen to hemoglobin, it is difficult to determine more than the overall binding enthalpy even with calorimetric methods.

The various general forces contributing to protein-ligand interactions have associated with them a fairly well described set of thermodynamic parameters. Using model systems, the sign and relative magnitude of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ have been determined. Table 1 summarizes these results.

IV. EXPERIMENTAL SYSTEMS

We are now in a position to examine in detail those nucleotide systems for which thermodynamic descriptions have been obtained. In this section, we will concentrate on the results obtained and the conclusions based on these results. The values reported in each study, along with pertinent experimental information are summarized in Table

TABLE 1
THERMODYNAMIC EFFECTS OF THE FORCES WHICH STABILIZE PROTEIN-LIGAND INTERACTIONS AT 25°C[26]

Type	Effect*	Comments
van der Waals	$\Delta H^{\circ\prime}$ small	Nonspecific, nondirected
	$\Delta S^{\circ\prime}$ small	
H-Bond	$\Delta H^{\circ\prime} \ll 0$	Specific, directed; orientation determines strength
Nonpolar environment	$\Delta S^{\circ\prime} < 0$	
Polar environment	$\Delta H^{\circ\prime} \lesssim 0$	
	$\Delta S^{\circ\prime} < 0$	
Ionic (electrostatic)	$\Delta H^{\circ\prime} \leq 0$	Specific, directed
	$\Delta S^{\circ\prime} \gg 0$	
Hydrophobic	$\Delta H^{\circ\prime} > 0$	Nonspecific, nondirected, occurs between apolar regions of molecules in a polar environment
	$\Delta S^{\circ\prime} \gg 0$	

* Double inequalities indicate that the associated parameter provides the greater driving force.

2. Frequent reference to this table will be helpful in understanding the various arguments and conclusions.

A. Dehydrogenases

Thermodynamic parameters for the binding of the coenzyme nicotinamide adenine dinucleotide (NAD) to glyceraldehyde-3-phosphate dehydrogenase from yeast and from rabbit muscle have been investigated, both by fluorometric titration and calorimetrically, by Velick et al.[13] The investigation was carried out at various temperatures in phosphate buffer, pH 7.3. Both methods yielded consistent thermodynamic parameters. The four binding sites of the yeast enzyme were observed to be identical and independent. At 5°C, binding is driven almost entirely by the large entropy change (see Table 2 below) while, at 25 and 40°C, the enthalpy change is the main contribution to the driving force. It is interesting to note that entropy-enthalpy compensation effects are acting in such a way that $\Delta G^{\circ\prime}$ remains essentially constant over the temperature range studied.

The rabbit muscle enzyme shows an apparent negative cooperativity between NAD binding sites. $\Delta H^{\circ\prime}$ varied from −22 kcal (mol NAD)$^{-1}$ for the first site to −14 kcal (mol NAD)$^{-1}$ for the fourth at 25°, while $\Delta S^{\circ\prime}$ varied from −40 to −29 cal (mol NAD)$^{-1}$ deg^{-1} from the first to the last site. Heat capacity changes for both enzymes were calculated from the net enthalpy changes over all sites at 5 and 25°; ΔC_p = −520 cal (mol NAD)$^{-1}$ deg^{-1} for the yeast enzyme and −220 cal (mol NAD)$^{-1}$ deg^{-1} for the rabbit enzyme. These very large negative values are difficult to interpret, but are characteristic of many ligand binding reactions (Rialdi and Biltonen[2]).

The authors found it difficult to interpret the data in terms of currently available thermodynamic theories. The large negative values of ΔC_p for both enzymes suggest the formation of significant hydrophobic interactions. However, the formation of hydrophobic bonds is expected to bring about a positive change in enthalpy as well as a large positive change in entropy. The observed $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ contradict these expected changes. Alternatively, the large negative values of $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$, and $\Delta C_p^{\circ\prime}$ suggest a protein folding process upon binding. Yet the dehydrogenases do not exhibit extensive conformational changes as measured by optical or hydrodynamic methods. The au-

thors also discounted the possibility of a dimer-tetramer equilibrium[27] influencing the observed thermodynamic parameters, reasoning that both apo and holo enzyme would remain in the tetrameric form at the concentrations used. Consistency of $\Delta H^{\circ\prime}_{vH}$ with the calorimetrically determined $\Delta H^{\circ\prime}$ appears to support this conclusion. It should also be noted that the observation of identical and independent binding sites for the yeast enzyme conflicts with that of Kirschner et al.,[28] and that the binding of NAD to the yeast enzyme is sigmoidal and fits the allosteric model of Monod et al.[29]

Hinz and Jaenicke[30] examined the binding of NADH to pig skeletal muscle lactate dehydrogenase in phosphate buffer, pH 7.0, using calorimetric and equilibrium dialysis techniques. They, too, observed a very large negative heat capacity change, $\Delta C_P = -1300$ cal (mol enzyme)$^{-1}$ deg^{-1} (-325 cal [mol NADH]$^{-1}$ deg^{-1}). Their enthalpy and entropy data for the binding reaction are also qualitatively very similar to the yeast glyceraldehyde-3-phosphate dehydrogenase data of Velick et al.[13] Similarities between the thermodynamic properties of the two enzymes suggest a similarity in the binding sites. Indeed, comparison of the coenzyme binding site of horse liver alcohol dehydrogenase with lactate and malate dehydrogenases using X-ray techniques has led to the suggestion (Brandon et al.[31]) that the binding site substructure may be common to most dehydrogenases for the binding of nucleotides, NAD in particular.

Subramanian and Ross[32,33] continued this study of the interaction of nucleotides (and similar compounds) with the dehydrogenases in a series of studies which supported the contention[34,35] that the binding domain of a number of NAD-dependent dehydrogenases has been conserved. The results of high resolution X-ray crystallographic analysis suggest that the binding domain, which consists of six parallel stands of pleated sheet and four lengths of helix in LDH, GAPDH, and liver ADH is conserved. The implications of this conclusion are that the binding domain was present in precellular evolution and remained unchanged during evolution. In an attempt to further characterize the similarities and differences among the binding sites for nucleotides, Subramanian and Ross[32] examined the binding of NADH, ADP-ribose, 5-iodosalicylate (a competitive inhibitor), and NAD to the enzymes liver ADH, yeast ADH, mitochondrial MDH (m-MDH), rabbit muscle LDH, beef heart LDH, and bovine GDH. They used batchwise incremental thermal titration methods to follow the binding of the various ligands to the enzyme. In this study, 0.1 *M* sodium phosphate, pH 7.6 buffer was used. No attempt was made to determine the involvement of protons in the reaction. The small heat ionization of phosphate, however, suggests that the main conclusions are probably valid.

