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Enthalpy–Entropy Compensation and Heat Capacity Changes for Protein–Ligand Interactions: General Thermodynamic Models and Data for the Binding of Nucleotides to Ribonuclease A†

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ABSTRACT: General thermodynamic models are presented that can account for the existence of heat capacity changes and compensation between the enthalpy and entropy changes in protein–ligand interactions. The models involve the coupling between some type of transition in the state of the protein (or ligand) and the binding process. The coupled transition may be a proton dissociation, the binding of a second ligand, a change in the degree of aggregation, or a conformational change in either the protein or ligand. Both mandatory coupling and nonmandatory coupling between the binding process and the transition are considered. The model is also extended to include a multistate transition of the protein. Computer simulations show that apparently linear compensation plots (plots of $\Delta H°$ vs. $\Delta S°$) with a slope approximately equal to the experimental temperature are to be expected for the binding of a ligand to a protein when such coupled reactions exist. Also heat capacity changes, which may be either positive or negative, are to be expected to accompany the reaction. Experimental thermodynamic data for the binding of cytidine 3′-phosphate to ribonuclease A are presented. These data demonstrate apparent enthalpy–entropy compensation when pH and ionic strength are varied. A negative heat capacity change, ranging from −145 (at μ = 1.0 M) to −225 cal/(mol·deg) (at μ = 0.05 M), is also observed for this protein–ligand interaction. The apparent compensation and heat capacity change data are interpreted according to the models presented.

The compensation between the enthalpy change, $\Delta H°$, and the entropy change, $\Delta S°$, for the binding of ligands to proteins has been observed for many systems, and the molecular basis for such compensating behavior has been a matter of much interest. An extensive discussion of enthalpy–entropy compensation data and possible interpretations of this effect have been provided by Lumry & Rajender (1970).

Also a number of studies in recent years have demonstrated that the binding of a ligand to a protein is accompanied by a change in the heat capacity, ΔC_p°, for the system (Hinz et al., 1971; Suurkuusk & Wadsö, 1972; Schmid et al., 1976; Niekamp et al., 1977; Fisher et al., 1981). The basis for the heat capacity changes has been proposed to be related to changes in the solvation of the ligand and protein upon binding (Sturtevant, 1977; Eftink & Biltonen, 1980a).

In this report, we discuss some simple models that provide a possible explanation of both enthalpy–entropy compensation and heat capacity changes in terms of ligand-induced changes in the state of the protein. We will use these models in considering thermodynamic data for the interaction of cytidine 3′-phosphate (3′-CMP)[1] with ribonuclease A (RNase A).

Theory

Studies suggest that the globular structure of proteins in solution is quite fragile, being marginally stabilized by a large number of individually weak intramolecular interactions (hydrogen bonds, van der Waals contacts, etc.) (Lumry & Biltonen, 1969; Lumry & Rosenberg, 1976; Ikegami, 1977). Rapid fluctuations in the structure of proteins have been sensed by a number of techniques. These fluctuations may involve the rotation of side chains, vibration of bonds, and the making/breaking of hydrogen bonds and van der Waals contacts. As a result of these structural fluctuations, a protein can be considered a macroscopic ensemble of a large number of closely related microstates.

Proteins may also exist in different macroscopic structural states, each macrostate having a distinctly different folding pattern (in at least a part of the structure). The interconversion of macrostates [Lumry & Biltonen's (1969) "refolding transition"] can be considered to be a two-state process and may occur slowly [an example is the alkaline transition of α-chymotrypsin (Stoesz & Lumry, 1978)]. Different states of aggregation of a protein (i.e., monomer ↔ dimer equilibrium) may also exist. In addition to these different structural states, a protein may also exist in various states of protonation. All of the different states (structural, aggregation, protonation)

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[1] Abbreviations: 3′-CMP, cytidine 3′-phosphate; 2′-CMP, cytidine 2′-phosphate; RNase A, bovine pancreatic ribonuclease A; μ, ionic strength.

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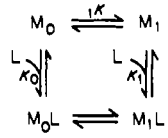
Scheme I



of a protein may be characterized by a different enthalpy level.

The primary function of proteins is to interact with other molecules (ligands), whether the molecule is a substrate, transition state, effector, hormone, ion, lipid, nucleic acid, or another protein. If different states of a protein exist, it is likely that they will have different abilities to interact with ligand. The ligand, by preferentially binding to some specific state(s) of the protein, may affect a shift in the distribution of the states of the protein. When the delicate equilibria are perturbed in this manner, the total intrinsic Gibbs energy available for the interaction between the ligand and the protein (i.e., the Gibbs energy for binding to the optimal state of the protein) will be partitioned among these various modes (Weber, 1972). Thus, the observed Gibbs energy change for ligand binding will be more positive than the intrinsic Gibbs energy change. Since only sensitive equilibria can be perturbed, however, the deficit between the observed and the intrinsic Gibbs energy change will be small. However, contributions made by the coupled equilibrium shifts to the observed entropy change and enthalpy change (and the temperature dependence of the enthalpy change) may be quite significant.

Below we present models which describe how such coupled equilibrium shifts may contribute to the observed thermodynamic parameters and may give rise to enthalpy–entropy compensation and heat capacity changes for protein–ligand interactions.

Mandatory and Nonmandatory Coupling between a Two-State Transition and the Binding of a Ligand. Consider a macromolecule which exists in equilibrium between two states, M_0 and M_1 (which may differ in their structure, degree of aggregation, state of protonation, etc.). Further, as illustrated in Scheme I, consider ligand, L, to bind to these two states of the macromolecule with association constants K_0 and K_1. In general, since the two states of the macromolecule differ, it is likely that these two association constants will also differ from one another. It will be convenient to introduce the parameter γ as the ratio of the two association constants (i.e., $\gamma = K_1/K_0$). If $\gamma = 0$, the ligand binds only to the M_0 state of the macromolecule, and there is a *mandatory* coupling between the binding of ligand and the two-state $M_0 \leftrightarrow M_1$ transition. If $\gamma > 0$, the ligand is able to interact with both states of the macromolecule to a certain extent, and a mixture of M_0L and M_1L complexes will form. In this case the coupling between the binding process and the two-state transition can be considered to be *nonmandatory*.

The apparent ligand association constant, K(app), for the above system is given by eq 1. From this general expression one can obtain [from the derivative of $-R \ln K$(app) with respect to T^{-1} and the derivative of $-RT \ln K$(app) with respect to T] eq 2 and 3 for the apparent standard enthalpy change, $\Delta H°$(app), and apparent standard entropy change, $\Delta S°$(app), for ligand binding. Also from the temperature derivative of $\Delta H°$(app) one can obtain an expression for the apparent heat capacity change (eq 4). Here we assume that there is no intrinsic heat capacity change for either the two-state transition or the individual binding processes (i.e., $d\Delta H_0^\circ/dT = d\Delta H_1^\circ/dT = d_1\Delta H°/dT = 0$). This assumption may not necessarily be true in a real system, but the point of our model is to demonstrate that heat capacity changes can result from ligand-induced shifts in the equilibria of protein states.