The results of this study are summarized in Table 2 below. Comparing the values for $\Delta H^{\circ\prime}$ observed in this report for NADH with values reported earlier for NAD^+ binding, Subramanian and Ross[32] conclude that the values lie in a small range centered on -7 to -8 kcal mol^{-1}. Furthermore, using free-energy values derived from these experiments, they conclude that NADH binds to all dehydrogenases 2 to 4 kcal mol^{-1} more negatively than does NAD. They attribute these differences to the absence of a positive charge on the nitrogen in the nicotinamide ring of NADH and the hydrophobicity of the NADH binding sites. They discount the importance of the changed conformation of NADH with respect to the oxidized form of the coenzyme. It is worth noting that the data of Subramanian and Ross[32] compare well with other published data insofar as such a comparison is possible.[36] In consideration of all of the data, Subramanian and Ross argue that ". . . it is quite significant that the structure-function correlation is manifest in the realm of energetics, too."[32]

Horse liver ADH is treated separately by Subramanian and Ross[32] since it exhibits binding parameters somewhat different from those of the other dehydrogenases. The binding of NAD^+ is primarily entropically driven. This is consistent with a postulated protein conformational change which occurs upon binding of the coenzyme.

Table 2
THERMODYNAMIC PARAMETERS OF NUCLEOTIDE BINDING

Protein[a]	Ligand	Conditions	T(°C)[b]	n[c]	n_{H+}[d]	K'_c[e]	$\Delta G°'$[f]	$\Delta H°'$[g]	$\Delta S°'$[h]	Ref.
Yeast ADH	NAD+	0.1 *M* NaPi, pH 7.6	25			0.00144	−4.3	− 8.9	−15.4	32,33
B.H. LDH	NAD+	0.1 *M* NaPi, pH 7.6	25			0.00135	−4.3	− 8.5	−14.2	32,33
R.M. LDH	NAD+	0.1 *M* NaPi, pH 7.6	25			0.00065	−3.8	− 6.3	− 8.3	32,33
P.H.m-MDH	NAD+	0.1 *M* NaPi, pH 7.6	25			0.00053	−3.7	−10.2	−21.7	32,33
H.L. ADH	NAD+	0.1 *M* imidazole, pH 7.6	25			(0.00232)	−4.6	− 1.0	+12.1	32,33
B.L. GDH	NAD+	0.1 *M* NaPi, pH 7.6	25	4			−3.7	−15.0	−38.0	32,33
Yeast ADH	NADH	0.1 *M* NaPi, pH 7.6	25			0.04	−6.3	− 9.5	−10.7	32,33
B.H. LDH	NADH	0.1 *M* NaPi, pH 7.6	25			2.85	−8.8	− 9.7	− 3.0	32,33
R.M. LDH	NADH	0.1 *M* NaPi, pH 7.6	25			0.28	−7.4	− 6.9	+ 1.7	32,33
P.H.m-MDH	NADH	0.1 *M* NaPi, pH 7.6	25			1.00	−8.2	−12.1	−13.1	32,33
H.L. ADH	NADH	0.1 *M* NaPi, pH 7.6	25			2.85	−8.8	0.0	+29.5	32,33
Yeast ADH	ADP-rib	0.1 *M* NaPi, pH 7.6	25			0.00166	−4.4	−11.2	−22.8	32,33
B.H. LDH	ADP-rib	0.1 *M* NaPi, pH 7.6	25			0.00515	−5.0	− 8.5	−11.7	32,33
R.M. LDH	ADP-rib	0.1 *M* NaPi, pH 7.6	25			0.00224	−4.6	− 7.6	−10.0	32,33
P.H.m−MDH	ADP-rib	0.1 *M* NaPi, pH 7.6	25			0.00855	−5.4	− 9.2	−12.8	32,33
H.L. ADH	ADP-rib	0.1 *M* NaPi, pH 7.6	25			0.00869	−5.4	− 6.0	− 2.2	32,33
Yeast	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	5	4		(0.655)[i]	(−7.4)[i]	− 1.9	+19.9	13
GPDH	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	25	4		(0.236)[i]	−7.43 (−7.33)[i]	−12.4	−16.8	13
	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	40	4		(0.105)[i]	−6.99 (−7.19)[i]	−20.1	−41.6	13
Rabbit Muscle	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	5	4		site 1	−12.2	−16.0	−13.5	13
GPDH	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	5	4		site 2	−11.4	−12.4	− 3.4	13
	NAD+	0.05 *M* KPi, 0.05 *M* KCl 2m *M* EDTA, pH 7.3	5	4		site 3	− 7.0	−11.7	−13.0	13

	NAD^+	0.05 *M*KPi, 0.05 *M*KCl 2m *M*EDTA, pH 7.3	5	4		site 4	− 6.0	−11.7	−24.1	13
	NAD^+	0.05 *M*KPi, 0.05 *M*KCl 2m *M*EDTA, pH 7.3	25	4		site 1	− 8.72	−22.0	−40.4	13
	NAD^+	0.05 *M*KPi, 0.05 *M*KCl 2m *M*EDTA, pH 7.3	25	4		site 2	− 8.72	−16.7	−22.6	13
	NAD^+	0.05 *M*KPi, 0.05 *M*KCl 2m *M*EDTA, pH 7.3	25	4		site 3	− 8.72	−16.7	−22.6	13
	NAD^+	0.05 *M*KPi, 0.05 *M*KCl 2m *M*EDTA, pH 7.3	25	4		site 4	− 5.79	−14.4	−29.2	13
Pig skeletal muscle LDH	NADH	0.2 *M*KPi, pH 7.0	25	4		0.090	−6.73	8.1	− 4.6	30,36
	NADH	0.2 *M*KPi, pH 7.0	5	4			−6.73[r]	− 1.05[r]	+ 20.42[r]	30,36
	NADH	0.2 *M*KPi, pH 7.0	10	4		(0.159)[s]	−6.82	− 2.67	+ 14.66	30,36
	NADH	0.2 *M*KPi, pH 7.0	15	4		(0.125)	−6.88	− 4.30	+ 8.95	30,36
	NADH	0.2 *M*KPi, pH 7.0	20	4			−6.91	− 5.92	+ 3.38	30,36
	NADH	0.2 *M*KPi, pH 7.0	25	4		(0.116)	−6.91	− 7.55	− 2.15	30,36
	NADH	0.2 *M*KPi, pH 7.0	30	4			−6.89	− 9.17	− 7.52	30,36
	NADH	0.2 *M*KPi, pH 7.0	35	4			−6.84	−10.79	−12.82	30,36
	NADH	0.2 *M*KPi, pH 7.0	40	4			−6.76	−12.42	−18.07	30,36
	NADH	0.2 *M*KPi, pH 7.0	45	4			−6.66	−14.04	−23.2	30,36
	NADH	0.2 *M*KPi, pH 7.0	50	4			−6.53	−15 66	−28.25	30.36
	Adenosine	0.1 *M*KPi, pH 7.6	25	1		0.0029	−4.7	− 8.5	−12.6	37
GDH	AMP	0.1 *M*KPi, pH 7.6	25	1		0.053	−6.4	− 5.8	+ 2.3	37
	ADP	0.1 *M*KPi, pH 7.6	25	1	0	0.26	−7.4	−12.4	−17.0	37
	ATP	0.1 *M*KPi, pH 7.6	25	1		0.014	−5.6	−10.2	−15.0	37
	dAdenosine	0.1 *M*KPi, pH 7.6	25	1s—3w[t]		0.001	−4.1	− 6.8	− 8.9	37
	dAMP	0.1 *M*KPi, pH 7.6	25	1		0.053	−6.4	− 5.8	+ 2.3	37
	dADP	0.1 *M*KPi, pH 7.6	25	1		0.027	−6.0	−11.8	−19.0	37
	dATP	0.1 *M*KPi, pH 7.6	25	1		0.0051	−5.0	− 6.9	− 6.0	37
	AMP	0.1 *M*KPi, pH 7.6	15	1		(0.027)	−6.0	− 6.6	− 2	37
	ADP	0.1 *M*KPi, pH 7.6	15	1			−6.3	−11.2	−17	37
	ADP	0.5 *M*NaCl, 0.1 *M*KPi, pH 7.6	25	1			−5.7	−12.0		37
	3′, 5′cAMP	0.1 *M*NaCl, pH 7.6	25				−4.6	− 3.9	+ 2	37
	Purine riboside	0.1 *M*NaCl, pH 7.6	25				>−2			37