$$K(\text{app}) = \frac{K_0(1 + \gamma_1 K)}{1 + {}_1K} \tag{1}$$

$$\Delta H°(\text{app}) = \Delta H_0^\circ + \frac{\gamma_1 K({}_1\Delta H° + \Delta H_1^\circ - \Delta H_0^\circ)}{1 + \gamma_1 K} - \frac{{}_1\Delta H°\,{}_1K}{1 + {}_1K} \tag{2}$$

$$\Delta S°(\text{app}) = \Delta S_0^\circ + R \ln\left(\frac{1 + \gamma_1 K}{1 + {}_1K}\right) + \frac{\gamma_1 K({}_1\Delta H° + \Delta H_1^\circ - \Delta H_0^\circ)}{T(1 + \gamma_1 K)} - \frac{{}_1\Delta H°\,{}_1K}{T(1 + {}_1K)} \tag{3}$$

$$\Delta C_p^\circ(\text{app}) = \frac{\gamma_1 K({}_1\Delta H° + \Delta H_1^\circ - \Delta H_0^\circ)^2}{RT^2(1 + \gamma_1 K)^2} - \frac{{}_1K({}_1\Delta H°)^2}{RT^2(1 + {}_1K)^2} \tag{4}$$

In these equations ${}_1K$, ${}_1\Delta H°$, and ${}_1\Delta S°$ pertain to the $M_0 \leftrightarrow M_1$ transition and K_0, ΔH_0°, ΔS_0° and K_1, ΔH_1° pertain to the binding of L to states M_0 and M_1, respectively.

In the specific case of mandatory coupling mentioned above (i.e., $\gamma = 0$), the above equations simplify considerably to eq 5–8.

$$K(\text{app}) = \frac{K_0}{1 + {}_1K} \tag{5}$$

$$\Delta H°(\text{app}) = \Delta H_0^\circ - \frac{{}_1\Delta H°\,{}_1K}{1 + {}_1K} \tag{6}$$

$$\Delta S°(\text{app}) = \Delta S_0^\circ - R \ln(1 + {}_1K) - \frac{{}_1\Delta H°\,{}_1K}{T(1 + {}_1K)} \tag{7}$$

$$\Delta C_p^\circ(\text{app}) = -\frac{({}_1\Delta H°)^2\,{}_1K}{RT^2(1 + {}_1K)^2} \tag{8}$$

By combining eq 2 and 3 [solving eq 3 for ${}_1\Delta H°\,{}_1K/(1 + {}_1K)$ and substituting this into eq 2] one obtains the following relationship between $\Delta H°$(app) and $\Delta S°$(app):

$$\Delta H°(\text{app}) = \Delta H_0^\circ - T\Delta S_0^\circ + RT \ln\left(\frac{1 + {}_1K}{1 + \gamma_1 K}\right) + T\Delta S°(\text{app}) \tag{9a}$$

$$\Delta H°(\text{app}) = \Delta G_0^\circ + RT \ln\left(\frac{1 + {}_1K}{1 + \gamma_1 K}\right) + T\Delta S°(\text{app}) \tag{9b}$$

where $\Delta G_0^\circ = \Delta H_0^\circ - T\Delta S_0^\circ$. Note that eq 9 is similar to the expression normally taken to describe enthalpy–entropy compensation, $\Delta H°(\text{app}) = \alpha + T_c\Delta S°(\text{app})$, where T_c is the "compensation temperature" which is usually found to be approximately equal to the experimental temperature.[2] Comparison of this equation with eq 9b indicates that for the above model $\alpha = \Delta G_0^\circ + RT \ln[(1 + {}_1K)/(1 + \gamma_1 K)]$. Again, for the simple case of mandatory coupling between ligand binding and the $M_0 \leftrightarrow M_1$ transition, the relationship between $\Delta H°$(app) and $\Delta S°$(app) obtained from the above model becomes

$$\Delta H°(\text{app}) = \Delta G_0^\circ + RT \ln(1 + {}_1K) + T\Delta S°(\text{app}) \tag{10}$$

[2] In cases where $\Delta H°$(app) and $\Delta S°$(app) are determined from a van't Hoff plot, the experimental temperature refers to the mean harmonic temperature; for calorimetrically determined $\Delta H°$(app) and $\Delta S°$(app), it is the temperature at which the heat effect is measured.

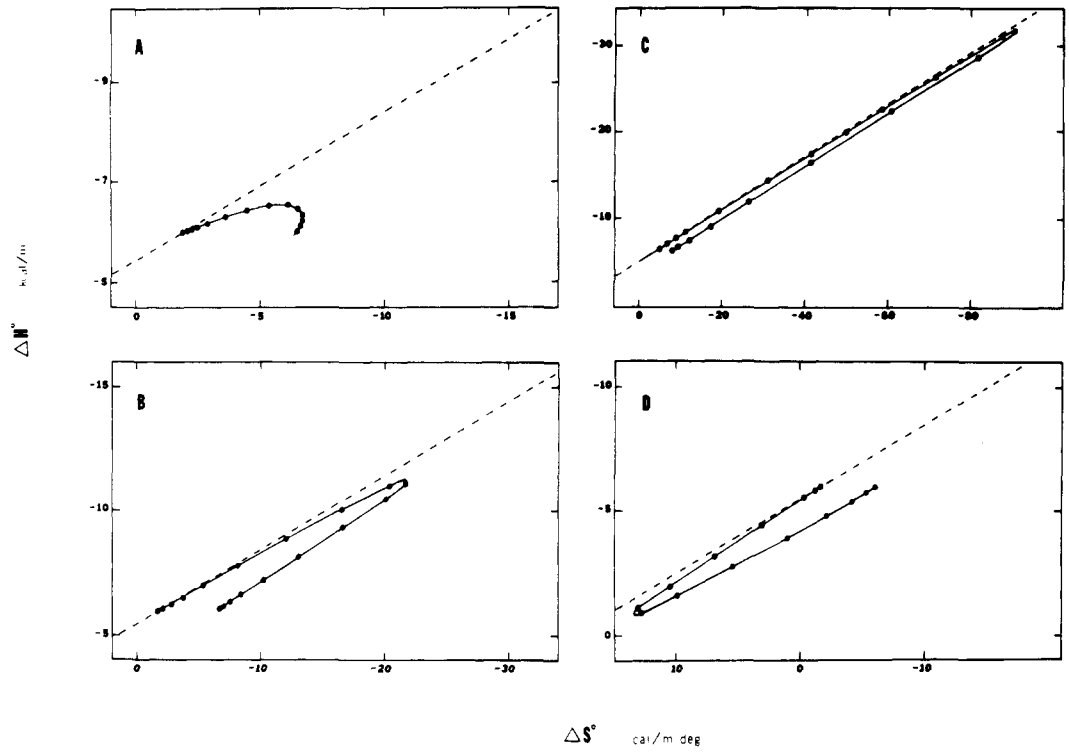


FIGURE 1: Simulated plots of $\Delta H°$(app) vs. $\Delta S°$(app) for the binding of a ligand to a macromolecule in which there is nonmandatory coupling between a two-state transition and the binding process. See Scheme I. K_0 is assumed to be 10^4 M^{-1} (at 298 K) and $\Delta H_0^\circ = \Delta H_1^\circ$ is assumed to be −6 kcal/mol. γ is assumed to be 0.1. The different quadrants of the figure are for different values of ${}_1\Delta H°$, the enthalpy change for the coupled transition, as follows: (A) ${}_1\Delta H° = 1$ kcal/mol; (B) ${}_1\Delta H° = 10$ kcal/mol; (C) ${}_1\Delta H° = 50$ kcal/mol; (D) ${}_1\Delta H° = -10$ kcal/mol. In each quadrant the series of points were generated (using eq 2 and 3) by varying ${}_1K$ from 0.001 to 1000. (The points for smaller ${}_1K$ are closer to the dashed line.) The dashed guideline is for that combination of $\Delta H°$ and $\Delta S°$ giving $\Delta G° = -5.45$ kcal/mol ($=RT \ln K_0$). The slope of the guideline is 298 K. See the text and Scheme I for the identification of symbols. The assumption that $\Delta H_0^\circ = \Delta H_1^\circ$ is made to simplify the data simulation. A more realistic assumption might be for ΔH_1° to be more positive than ΔH_0°, just as ΔG_1° is more positive than ΔG_0°. However, the overall features of the "compensation" plots would be changed only slightly if this were the case.

which is also of the form of the compensation equation $\Delta H°(\text{app}) = \alpha + T\Delta S°(\text{app})$ with $\alpha = \Delta G_0^\circ + RT \ln (1 + {}_1K)$.