Table 2 (continued)
THERMODYNAMIC PARAMETERS OF NUCLEOTIDE BINDING

Protein[a]	Ligand	Conditions	T(°C)[b]	n[c]	n_{H^+}[d]	K'_c[e]	$\Delta G^{\circ\prime}$[f]	$\Delta H^{\circ\prime}$[g]	$\Delta S^{\circ\prime}$[h]	Ref.
Chicken liver dihydrofolate reductase	NADP+	0.1 *M* KPi, pH 7.4	25	1		0.263[i]	−7.4	−0.6	+22.7	59
	NADPH	0.1 *M* KPi, pH 7.4	25	1	−0.1	3.22[i]	−8.9	−3.4	+18.4	59
	NADPH	0.2 *M* tris, pH 7.4	25	1	−0.1			−4.4		59
	NADH	0.1 *M* KPi, pH 7.4	25	1				0.0		59
	2′-AMP	0.1 *M* KPi, pH 7.4	25					−2.2		59
	2′-P-ADP-ribose	0.1 *M* KPi, pH 7.4	25					−0.3		59
TSase	dUMP	0.05 *M* tris or HEPES: KCl μ = 0.1,[m] pH 7.4	25	1	0.77	0.17	−7.1[n]	−5.4[n]	5.7[n]	15
	FdUMP	00.05 *M* tris or HEPES: KCl μ = 0.1,[m] pH 7.4	25	1	0.95	0.095	−6.8	−4.3	8.5	15
	dTMP	0.05 *M* tris or HEPES: KCl μ = 0.1,[m] pH 7.4	25	1	0.48	0.057	−6.5	0.7	24	15
	UMP	0.05 *M* tris or HEPES: KCl μ = 0.1,[m] pH 7.4	25			<0.0005	>−3.7			15
	dUMP	0.05 *M* bistris or cacodylate μ = KCl 0.1,[m] pH 6.6	25	0.7	0.55	∼0.6	−7.9	−6.6	4	15
	dUMP	0.05 *M* bistris or cacodylate μ = KCl 0.1,[m] pH 5.8	25	0.7	−0.50	∼1.0	−8.2	−3.1	17	15
ATCase	ATP(I)[o]	0.1 *M* Hepes, pH 7.8	25	6	0.32 (0.35)[p]		−5.2[n]	−10.4[n]	−17.4[n]	12
	ATP(I)	0.1 *M* Tris Ac, pH 7.8	25	6	0.32 (0.35)[p]		−5.04	−10.2	−17.2	12
	ATP(I)	0.1 *M* Tris Ac, pH 8.5	25	3	(∼0.28)[p]		−5.56	− 8.9	−11.2	12
	ATP(II)	0.1 *M* Tris Ac, pH 8.5	25	3	(−0.28)[p]		−4.18	− 8.9	−15.8	12

	CTP(I)	0.1 *M* Hepes, pH 7.8	25	3	0.32 (0.35)[p]	−6.9	−13.5	−22.1	12
	CTP(II)	0.1 *M* Hepes, pH 7.8	25	3	0.32 (0.35)[p]	−5.7	−13.5	−26.1	12
	CTP(I)	0.1 *M* Tris Ac, pH 7.8	25	3	0.32 (0.35)[p]	−6.6	−13.3	−22.6	12
	CTP(II)	0.1 *M* Tris Ac, pH 7.8	25	3	0.32 (0.35)[p]	−5.5	−13.3	−26.3	12
	CTP(I)	0.1 *M* Tris Ac, pH 8.5	25	3	(0.28)[p]	−5.56	− 8.9	−11.2	12
	CTP(II)	0.1 *M* Tris Ac, pH 8.5	25	3	(0.28)[p]	−4.18	− 8.9	−15.8	12
RNase A	3′-CMP	NaAcetate 0.05 *M*, pH 5.5	25	1	0.036	−6.19	−15.3	−30.6	48,55
	3′-CMP	0.15, pH 5.5	25	1	0.039	−6.24	−13.2	−23.5	48,55
	3′-CMP	0.50, pH 5.5	25	1	0.0053	−5.11	− 9.2	−14.0	48,55
	3′-CMP	1.20, pH 5.5	25	1	0.0015	−4.62	− 7.5	−10.7	48,55
	3′-CMP	3.00, pH 5.5	25	1	0.0021	−4.53	− 6.0	− 4.9	48,55
	3′-CMP	KCl 0.05 *M*, pH 5.5	25	1	0.038	−6.21	−15.8	−29.5	48,55
	3′-CMP	0.50, pH 5.5	25	1	0.0063	−5.20	− 9.8	−15.4	48,55
	3′-CMP	1.20, pH 5.5	25	1	0.0032	−4.64	− 7.0	−11.3	48,55
RNaseA	Cytidine	μ = 0.05,[m] pH 6.5	25	1	0.057	− 5.1[q]	−6.1[q]	− 3.0[q]	48,55
	Phosphate	μ = 0.05,[m] pH 6.5	25	1	53.5	−10.5[q]	−0.3[q]	36[q]	48,55
	Phosphate	μ = 0.05,[m] pH 5.5	25	1	8.3	− 9.4[q]	+ 0.1[q]	31[q]	48,55
	3′-CMP	μ = 0.05,[m] pH 4—6.5	25	1	89	−10.8[q]	−7.1[q]	12[q]	48,55
	2′-CMP	μ = 0.05,[m] pH 6.5	25	1	680	−12.0[q]	−6.6[q]	18[q]	48,55
	2′-CMP	μ = 0.05,[m] pH 5.5	25	1	409	−11.7[q]	−6.0[q]	19[q]	48,55
Phosphorylase b	AMP	0.04 *M* glycerophosphate, 0.1 *M* KCl, 0.03 *M* β-ME, pH 6.9	25	2 (per dimer)		−4.9	−13.2[r]	−27.9	60
	AMP	0.04 *M* glycerophosphate, 0.1 *M* KCl, 0.03 *M* β-ME, pH 6.9	18	2 hi affinity		−5.2	+ 2.6[r]	+ 26.8	60
	IMP	0.04 *M* glycerophosphate, 0.1 *M* KCl, 0.03 *M* β-ME, pH 6.9	25	2		−3.9	−11.1[r]	−24.2	60
	IMP	0.04 *M* glycerophosphate, 0.1 *M* KCl, 0.03 *M* β-ME, pH 6.9	18	2		−4.1	−11.1[r]	−24.1	60

Table 2 (continued)
THERMODYNAMIC PARAMETERS OF NUCLEOTIDE BINDING

Protein[a]	Ligand	Conditions	T(°C)[b]	n[c]	n_{H^+}[d]	K'_c[e]	$\Delta G°'$[f]	$\Delta H°'$[g]	$\Delta S°'$[h]	Ref.
met-Hb	ATP	0.1 *M* KCl, pH 6.2	5	10		0.00021	−2.94	−6.8	−13.7	47
	ATP	0.1 *M* KCl, pH 6.2	10	10		0.00018	−2.89	−6.8	−13.6	47
	ATP	0.1 *M* KCl, pH 6.2	14	10		0.00015	−2.84	−6.8	−13.6	47
	ATP	0.1 *M* KCl, pH 6.2	21	10		0.00011	−2.71	−6.8	−13.7	47
	ATP	0.1 *M* KCl, pH 7.2	5	4		0.00027	−3.08	−6.8	−13.6	47
	ATP	0.1 *M* KCl, pH 7.2	17	4		0.00016	−2.67	−6.8	−14.4	47
Glutamine synthetase	AMP	0.02 *M* imidazole, 0.1 *M* KCl, 1.0 m *M* $MnCl_2$, pH 7.07	25	12		0.008	−5.33	−2.0	+11	46
P.H. LDH	NADH	0.2 *M* KPi, pH 7.0	25	4.0		0.260	−7.39	−10.6	− 10.8	61
P.H. LDH	NAD^+	0.2 *M* KPi, pH 7.0	25	4.0		0.00308	−4.76	− 6.1	− 4.5	61
Aldolase	Pi	tris, HEPES, or diethylmalonic acid, μ = 0.013,[m] pH 7.5	37	6.6	1.2	0.00040	−3.7	−21.2	− 56.5	42
Aldolase	D-hexitol-1,6-diPi	0.1 *M* Tris	25	2.7—3	1.4	0.21	−7.23	1.3	28.4	42
(Na^+, K^+) ATPase	Pi	0.04 *M* imidazole, pH 6.2	24.5			0.00033	−3.46	−49.5	−154	62

HEAT CAPACITY CHANGE DATA

Protein/ligand	Temperature range (°C)	$\Delta C_p°'$[a]	Ref.
Yeast GPDH/NAD^+	5—40	−522	13
R.M. GPDH/NAD^+	5—25	−225	13
P.S.M. LDH/NADH	5—50	−325	30,36
P.H. LDH/NADH	10—35	−167	61
P.H. LDH/NAD	10—35	− 84	61
GDH/AMP	15—25	+ 80	37
GDH/ADP	15—25	−120	37

Note: Entries in this second section of the table refer to conditions given in the first section.

[a] The abbreviations used are: ADH, alcohol dehydrogenase; B.H. LDH, beef heart lactate dehydrogenase; R.M. LDH, rabbit muscle LDH; P.H. m-MDH, pig heart mitochondrial malate dehydrogenase; H.L. ADH, horse liver ADH; B.L. GDH, beef liver glutamate dehydrogenase; R.M. GPDH, R.M. glyceraldehyde phosphate dehydrogenase; PSM LDH, pig skeletal muscle LDH; TSase, *Lactobacillus casei* thymidylate synthetase; ATCase, aspartate transcarbamylase.

[b] T, temperature at which the data were collected.

[c] n, moles of ligand bound per mole of protein.

[d] n_H^+, moles of H^+ bound per mole of protein (a negative number represents a loss of H^+ upon product formation).

[e] K'_c, binding or association constant, scaled to units of 10^6 M^{-1}.

[f] $\Delta G°'$, kcal mol^{-1}.

[g] $\Delta H°'$, kcal mol^{-1}.

[h] $\Delta S°'$, cal mol^{-1} deg^{-1}.

[i] These parameters (in parentheses) were determined by fluorimetric titration.

[j] Data in this study were calculated from experimental data using $\Delta H°'_{298}$, $\Delta G°'_{298}$, and $\Delta C_p°'$.

[k] These data (in parenthesis) were determined from equilibrium dialysis experiments.

[l] Read this entry as one strong site and three weaker sites. The thermodynamic data refer to the strong site.

[m] μ, ionic strength of the solvent in M units.

[n] All $\Delta H°'$ and $\Delta S°'$ in this study have been corrected for proton effects.

[o] Roman numerals refer to class (*I* or *II*) at site. *Class I + Class II = six sites.*

[p] Numbers in parentheses in n_H^+ column refer to pH stat measurements.

[q] All of these thermodynamic values correspond to binding of the hypothetical *fully ionized* ligand to the hypothetical *fully protonated* enzyme. These values are different from values corrected for proton effects (see note n).

[r] The measured $\Delta H°'$ at 18°C includes a contribution from the ligand induced association, dimer → tetramer. The values for the association are $\Delta G°'$ = −7.1 kcal mol^{-1}, $\Delta H°'$ = −85 kcal mol^{-1}, and $\Delta S°'$ = −268 cal mol^{-1} deg^{-1}. All $\Delta H°'$ values in this study are average van't Hoff measurements based on hydrodynamic data.

[s] $\Delta C_p°'$, cal mol^{-1} deg^{-1}.

The overall conclusion of this rather extensive study is that the energetics of binding of nucleotide and nucleotide analogs to dehydrogenases follow the super-secondary structure features of the NAD^+ binding site. Small differences in the absolute values of binding parameters are attributed to specific individual interactions. It is difficult to assess the validity of this rather sweeping conclusion by Subramanian and Ross. The recent study by Johnson and Rupley[58] does support this conclusion in that the trends in the data obtained by these workers are similar to those observed for the LDH system. While it is unlikely that the similarity of the various trends in these data are simply fortuitous, there is sufficient scatter in the data for one to at least be wary of over-interpretation. Further testing of this hypothesis is necessary using very carefully selected systems.