The above model and eq 9 and 10 provide a thermodynamic basis for compensation in the apparent enthalpy changes and entropy changes for ligand binding due to an induced shift in the position of a $M_0 \leftrightarrow M_1$ transition upon binding. In particular the above model predicts that enthalpy–entropy compensation will be observed for a system when the binding of a ligand is studied as a function of some condition (pH, ionic strength, macromolecule concentration, etc.) which causes a variation in the value of ${}_1K$. For example in Figure 1 we present some simulated data for a system with nonmandatory coupling (eq 9) in which $\Delta H_0^\circ = \Delta H_1^\circ = -6$ kcal/mol, $\Delta G_0^\circ = -5.45$ kcal/mol ($K_0 = 10^4$ M^{-1}), and $\gamma = 0.1$ (i.e., L binds 10-fold better to M_0 than to M_1). These parameters were inserted into eq 2 and 3 to calculate $\Delta H°$(app) and $\Delta S°$(app) as a function of ${}_1K$. The different quadrants of Figure 1 are for different values of ${}_1\Delta H°$. In each plot ${}_1K$ is allowed to vary from 0.001 to 1000. A dashed guideline is drawn in each plot through those $\Delta H°$, $\Delta S°$ pairs that give $\Delta G_0^\circ = -5.45$ kcal/mol, the intrinsic Gibbs energy change for binding to state M_0 (the slope of the guideline is 298 K). The farther perpendicularly that a point lies below this guideline, the poorer the binding of the ligand to the macromolecule.

Note the characteristic hairpin curve pattern observed in these simulations. At low ${}_1K$, L binds predominantly to the existing state of the macromolecule, M_0, with $\Delta H°(\text{app}) = \Delta H_0^\circ$; at high ${}_1K$, L binds to the existing M_1 state with $\Delta H°(\text{app}) = \Delta H_1^\circ$. At intermediate ${}_1K$ values, L will bind to both states, but since the affinity for M_0 is greater, ligand binding will induce a $M_1 \rightarrow M_0$ transition and the $\Delta H°$(app) will include a contribution from ${}_1\Delta H°$. Such hairpin curves have been experimentally observed for the binding of ligands to hemoglobin (Anusiem et al., 1966, 1968), chymotrypsin (Lumry & Rajender, 1970), and ribonuclease (see following data section) when, in each case, pH is varied.

The limbs of these curves (the ${}_1K = 0$ to $1/\gamma$ limb and the ${}_1K = 1/\gamma$ to ∞ limb, where the apex of the curve occurs at ${}_1K = 1/\gamma$) can appear to be approximately linear, particularly when ${}_1\Delta H°$ is large. The slopes of these limbs are slightly greater than and less than the experimental temperature (depending also on the magnitude of ${}_1\Delta H°$).

In Figure 2 are simulated $\Delta H°$(app) vs. $\Delta S°$(app) plots for the case of mandatory coupling between the binding of ligand and the $M_0 \leftrightarrow M_1$ transition. The different quadrants of the figure are for different values of ${}_1\Delta H°$, and in each plot, ${}_1K$ is allowed to vary from 0.001 to 1000. Again a dashed guideline of $\Delta G_0^\circ = -5.45$ kcal/mol is drawn (with slope 298 K). Note that the simulated compensation plots again appear linear over large stretches, particularly when ${}_1\Delta H°$ is large (positive or negative). The apparent compensation temperature, T_c, that would be determined from such data for a system with mandatory coupling would be either slightly larger or smaller than the experimental temperature, depending on whether ${}_1\Delta H°$ is positive or negative. Also, the larger the magnitude of ${}_1\Delta H°$, the closer the apparent T_c will be to the experimental temperature.

Above we have considered a thermodynamic basis for enthalpy–entropy compensation in ligand binding resulting from a shift in the macroscopic state of a macromolecule (i.e., shift in ${}_1K$). Experimentally, this equilibrium shift, and the resulting

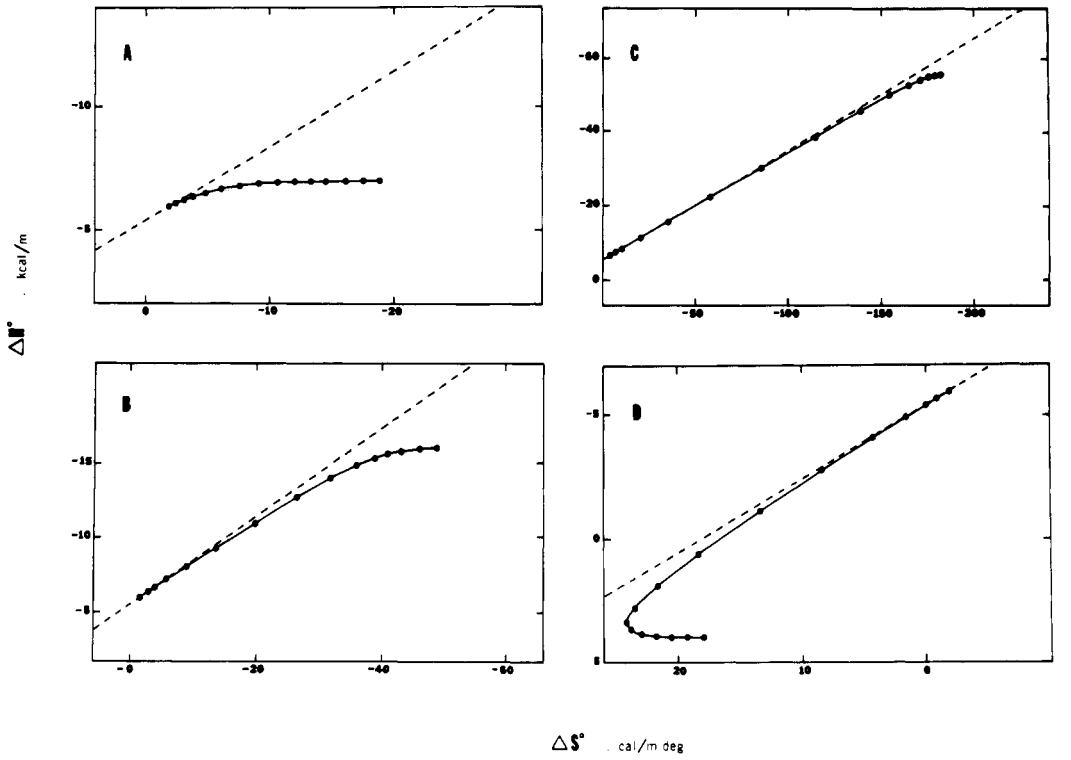


FIGURE 2: Simulated plots of $\Delta H°$(app) vs. $\Delta S°$(app) for the binding of a ligand to a macromolecule in which there is mandatory coupling between a two-state transition and the binding process. See Scheme I and eq 6 and 7. (A) ${}_1\Delta H° = 1$ kcal/mol; (B) ${}_1\Delta H° = 10$ kcal/mol; (C) ${}_1\Delta H° = 50$ kcal/mol; (D) ${}_1\Delta H° = -10$ kcal/mol. K_0 and $\Delta H°_0$ are as given in Figure 1. ${}_1K$ is varied from 0.001 to 1000. See the text for further explanation.

compensation, can be caused by a change in pH, ionic strength, solvent composition, or macromolecule concentration. Compensation has also been frequently demonstrated in studies in which the experimental conditions are fixed and the structure of the ligand is varied (congener series) (Lumry & Rajender, 1970; Belleau & Lavoie, 1968). As the chemical structure of the ligand is changed, the intrinsic thermodynamic parameters ($\Delta H°_0$, $\Delta S°_0$, etc.) for binding to the macromolecule may also change. That linear compensation is observed for such congener series may be coincidental. As a ligand enters the potential surface of a macromolecule's binding site, the more strongly it is bound (the more negative the ΔH of binding), the more its rotational and translational freedom will be restricted (the more negative the ΔS). This "extrathermodynamic" basis for compensation has been discussed previously by such authors as Frank & Evans (1945) and Leffler & Grunwald (1963).

A possible thermodynamic basis for linear compensation for a congener series can, however, be qualitatively demonstrated by using the above nonmandatory ligand binding model. Consider a series of ligands in which each member, i, is characterized by $\Delta H°_{0,i}$, $\Delta H°_{1,i}$, $K_{0,i}$, and γ_i. The variation of γ_i within the series can provide a basis for compensation. Consider the simulated data in Figure 3 in which we assume that $\Delta H°_{0,i} = \Delta H°_{1,i} = -6$ kcal/mol, $\Delta G°_0 = -5.45$ kcal/mol for each ligand, ${}_1K = 8$, and ${}_1\Delta H° = 10$ or -10 kcal/mol. For the series of ligands, γ_i is varied from 0.02 to 0.2 (although each ligand has the same $\Delta H°_0$, $\Delta H°_0$, they will have different overall Gibb's energies for binding due to the variation in γ_i). As can be seen in Figure 3 an apparent linear compensation plot can be generated with a slope just above or below the experimental temperature. The larger the ${}_1\Delta H$ for the $M_0 \leftrightarrow M_1$ transition, the closer the slope of the line will be to the experimental temperature. Therefore a basis for linear enthalpy–entropy compensation can be demonstrated by using a model in which there is nonmandatory coupling between the binding of ligand and a two-state transition in the protein.

For the model presented in Scheme I a change in heat capacity will also be observed for ligand binding (if ${}_1\Delta H° \neq 0$) as described by eq 4 and 8. In Figure 4 we present simulated apparent $\Delta C°_p$ values for systems with mandatory and nonmandatory coupling generated by a variation in ${}_1K$. For the case of mandatory coupling, the $\Delta C°_p$(app) will always be negative and will be of largest absolute magnitude when ${}_1K$ is unity. At this point $\Delta C°_p$(app) is equal to $-{}_1\Delta H°^2/(4RT^2)$, and thus, from the magnitude of $\Delta C°_p$(app) one can estimate the magnitude of ${}_1\Delta H°$. For nonmandatory coupling both positive and negative values of $\Delta C°_p$(app) are predicted.

Note that the $\Delta C°_p$(app) values due to the coupled $M_0 \leftrightarrow M_1$ transition will also be temperature dependent, as we have discussed previously (Eftink & Biltonen, 1980a). Figure 5 illustrates the way in which the $\Delta C°_p$(app), as well as $\Delta H°$(app), for a macromolecule–ligand interaction will vary with temperature as a result of mandatory coupling to a $M_0 \leftrightarrow M_1$ transition of the macromolecule.

Ligand-Induced Multistate Transition. Up to this point we have considered the protein to exist in only two functional states. As discussed at the beginning of this section, evidence suggests that each macroscopic state of a protein can exist as a distribution of rapidly interconverting microstates. If a ligand has a different affinity for the different microstates, a ligand-induced shift in the distribution of microstates will occur, and the $\Delta H°$(app) and $\Delta S°$(app) will include a contribution from this multistate transition.

Before developing a model to describe such a system, let us consider the thermodynamic basis for the existence of these microstates. The partial specific heat capacity, C_p, of globular proteins has been found to be about 0.32 cal/(g·deg) [e.g., for chymotrypsin (Suurkuusk, 1974)]. A large contribution to the C_p must be due to vibrational and rotational contributions from the peptide bonds and residue side chains and from interactions between the protein and water (Kanehisa & Ik-

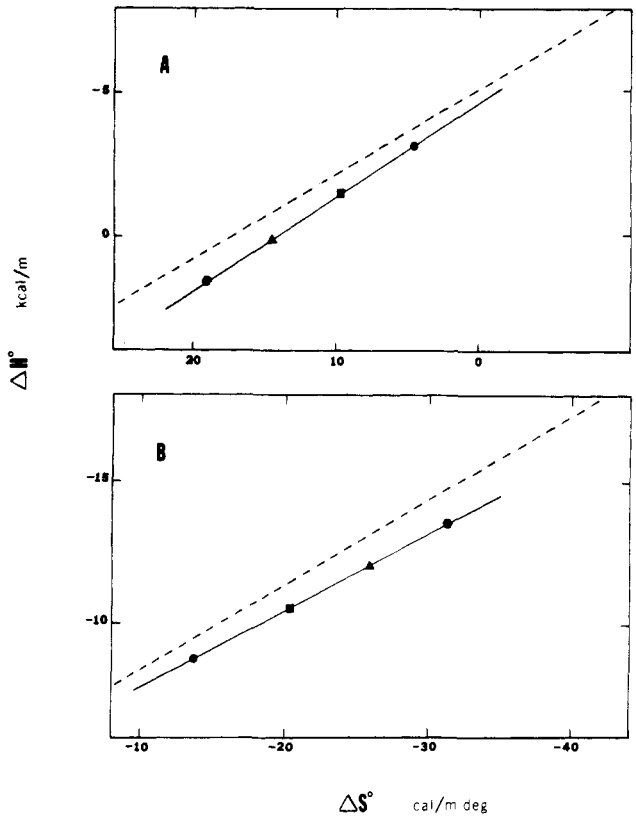


FIGURE 3: Simulated data demonstrating a possible basis for linear enthalpy–entropy compensation for a congener series of ligands binding to a macromolecule. A nonmandatory transition is assumed to be coupled to the binding process. See Scheme I. For each ligand $\Delta H^\circ_0 = -6$ kcal/mol and $\Delta G^\circ_0 = -5.45$ kcal/mol. ${}_1K$ is assumed to be 8 in all cases, and ${}_1\Delta H^\circ$ is equal to −10 kcal/mol in (A) and 10 kcal/mol in (B). For the series of ligands γ_l is varied as follows: (●) $\gamma = 0.2$; (■) $\gamma = 0.1$; (▲) $\gamma = 0.05$; (●) $\gamma = 0.02$. The sopes of the ΔH°(app) vs. ΔS°(app) plots are seen to be just above the experimental temperature (298 K) in (A) and just below in (B).