Subramanian et al.[37] studied the binding of adenosine, AMP, ADP, ATP, and their deoxyribo forms to L-glutamate dehydrogenase calorimetrically, in phosphate buffer, pH 7.6, at 15 and 25°C. ADP and dAMP bind most tightly, with $\Delta G°'$ becoming less negative as the phosphates are added or removed. No significant variation in the thermodynamic parameters was observed between ribo- and deoxy-ribo forms. The binding of ADP and dADP at 25°C produced the most negative enthalpy changes, $\Delta H°' = -12.4$ and -11.8 kcal mol^{-1}, respectively, as well as the most negative entropy changes, $\Delta S°' = -17.0$ and -19.0 cal mol^{-1} deg^{-1}, respectively. Alternatively, AMP and dAMP produced the least negative enthalpy and entropy changes. The variation was attributed partly to two-step binding of ADP in contrast to simple binding for AMP. The authors also examined the binding of ADP in tris buffer, and concluded that complex formation is not accompanied by a net transfer of protons. Their extrapolation of this observation to all other ligands examined may have been unjustified, however, especially since differences in the higher pKs of the phosphate groups exist. A decrease in the binding of ADP due to addition of 0.5 *M* NaCl was also observed. The effect was attributed entirely to a decrease in $\Delta S°'$ since no change in $\Delta H°'$ was detected. In addition, removal of the exocyclic amino group of adenosine led to a significant decrease in binding affinity, suggesting a significant contribution of this group to the binding.

It was concluded that only AMP exhibits "classical hydrophobic bonding."[37] Nevertheless, the significantly negative $\Delta H°'$ as well as the very small, albeit positive, value of $\Delta S°'$ for binding of this nucleotide appears to indicate that other forces also contribute substantially to the interaction. Indeed, ΔC_P calculated from the results of experiments at 15 and 25° is 80 cal mol^{-1} deg^{-1}, an unlikely value for a purely hydrophobic interaction.

Interpretations of binding of aromatic compounds in terms of hydrophobic forces must be tempered in part by reference to the work on thymine. Alvarez and Biltonen[58] argue that hydrophobic bonds do not appear to be a major source of stability (at least in nucleic acids) but that solute-solvent hydrogen bonds do make a major contribution. While the actual situation probably lies between these two extremes, any attempt to rigorously partition the binding free energy between hydrophobic interactions and hydrogen bonds lies in the realm of speculation.

B. Aldolase

Hinz et al.[38] examined the interaction between rabbit muscle aldolase and the substrate-like inhibitor, D-hexitol-1,6-diphosphate, using four different buffer systems, at pH 7.5 and various temperatures. Apparently, 1.4 mol of protons are absorbed when the enzyme is saturated with the inhibitor.[39] Thermodynamic values, corrected for proton transfer, at 25°C are $\Delta G°' = -7.23$ kcal (mol ligand)$^{-1}$, $\Delta H°' = 1.3$ kcal (mol ligand)$^{-1}$, $\Delta S°' = 28.4$ cal (mol ligand)$^{-1}$ deg^{-1}, and $\Delta C_P = -1100$ cal (mol ligand)$^{-1}$ deg^{-1}. The authors offered an intresting rationalization for the large negative value of

ΔC_P. They point to the well-known phenomenon that the reversible unfolding of most proteins is accompanied by a large positive increase in heat capacity. Kauzmann[40] and Tanford[41] have attributed this phenomenon to the exposure of hydrophobic protein residues to the solvent upon unfolding, resulting in an increase in the structuring of nearby water molecules. The large negative ΔC_P observed for inhibitor binding could be due at least in part to the removal from solvent contact of hydrophobic residues when the inhibitor is bound. Loss of degrees of freedom by both the inhibitor and enzyme are also likely to contribute to ΔC_P. However, to be appreciable this would require the loss of very many degrees of freedom per inhibitor bound.

In a more recent study, Crowder et al.[42] examined the interactions of various other substrate analogs with aldolase at 37°C, pH 7.5. In each case, protons were absorbed upon binding of the inhibitor. Binding of phosphate in particular led to some very unusual changes in enthalpy and entropy: $\Delta H^{\circ\prime} = -21.2$ kcal (mol ligand)$^{-1}$ and $\Delta S^{\circ\prime} = -56.5$ cal (mol ligand)$^{-1}$ deg^{-1}. Also, 1.2 mol of protons were absorbed per mole of phosphate. (Crowder et al.[42] also examined the binding of a variety of sugar derivatives. These data lie outside the bounds of this review and have not been included.)

C. Phosphorylase b

Ho and Wang[43] investigated the binding of the activators AMP and IMP to skeletal muscle glycogen phosphorylase b at 18 and 25°C in glycerol phosphate buffer, pH 6.9. For the binding of AMP at 25°C, $\Delta G^{\circ\prime} = -4.9$ kcal mol^{-1} $\Delta H^{\circ\prime} = -13.2$ kcal mol^{-1} and $\Delta S^{\circ\prime} = -27.9$ cal mol^{-1} deg^{-1}. At 18°C both $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are significantly different, changing to $+2.6$ kcal mol^{-1} and $+26.8$ cal mol^{-1} deg^{-1}, respectively. While changes are compensatory, with $\Delta G^{\circ\prime}$ only changing 0.3 kcal mol^{-1}, Ho and Wang suggest that different modes of binding occur at each temperature. The heat per mole of enzyme also increases as the enzyme concentration is increased, but this effect was observed only near 18°C. According to Ho and Wang, this supports the observation that, at 18°C (but not at 25°C) binding of AMP induces an association of phosphorylase b dimers to tetramers, as had been demonstrated by Kastenschmidt et al.[44] In contrast, IMP, which does not induce aggregation, binds with $\Delta G^{\circ\prime} \sim -4.0$ kcal mol^{-1}, $\Delta H^{\circ\prime} = -11.1$ kcal mol^{-1}, and $\Delta S^{\circ\prime} = -24$ cal mol^{-1} deg^{-1} at both temperatures.