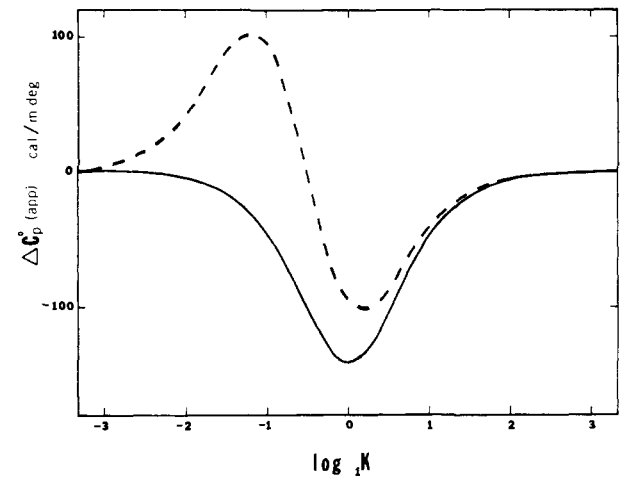


FIGURE 4: Apparent ΔC°_p for the binding of a ligand to a macromolecule in which there is mandatory (—) and nonmandatory (- - -) coupling between a two-state transition (having ${}_1\Delta H^\circ = \pm 10$ kcal/mol) and the binding process. See Scheme I. The dependence of ΔC°_p(app) on the value ${}_1K$ is shown. The data were simulated by using eq 4 and 8 and the parameters given in the legend of Figures 1 and 2.

egami, 1977; Suurkuusk, 1974; Yang & Rupley, 1979). While it is true that the C_p of a protein is, to a first approximation, equal to the sum of the heat capacities of the individual amino acids (Suurkuusk, 1974), it is also likely that a small portion of the C_p of a globular protein is due to the existence of various microscopic states which differ only slightly in terms of the number of intact intramolecular hydrogen bonds or van der Waals interactions.

Conventional reasoning has been that all possible intramolecular interactions, particularly hydrogen bonds, within the globular structure of a protein must be made in order to ensure the stability of the native state (Tanford, 1962; Finney et al., 1980). However, this argument is not entirely sound. The most stable microscopic states of a protein near 298 K may in fact be ones in which a number of intramolecular interactions are broken. Consider a protein having N possible noncovalent intramolecular bonds. For simplicity let all such bonds have an interaction energy, ϵ. Under the assumption that no significant increase in "chain" entropy accompanies the breaking of such an intramolecular bond (a reasonable assumption as long as the number of broken bonds is small and the protein remains in a globular "native" structure), ϵ can also be considered an interaction Gibbs energy. Further let us assume that there is one unique structure of the protein in which all the intramolecular interactions are made. The probability that any individual bond will be broken is $p = \exp[-\epsilon/(RT)]$, and if $\epsilon > RT$, p will be small. However, the probability that one bond is broken in the entire protein is $p_1 = N\exp[-\epsilon/(RT)]$. The degeneracy term, which is N in this case, must be introduced since there are N bonds that may be broken. Even if the probability of breaking an individual bond is small (ϵ is large), the degeneracy term serves to increase the probability of finding one broken intramolecular interaction somewhere within the protein. Similarly the probability that any two (three, etc.) interactions are broken can be described if the appropriate degeneracy term is known. If the interactions are made and broken independently and if each intramolecular interaction is unique in its position in an asymmetric protein, the degeneracy term, ω_i, will be

$$\omega_i = \frac{N!}{(N-1)!i!}$$

The probability of finding a microstate of the protein having i broken intramolecular interactions will then be

$$p_i = \frac{N!e^{-i\epsilon/(RT)}}{(N-i)!i!} \qquad (11)$$

If we assume a reasonable value of $\epsilon = 1.5$ kcal/mol for the average energy of the intramolecular interaction [which may be a hydrogen bond or van der Waals bond; see Kanehisa & Ikegami (1977)] and assume that there are $N = 100$ such interactions in a model protein, the population density of various microstates shown in Figure 6 is obtained for $T = 298$ K. As can be seen, due to the weighting factor (combinatorial entropy term) the most populated microstate of the model protein will not be that in which all of the interactions are made ($i = 0$); instead a microstate having several ($i = 7$ in this case) broken interactions will be the most populated structure. The mean energy of the protein will be 11.2 kcal above that for the most structured ($i = 0$) microstate, and the heat capacity contribution to the protein from the existence of these microstates will be 99 cal/(mol·deg). For a typical 2.5×10^4-dalton molecular weight protein, this value will be 3.5×10^{-4} cal/(g·deg). The existence of microstates resulting from the lability of intramolecular interactions thus would be expected to make a very small contribution (0.1%) to the total heat capacity of a globular protein, as compared to the contribution due to vibrational and rotational energy levels.

One might question the choice of the ϵ and N values selected. As mentioned above the selection of $\epsilon = 1.5$ kcal/mol and $N = 100$ was made from consideration of Kanehisa & Ikegami's (1977) statistical thermodynamic analysis of the unfolding of