A possible point of confusion with this paper is that the molar heat *observed* for the reaction of AMP with the enzyme at 18°C is actually *negative*, and that the value, $\Delta H^{\circ\prime} = 2.6$ kcal mol^{-1}, results from subtraction of the contribution of dimer-tetramer association to the overall heat of reaction. Values derived for the latter association, in the presence of 0.5 m*M* AMP, are $\Delta G^{\circ\prime} = -7.1$ kcal/(mol enz)$^{-1}$, $\Delta H^{\circ\prime} = -85$ kcal/(mol enz)$^{-1}$ and $\Delta S^{\circ\prime} = -268$ cal (mol enz)$^{-1}$ deg^{-1}; the association is therefore entirely enthalpically driven, and is opposed by a very large negative entropy change ($T\Delta S^{\circ\prime} = -78$ kcal mol^{-1}). It is also interesting that the binding of IMP apparently takes place with $\Delta C_P^{\circ\prime} \sim 0$, while the intrinsic binding of AMP produces an apparently large negative $\Delta C_P^{\circ\prime}$. It is also noted that both AMP and IMP produce changes in the phosphorylase b conformation upon binding. Hence, the data suggest that these changes are similar at 25°C but not at 18°C.

It was noted that differences in $\Delta H^{\circ\prime}$ between AMP and IMP binding ($\Delta(\Delta H^{\circ\prime}) = -2.1$ kcal mol^{-1}) at 25°C could be accounted for by the fact that AMP contains an amino group reported capable of participating in hydrogen bonding to the enzyme (Okazaki et al.[45]). This argument, however, should be examined in the context of our brief discussion of the hydrogen bond.

D. Glutamine Synthetase

Ross and Ginsberg[46] examined the binding of the feedback inhibitors AMP and L-tryptophan to glutamine synthetase from *Escherichia coli* in imidazole buffer, at 25°C, pH 7.1. They reported that $\Delta H^{\circ\prime} = -2.0$ and -7.35 kcal mol^{-1} and $\Delta S^{\circ\prime} = 11$ and

-10.7 cal mol^{-1} deg^{-1} for the nucleotide and amino acid, respectively. When both AMP and L-tryptophan were bound to the enzyme, $\Delta H°'$ had the value -9.6 kcal mol^{-1}, suggesting that both effectors can bind simultaneously and independently of each other. AMP binding is entropically driven ($T\Delta S$ = 3.3 kcal mol^{-1}), although the small decrease in enthalpy also aids in the binding.

It should be noted that the authors made no conclusions about proton transfer during the binding process. In view of the large ionization enthalpy of imidazole (8.6 kcal mol^{-1}), uncertainty of proton transfer markedly affects any interpretation of the reported data.

E. Methemoglobin

The binding of ATP to human methemoglobin in KCl from 5 to 21°C at pH 6.2 and 7.2, using ultracentrifugation was investigated by Janig et al.[47] $\Delta H°'$ was derived by van't Hoff analysis (Equation 10). Under all combinations of temperature and pH investigated, $\Delta G°' \sim -3$ kcal mol^{-1}, $\Delta H_{vH}°' \sim -7$ kcal mol^{-1} and $\Delta S°' \sim -14$ cal mol^{-1} deg^{-1}. While many apparently nonspecific binding sites were observed at pH 6.2, binding at pH 7.2 was more straightforward, with four seemingly identical and independent ATP binding sites observed at this pH. The study is particularly interesting in that, in contrast to the other studies discussed, $\Delta H_{vH}°'$ and $\Delta S°'$ are independent of temperature from 5 to 21°C. It will be very interesting to compare $\Delta H_{vH}°'$ with calorimetrically determined $\Delta H°'$ values over the same temperature range when the calorimetric data become available.

The authors also note that ATP binding is accompanied by a change in the ionization states of both nucleotide and protein and results in the binding of protons. The contribution of the ionization reactions to $\Delta H°'_{vH}$ under the unbuffered conditions employed is difficult to interpret in view of the self-buffering capacity of proteins.

F. Aspartate Transcarbamylase

Using both equilibrium dialysis and flow microcalorimetry, Allewell et al.[12] examined the binding of the feedback inhibitor, CTP, and the activator, ATP, to *E. coli* aspartate transcarbamylase. Experiments were performed in tris-acetate and HEPES buffers, pH 7.8 and pH 8.5, at 25°C. Binding of both nucleotides was accompanied by the binding of protons. The number of moles of protons bound per mole of nucleotide bound increased with decreasing pH, from 0.28 at pH 8.3 to 0.35 at pH 7.8, as measured by direct pH-stat titration. At pH 6.74, at a nonsaturating concentration of CTP, 4.1 mol of protons were absorbed per mole of enzyme.

Binding of both ATP and CTP was described as involving two different classes of sites. All $\Delta H°'$ and $\Delta S°'$ values for the binding of both nucleotides to both classes of sites, corrected for proton transfer, were in the approximate ranges, -9 to -13 kcal (mol nucleotide)$^{-1}$ and -17 to -25 cal (mol nucleotide)$^{-1}$ deg^{-1}, respectively. All interactions are therefore enthalpy driven, with the entropy change being unfavorable. The large negative entropy changes were interpreted as consistent with a decrease in the flexibility of the enzyme upon nucleotide binding.

The authors noted that the binding of nucleotides to proteins in general results in significant negative values for both $\Delta H°'$ and $\Delta S°'$. This should be quite apparent from the studies reviewed thus far. They also noted that two exceptions to this trend are the binding of AMP to glutamine synthetase and to phosphorylase b, as previously discussed, and they suggested that these anomalies stem from the known ability of AMP to induce changes in quaternary structure in these systems. However, as was pointed out earlier, the value, $\Delta H°'$ = 2.6 kcal (mol^{-1}), reported by Ho and Wang[43] for ATP binding to phosphorylase b at 18°C was obtained from the observed $\Delta H°'$, which was then corrected for tetramer formation by the enzyme. The *observed* $\Delta H°'$ for this in-

teraction was large and negative, as judged by their Figure 1 (Ho and Wang[43]). Also, the reader will recall that in the study of AMP binding to glutamine synthetase by Ross and Ginsberg,[46] where the *observed* $\Delta H^{\circ\prime} = -2.0$ kcal mol^{-1} and $\Delta S^{\circ\prime} = 11$ cal mol^{-1} deg^{-1}, proton transfer was not ruled out. Hence, the suggestion of Allewell et al.,[12] i.e., that a small $\Delta H^{\circ\prime}$ of ligand binding may be characteristic of systems which undergo changes in quaternary structure, remains speculative and not critically tested at this time.

G. Ribonuclease A

Perhaps the most extensive examination of the thermodynamics of protein-nucleotide interactions to date involves the binding of the inhibitor 3′-CMP to ribonuclease A (RNase). Bolen et al.[48] examined calorimetrically the effect of ionic strength on the binding in unbuffered acetate and KCl solutions at pH 5.5, 25°C. No differences were observed between acetate and KCl but salt concentration (ionic strength) was a factor. At low sodium acetate concentration (0.05*M*), $\Delta H^{\circ\prime} = -15.3$ kcal mol^{-1} and $\Delta S^{\circ\prime} = -30.6$ cal mol^{-1} deg^{-1}. As the salt concentration increased to 3.0 *M*, $\Delta H^{\circ\prime}$ also elicited a significant increase to −6.0 kcal mol^{-1}. The increase in $\Delta H^{\circ\prime}$ was counteracted by a simultaneous increase in $\Delta S^{\circ\prime}$ to −4.9 cal mol^{-1} deg^{-1} under the same conditions. As a result of this entropy-enthalpy compensation $\Delta G^{\circ\prime}$ varied by only 1.7 kcal mol^{-1}, changing from −6.2 kcal mol^{-1} at low salt concentration to −4.5 kcal mol^{-1} at high salt concentration.