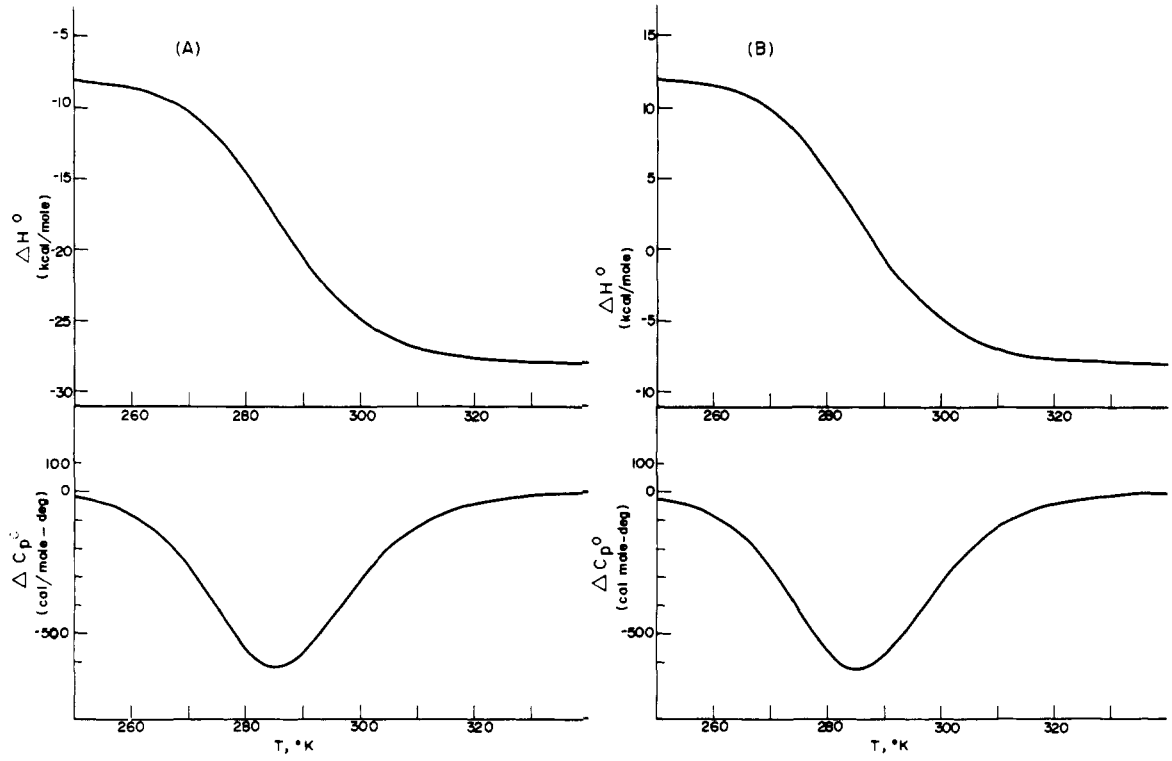


FIGURE 5: Temperature dependence of $\Delta H°$(app) and ΔC_p°(app) for the binding of a ligand to a macromolecule in which there is mandatory coupling between a two-state transition and the binding process. (A) ${}_1\Delta H° = 20$ kcal/mol, ${}_1\Delta S° = 70$ cal/(mol·deg); (B) ${}_1\Delta H° = -20$ kcal/mol, ${}_1\Delta S° = -70$ cal/(mol·deg) (for both cases, ${}_1K = 1$ at 286 K.) In both (A) and (B) it is assumed that $\Delta H_0^\circ = -8$ kcal/mol and $\Delta S_0^\circ = 0$.

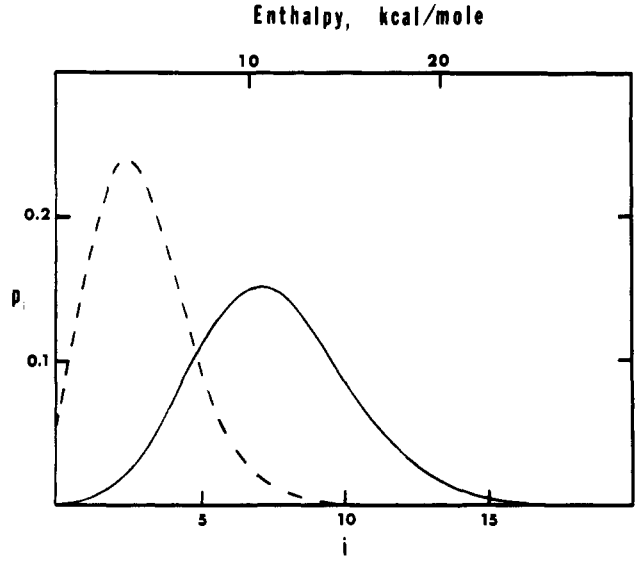


FIGURE 6: Population density of the microstates of a protein, as generated by the model described in the text. The solid line is for protein, and the dashed line is for a protein–ligand complex. In generating the distribution for the protein–ligand complex it is assumed that the ligand binds preferentially to the lower i microstates according to $\gamma = e^{-i}$.

proteins. The enthalpy change for the breaking of a hydrogen bond is usually thought to be about 4 kcal/mol, a value larger than the selected value for ϵ. The enthalpy change for the loss of a van der Waals contact between two groups, on the other hand, would be expected to be less than 1.5 kcal/mol. As pointed out by Finney et al. (1980), many internal intramolecular hydrogen bonds in a globular protein may be distorted, thus requiring less energy to be broken than the above figure of 4 kcal/mol. Also the breaking of certain hydrogen bonds, particularly those involving solvent molecules, may be accompanied by an increase in rotational and/or translational freedom (i.e., increase in entropy). This would result in an increase in the probability of such bonds being broken in spite of the enthalpy change being as large as 4 kcal/mol, thus allowing such bonds to contribute to the heat capacity of a protein. On the basis of these considerations it is our view that the ϵ and N values selected provide a reasonable description of the nature of intramolecular interactions in globular proteins.

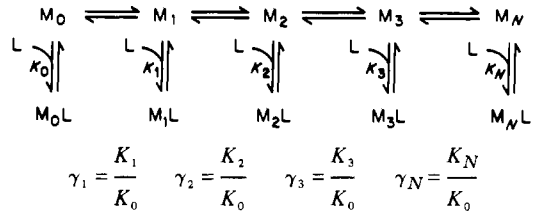
If more than one macrostate of a protein exists, a distribution of microstates for each macrostate would need to be considered.

The statistical mechanical model for a protein presented above is admittedly a simple one. Ikegami (1977) has presented a more developed model which includes cooperativity between neighboring intramolecular interactions. The present model, however, does allow a clear demonstration of the possible effect of a ligand on the distribution of microstates of a protein, as discussed below.

Accepting the existence of such microstates we now address the question of whether they all bind ligand with equal affinity. The binding of a ligand will be critically dependent on the proper alignment of the amino acid residues that constitute the binding pocket of the protein, and slight alterations in the position of these residues would be expected to alter the affinity of the ligand. However, conformational fluctuations in other regions of the protein might be tolerated so long as the binding site is not disturbed. Nevertheless, some variation in ligand binding ability is expected as the number of broken intramolecular bonds increases.

In order to describe the binding of a ligand to a protein possessing a distribution of microstates, consider Scheme II in which M_0, M_1, M_2, ..., M_i refer to different microstates (indexed in terms of their energy, the relative population of each microstate being given by eq 11), and γ_i refer to the relative association constant of ligand to microstate i. Since each microstate is actually a set of structures, the γ_i values

Scheme II



$$\gamma_1 = \frac{K_1}{K_0} \quad \gamma_2 = \frac{K_2}{K_0} \quad \gamma_3 = \frac{K_3}{K_0} \quad \gamma_N = \frac{K_N}{K_0}$$

will be an average over the set. That is, for microstate M_i some structures having i broken intramolecular interactions will have high affinity for ligand. Other structures, for which the broken interactions disrupt the binding site, will have lower affinity for ligand.

From such a model the following expressions can be written for the apparent association constant, enthalpy change, entropy change, and heat capacity change for ligand binding (where the summations are $i = 1$ to N).

$$K(\text{app}) = \frac{K_0(1 + \sum\gamma_i p_i)}{1 + \sum p_i} \tag{12}$$

$$\Delta H°(\text{app}) = \Delta H_0° + \frac{\sum i\gamma_i \epsilon p_i}{1 + \sum\gamma_i p_i} - \frac{\sum i\epsilon p_i}{1 + \sum p_i} \tag{13}$$

$$\Delta S°(\text{app}) = \Delta S_0° + R \ln\left(\frac{1 + \sum\gamma_i p_i}{1 + \sum p_i}\right) + \frac{\sum i\gamma_i \epsilon p_i}{T(1 + \sum\gamma_i p_i)} - \frac{\sum i\gamma p_i}{T(1 + \sum p_i)} \tag{14}$$

$$\Delta C_p°(\text{app}) = \frac{(\sum i^2\epsilon^2\gamma_i p_i)(1 + \sum\gamma_i p_i) + (\sum i\epsilon\gamma_i p_i)^2}{RT^2(1 + \sum\gamma_i p_i)^2} - \frac{(\sum i^2\epsilon^2 p_i)(1 + \sum p_i) + (\sum i\epsilon p_i)^2}{RT^2(1 + \sum p_i)^2} \tag{15}$$

With this model one can demonstrate that the preferential binding of ligand to certain microstates of a protein will lead to a shift in the distribution of the microstates. For example, if the lowest energy microstates are assumed to bind ligand most strongly, with γ_i arbitrarily assumed to decrease with i according to $\gamma_i = e^{-i}$, the resulting ligand-induced shift in microstate distribution will be that shown in Figure 6 (dashed line). The reduction in width of the distribution implies a decrease in heat capacity upon ligand binding. From eq 15 this $\Delta C_p°$(app) can be calculated to be −52 cal/(mol·deg) (a "within-state" heat capacity change). The $\Delta C_p°$ values resulting from such a ligand-induced shift in distribution would be small as compared to the $\Delta C_p°$ values that could result from a two-state transition (Scheme I). Nevertheless the $\Delta C_p°$ values for the present model can be significant.

In addition the ligand-induced shift in microstate distribution would result in a lowering of the mean energy of the protein itself. This decrease in energy would contribute to the apparent $\Delta H°$ for ligand binding, as indicated by eq 13. Furthermore, the distribution of microstates for the M–L complex would be predicted to be less skewed (weighted less toward the higher energy microstates—the third moment of the energy distribution smaller) if the ligand binds preferentially to the lower energy microstates.

Enthalpy–entropy compensation could also result from a ligand-induced shift in microstate distribution if the distribution of the protein can be altered by changing pH, ionic strength, or solvent composition. As these conditions are varied, changes in the distribution of the free protein would not be expected to be large, however, and compensation phenomena resulting from this model would not be anticipated to be as pronounced as that found for the above two-state models.

We have speculated that γ_i decreases with increasing i. This is expected if the lower energy microstates are more structured and present an optimal binding surface for the ligand. However, this may not be the case. It may not be unreasonable for γ_i to increase with i or to go through a maximum or minimum. If two different ligands are able to bind simultaneously to a protein and if the γ_i for each ligand shows a similar dependence on i, positive cooperative binding will occur. If the γ_i for one ligand increases with i and γ_i for the second ligand decreases with i, negative cooperativity will occur (Ikegami, 1977).

For cases in which γ_i decreases with increasing i, the binding of ligand will greatly reduce the population of the higher energy microstates, and it would be expected that this would result in a decrease in the mobility of the protein as judged by such methods as isotope exchange, fluorescence quenching, or other experiments that sense the dynamic character of a protein matrix.

Materials and Methods

Some of the data considered in the following section is taken from the literature. The temperature-dependence data for the RNAse–nucleotide interaction have been presented only in abstract form (Eftink & Biltonen, 1980b), and so the procedures for such experiments are here described.

Phosphate-free, lyophilized RNase A, 3′-CMP, and 2′-CMP were obtained from Sigma Chemical Co. and used without further purification. The protein and ligands were dissolved in 0.05 M sodium acetate buffer solutions. Ionic strength was varied by the addition of NaCl. Protein and ligand concentrations were determined spectroscopically, as described previously (Bolen et al., 1971).

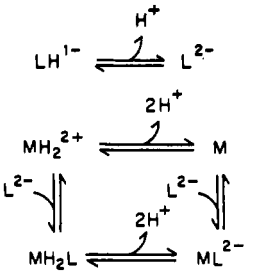
Calorimetric measurements were made on an LKB batch microcalorimeter by using a procedure previously described (Bolen et al., 1971). Heats of dilution of both the ligand and protein were measured and subtracted from the heat of reaction. An electrical calibration was performed at each temperature. The temperature within the microcalorimeter was determined by use of a thermistor probe and Yellow Springs Instrument Co. digital thermometer. The final concentration (after mixing) of the protein was typically about 8×10^{-5} M and that of nucleotide was about 5×10^{-3} M. This nucleotide concentration was sufficient to 98% saturate the protein at 24 °C and 96% saturate the protein at 43 °C. From knowledge of the association constant at pH 5.0 (and its temperature dependence) a small correction to 100% saturation was made.

The pH of the protein and nucleotide solutions was carefully matched by using a Radiometer 4B pH meter. Since the heat of ionization of acetate is approximately zero, the contribution to the observed heat effect due to protonation of the buffer was insignificant. Another feature of the acetate buffer is that the pH of this buffer solution remains practically constant as a function of temperature.

Data and Interpretation

In this section we consider some thermodynamic data for the binding of 3′-CMP to RNase A which appears to demonstrate enthalpy–entropy compensation as pH and ionic strength are varied. We will discuss a thermodynamic basis for the apparent compensation in terms of the coupling between ligand binding and a two-state transition of the protein (the protonation of groups on the protein). Also we will present

Scheme III



studies of the heat capacity change for 3′-CMP binding and will consider various origins of this parameter in terms of the schemes presented above.

Enthalpy–Entropy Compensation in the Binding of 3′-CMP to RNase A. The binding of 3′-CMP to RNase A has been thoroughly studied as a function of pH and ionic strength (Bolen et al., 1971; Flogel & Biltonen, 1975a,b). The pH dependence of the $\Delta G°$(app) and $\Delta H°$(app) for 3′-CMP binding [see Figures 3 and 4 in Flogel & Biltonen (1975b)], along with proton uptake and NMR data, has been used to arrive at Scheme III for the binding of 3′-CMP to RNase A. According to this scheme only the dianionic form of 3′-CMP binds to the protein, and its binding induces a shift in the pK_as of the two active site histidine residues (His-12 and -119) from an average value, $p\bar{K}_a$ = [pK_a(His-12) + pK_a(His-119)]/2, of 5.4 for the free protein to an average value of 7.1 for the complex at an ionic strength of 0.05 M. Also, there is a slight coupling between ligand binding and the dissociation of His-48, but the involvement of this residue in the thermodynamics of ligand binding is minimal and can be neglected.

If the apparent $\Delta H°$ and $\Delta S°$ values for 3′-CMP binding as a function of pH are plotted vs. one another without consideration of trends, a plot (Figure 7A) is obtained that appears to demonstrate linear enthalpy–entropy compensation. Inspection of the data shows that the apparent $\Delta H°$ and $\Delta S°$ values become more negative as the pH is increased from 4 to 6.5 but more positive as pH is increased from 7 to 9 (a hairpin curve). If a correction is made for the dissociation of the phosphate group of 3′-CMP, that is, if one considers the apparent $\Delta H°$ and $\Delta S°$ for the binding of the dianionic form of 3′-CMP over the entire pH range (a manipulation easily done since the pK_a of the phosphate group is known and the heat of protonation of the phosphate is approximately zero), a more obvious hairpin curve is obtained (Figure 7B).

Having previously determined the binding scheme for 3′-CMP, we can relate the origin of the apparent compensation to the pH-dependent shift in the state of protonation of the active site histidine residues. Since the ligand can bind to the state of the protein both in which the histidines are protonated and in which they are unprotonated, but preferentially to the former, the 3′-CMP–RNase A interaction is an example of a system in which nonmandatory coupling exists between ligand binding and a two-state transition in the protein. (Compare Scheme III to Scheme I and see eq 1–4.) The transition of the protein (proton dissociation of His-12 and -119) can be described by an average pK_a of 5.4 and $\Delta H°$ for proton ionization of 13 kcal/mol (6.5 kcal/mol for each histidine). In terms of the parameters used in eq 1–4, $_1K$ equals $10^{-2p\bar{K}_a}/[H^+]^2$, $_1\Delta H°$ equals 13 kcal/mol, and K_0, $\Delta H°_0$, and $\Delta S°_0$ refer to the binding of the dianionic ligand to the diprotonated state of the protein. The greater affinity of the dianionic ligand for the diprotonated state of the protein can be described by a γ value (association constant to unprotonated form/association constant to diprotonated form) of 2.3×10^{-4}.