RNase contains four histidine residues. Meadows and Jardetzky[49] in an elegant nuclear magnetic resonance study, determined that the pKs of two of these residues increased in the presence of 3′-CMP. These are histidine 12, which exhibits an increase from 6.2 to 7.0, and histidine 119 which exhibits an increase from 5.8 to 7.4. A model of the binding was offered to explain these results. According to the model, histidines 12 and 119 lie in or near the nucleotidyl phosphate locus of the active site. Upon binding, a strong electrostatic interaction is formed between both histidines and the nucleotidyl phosphate. This would result in an increase in the pKs of both histidines, as observed, as well as a decrease in the pK of the nucleotidyl phosphate. The model requires that the phosphate group be in the dianionic form to be most effective in binding, and that one or both histidines must be protonated. It predicts that the binding of 3′-CMP should be strongly pH dependent, falling off at high pH, where histidine protonation becomes unfavorable, as well as at low pH, where nucleotidyl phosphate ionization becomes unfavorable. This is confirmed by earlier data (Cathou and Hammes)[50] showing maximal binding at pH 5.6 as well as a decrease in binding with increased pH which parallels the titration of histidines 12 and 119. However, the low pH maximum for 3′-CMP binding suggests the interaction of yet another ionizable group.

In a related study, Roberts et al.[51] reported thermal data for the ionization of histidines 12, 119, and 105. From the temperature dependence of the pKs, they determined the van't Hoff enthalpy and entropy changes associated with protonation of these residues. All values were temperature dependent. At 20°C, $\Delta H^{\circ\prime}_{vH,12} = -4.0$ kcal mol^{-1} and $\Delta S^{\circ\prime}_{12} = 15.3$ cal mol^{-1} deg^{-1} for histidine 12, and $\Delta H^{\circ\prime}_{vH,119} = -6.3$ kcal mol^{-1} and $\Delta S^{\circ\prime}_{119} = 5.5$ cal mol^{-1} deg^{-1} for histidine 119. These values also apply at 25°C. At 41°C, the entropy of *deprotonation* of both residues is very large and positive. It was suggested that this could represent a slight unfolding of the protein at this temperature as the histidine protons are released. This is supported by Hirs,[52] who noted a localized unfolding of the enzyme involving the phosphate binding site.

Using calorimetric and potentiometric techniques, Flögel and Biltonen[53] also examined the proton associated behavior of RNase, as well as that of its 3′-CMP complex. Experiments were conducted at 25°C, $\mu = 0.05$. Although their results vary somewhat

from the data of Meadows and Jardetzky[49] and Roberts et al.,[51] many qualitative similarities exist. The binding of 3′-CMP causes an increase in the pK of histidines 12 and 119 and the corresponding changes in $\Delta G°'$ were attributed solely to increases in $\Delta S°'$, with $\Delta H°'$ remaining essentially unchanged at −6.5 kcal mol^{-1}. Radically different behavior, compared with the other histidines, was reported for histidine 48. Proton binding to this residue was characterized by $\Delta H°'_{48} = -24$ and −20 kcal mol^{-1} and $\Delta S°'_{48} = -50$ and −35 cal mol^{-1} deg^{-1} for the free and complexed enzyme, respectively. They concluded that the unusual heat of protonation associated with this residue is due to a rearrangement of the conformation of the enzyme between ionization states. They also concluded that only the dianionic phosphate form of 3′-CMP binds appreciably to RNase in the pH range 4 to 9.

In two related papers (Flögel and Biltonen,[54] Flögel et al.[55]) attempts were made to rationalize the enthalpy and entropy changes associated with binding of 3′-CMP, in terms of the corresponding changes associated with histidine protonation. The principal driving force for the hypothetical binding of dianionic 3′-CMP to RNase in which all histidines are unprotonated was estimated to be a negative enthalpy change. This is opposed by a negative unitary entropy change. Insofar as both hydrophobic and electrostatic interactions are characterized by positive entropy changes, it was concluded that binding of the ribonucleoside moiety is stabilized predominantly by van der Waals interactions. On the other hand, the hypothetical binding of dianionic 3′-CMP to RNase in which all histidines are fully protonated is characterized by an enthalpy change of −6.7 kcal mol^{-1} and a unitary entropy change of 14 cal mol^{-1} deg^{-1}. On this basis, it was concluded that the primary driving forces for the binding reaction are electrostatic interactions between the phosphate group of the nucleotide and enzyme histidines, and van der Waals interactions between the protein matrix and the riboside moiety. Binding studies using inhibitor analogs support these conclusions (Flögel et al.[55]).

H. Thymidylate Synthetase

The interactions of nucleotides and their analogs with thymidylate synthetase (TSase) has been studied by Beaudette et al.[14,15] with an emphasis on determining a set of energy parameters for the binding reactions at 25°C. The enzyme used was isolated from *Lactobacillus casei* and the small molecules investigated included dUMP, FdUMP, dTMP, and UMP. Most of the data were collected at pH 7.4 in a variety of buffers of different $\Delta H°_{ion}$ to allow the determination of n_{H^+}.

The binding of dUMP to the enzyme at pH 7.4 is best described by the reaction:

$$\text{dUMP} + \text{TSase} + 0.77\ \text{H}^+ \rightleftarrows \text{dUMP–TSase}^+$$

for which $\Delta G°' = -7.1$ kcal mol^{-1}, $\Delta H°' = -5.4$ kcal mol^{-1} and $\Delta S°' = 5.7$ cal mol^{-1} deg^{-1}. As the pH of the medium is lowered the proton flux changes such that at pH 5.8, 0.5 mol of protons are *lost* per mole of dUMP bound. At pH 7.4, the predominant driving force of the binding is enthalpic; as the pH is lowered, entropic contributions become increasing more important.

The binding of FdUMP to TSase was interpreted to have a stoichiometry of 2:1[14] based on both binding and calorimetric studies. This figure was later revised[15] to 1:1 and is now consistent with the binding of dUMP. The best interpretation of all calorimetric data for this system suggests that at pH 7.4 the binding is described by the equation:

$$\text{FdUMP} + \text{TSase} + 0.95\ \text{H}^+ \rightleftarrows \text{FdUMP–TSase}^+$$

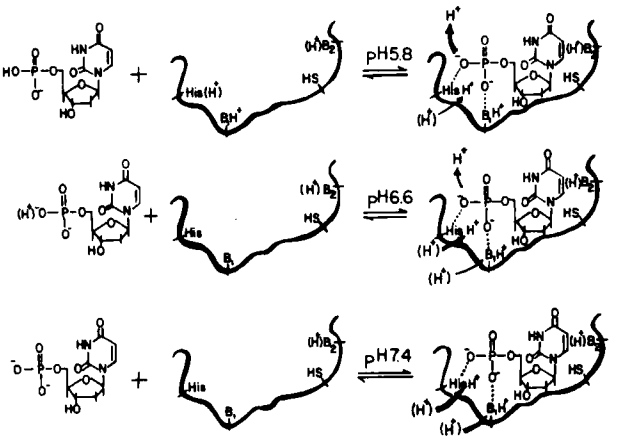

FIGURE 3. Model illustrating the binding of deoxyuridine monphosphate to a subunit of thymidylate synthetase at various pH values. B_1 and B_2 represent potential ionizable enzyme residues. His- represents a histidine residue proposed to lie in the phosphate locus of the nucleoide binding site. SH- is the active-site cysteine. Arrows indicate the direction and magnitude of the transfer of protons to or from the buffer. (From Beaudette, N. V., Langerman, N., and Kisliuk, R.L., *Arch. Biochem. Biophys.*, 179, 272, 1977. With permission.)

The thermodynamic parameters for this reaction are $\Delta G^{\circ\prime} = -6.8$ kcal mol^{-1}, $\Delta H^{\circ\prime} = -4.3$ kcal mol^{-1}, and $\Delta S^{\circ\prime} = 8.5$ cal mol^{-1} deg^{-1}. The similarity of these sets of parameters for dUMP and FdUMP is reminiscent of the situation for the NAD/NADH binding to the dehydrogenases or of 3′-CMP binding to RNase.

The binding of dTMP was also examined.[15] The reaction at pH 7.4 is best described as

$$\text{dTMP} + \text{TSase} + 0.48\ \text{H}^+ \rightleftarrows \text{dTMP–TSase}^+$$

with the set of parameters: $\Delta G^{\circ\prime} = -6.5$ kcal mol^{-1}, $\Delta H^{\circ\prime} = 0.7$ kcal mol^{-1}, and $\Delta S^{\circ\prime} = 24$ cal mol^{-1} deg^{-1}. Obviously, this binding is in marked contrast to that of dUMP and FdUMP. The explanation suggested by the authors is that the changes are due to the hydrophobic interaction between the 5-methyl group of dTMP and the protein. This interaction is absent in dUMP. No explanation is yet available for the reduction in the number of protons bound by this system.

No binding was observed, in either of two buffers, for UMP to the enzyme. This is interpreted as strong evidence for no interaction at all, rather than an interaction which is isoenthalpic.

Beaudette et al.[15] use the arguments of Flögel and Biltonen[53,54] to suggest the nature of the forces which are driving the binding of the various small molecules to TSase. From these arguments and from the proton flux data, they postulated a model for the interaction of dUMP (and by analogy, FdUMP) to TSase. Their model is shown in Figure 3. It is important to realize that their model is based on thermodynamic arguments, and to date, no specific information exists implicating histidine side chains in the interaction. The model does, however, suggest several lines of investigation which should be pursued to determine its validity.

V. COMPILATION OF THERMODYNAMIC DATA FOR BINDING

Table 2 presents a summary of a substantial set of data describing the binding of nucleotides and their analogs to a variety of proteins. Of necessity, some limits had to be placed on the data included in this table. Thus, we have limited entries to only those systems for which a value of $\Delta H^{\circ\prime}$ has been reported. The observed thermodynamic parameters are sensitive to the solution conditions as well as the temperature. Thus, a short indication of conditions is also reported. These data must be used judiciously, since frequently no proton correction is made and almost invariably the data refer to conditions other than the thermodynamic standard state.

For convenience, the data have been collected in a systematic notation and set of units. These are listed in the beginning of Table 2. The set of heat capacity data is quite small. For convenience, these data have been collected in a second section at the end of Table 2.

It is quite apparent that the literature dealing with the thermodynamic characterization of just one type of system, i.e., nucleotide-protein interactions, is extensive and diverse. While there are very few data which are blatantly wrong, care must be employed in interpreting this body of information. Thermodynamic parameters have historically been reported with both high precision and high accuracy. This was a result of the inherent accuracy of the experimental methods and availability of sufficiently large samples. This historical trend has not been maintained in the biochemical field because of the difficulty in obtaining large samples. Most biochemical thermodynamic data has a precision of about 10% and must be examined in this light.

Biochemical systems are inherently difficult to define precisely. All thermodynamic parameters must be referenced to a specific process to be meaningful and to avoid the misinterpretations which are possible if the process is not clearly stated. The phosphorylase system[43] is an example of this problem. It is recognized that a change in the quaternary structure of the enzyme occurs concomitant with nucleotide binding, but no definitive data exist to separate the effects. Thus, the data do not refer to simple binding. In the same sense, the work of Beaudette et al.[14,15] on thymidylate synthetase reactions are studied. Failure to do this leads to an erroneous interpretation of the experimental data. It is quite clear at this time that protein-ligand binding studies must be performed in several buffers at constant pH (and temperature and ionic strength).

Most workers in reaction calorimetry will routinely derive entropy change data from their experimental parameters. While this is perfectly legitimate, entropy is a difficult quantity to interpret. The classical textbook interpretation is in terms of "disorder". However, as argued by McGlashan,[56] this interpretation is only true for the special cases of perfect gases, mixtures of isotopes, and crystals at or near absolute zero. All too frequently negative entropy changes are observed in biological systems associated with a significant decrease in the number of degrees of freedom of the system as defined. Using the concept of "disorder" this observation is often interpreted in terms of the release of solvent molecules to bulk solution. However, no good theoretical basis currently exists to support this statement.

A serious difficulty is almost every protein-ligand thermodynamic study in the literature is the handling of the standard state. The generally accepted standard state for biological systems is the hypothetical 1 *M* solution (for all solute species) at a defined pH (usually near neutrality) and temperature (usually in the range of 0 to 50°C). To obtain thermodynamic parameters referenced to this standard state requires either an extrapolation to infinitely dilute solute concentration or some knowledge about the magnitude of solute activity coefficients. When this problem is acknowledged, (see Beaudette et al.[15]) it is usually stated that all solutes are assumed to behave ideally. Rarely, as in the work of Biltonen et al.[55] is the problem treated rigorously.

Biochemical thermodynamics is a field which is rapidly maturing. As biological systems are adequately defined, the detailed questions concerning energy changes will become amenable to experimental investigation. The methods are not difficult to employ and the information content in each experiment is large. The various studies of nucleotide binding clearly illustrate the variety of systems which can be studied and the information which can be gained.

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