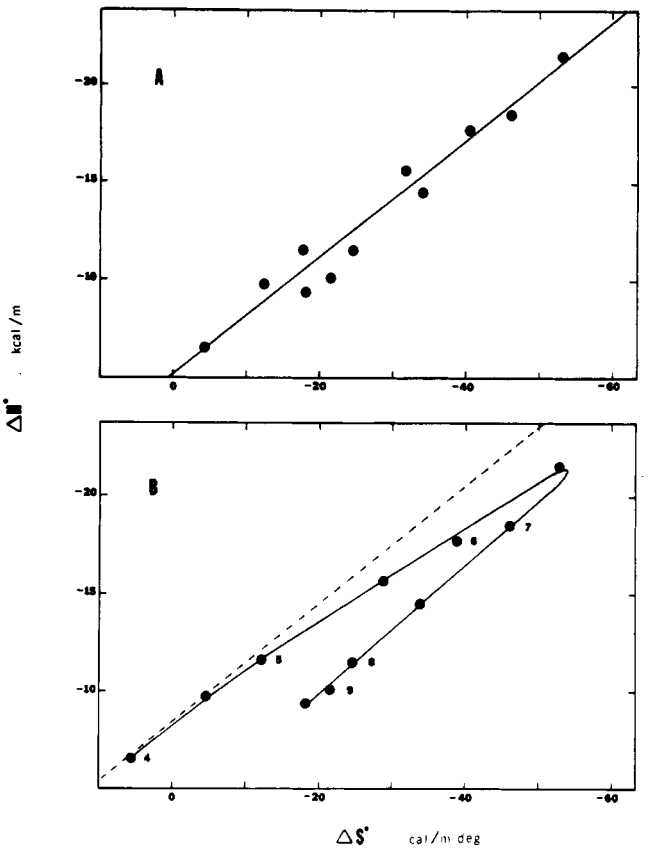


FIGURE 7: (A) Plot of the apparent $\Delta H°$ vs. the apparent $\Delta S°$ for the binding of 3′-CMP to RNase A at ionic strength 0.05 M, 25 °C. Various points are for data from pH 4.0 to 9.0. Data from Flogel & Biltonen (1975a). (B) Plot of $\Delta H°$ vs. the $\Delta S°$ for the binding of the dianionic state of 3′-CMP to the protein. (See the text and the original paper.) pH values are given beside the points in (B).

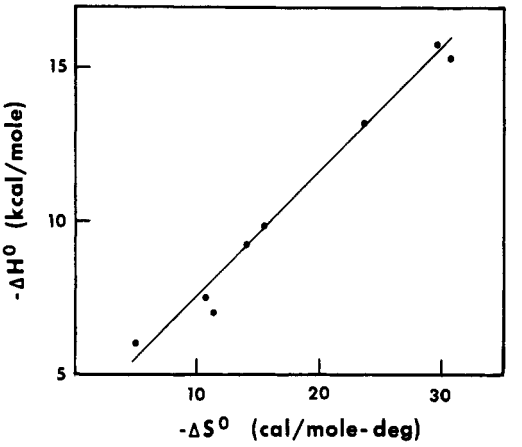


FIGURE 8: Plot of the apparent $\Delta H°$ vs. the apparent $\Delta S°$ for the binding of 3′-CMP to RNase A for data taken as a function of ionic strength. Temperature, 25 °C; pH 5.5. Data from Flogel et al. (1973).

[See Flogel & Biltonen (1975b) for the determined values of the association constants and $\Delta H°$ of binding to both states of the protein.]

Studies of the ionic strength dependence of the binding of 3′-CMP to RNase A have also been performed (Bolen et al., 1971; Flogel et al., 1973). If the $\Delta H°$(app) at pH 5.5 is plotted vs. $\Delta S°$(app) for several ionic strengths, an apparently linear compensation plot is obtained with a T_c = 400 ± 30 K (see Figure 8).

Again the basis for such compensation can be understood by considering the model for nonmandatory coupling along with some additional information concerning the effect of ionic

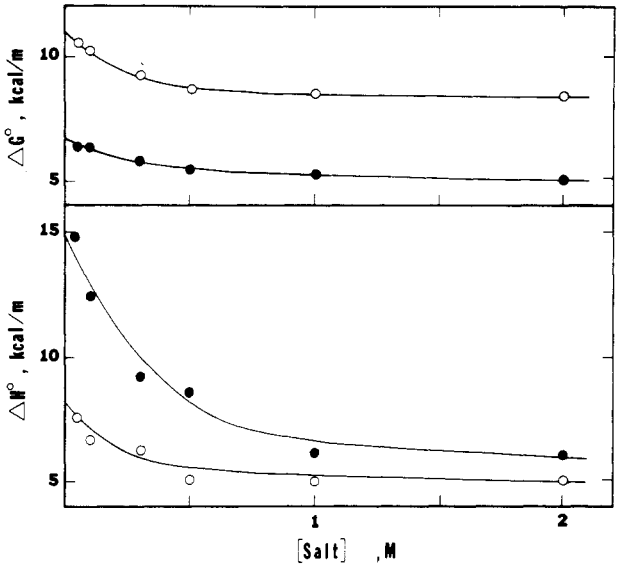


FIGURE 9: Ionic strength dependence of the Gibbs energy change and enthalpy change for the binding of 3′-CMP to RNase A at pH 5.5, 25 °C. The closed symbols are the apparent $\Delta G°$ and $\Delta H°$ for the binding process. The open symbols are the values for the binding of the ligand to the diprotonated state of the enzyme. These "corrected" ΔG_0° and ΔH_0° values were calculated by using eq 4 and 5 of Flogel & Biltonen (1975b), with values of the pK_a for the binding site histidine residues as described in the above text and with the heat of protonation of the histidine residues assumed to be −6.5 kcal/mol.

strength on the pK_as of the two active site histidine residues of RNase A. Increased ionic strength has been found by Ruterjans & Witzel (1969) to increase the pK_a of both active site histidine residues from an average value of 5.4 at an ionic strength of <0.05 M to 6.2 at an ionic strength of 0.4 M. When the Debye–Huckell theory is employed (Parsons & Raftery, 1972), the data of Ruterjans and Witzel can be extrapolated to obtain an average pK_a of 6.5 at an ionic strength of 2.0 M. Thus, in terms of the nonmandatory coupling scheme, an increase in ionic strength leads to a decrease in ${}_1K$ ($=10^{-2pK_a}/[H^+]^2$). In addition to decreasing ${}_1K$, increased ionic strength also affects the magnitude of K_0 (where M_0 is the high-affinity, diprotonated state of RNase A). As shown in Figure 9, ΔG_0° ($=-RT \ln K_0$) becomes more positive by 2–2.5 kcal/mol as the ionic strength increases from 0 to 2 M. The ΔG_0° values were determined from the $\Delta G°$(app) for 3′-CMP binding at each ionic strength by employing the model and equations described in Flogel & Biltonen (1975b) and the $p\bar{K}_a$ values as discussed above. The effect of salt in decreasing K_0 is expected since the additional affinity between the ligand and the diprotonated form of the protein is due to an electrostatic interaction between the dianionic phosphate group of 3′-CMP and the protonated histidine residues of RNase A.

Increased ionic strength thus leads to an increase (more positive) in $\Delta G°$(app) by affecting the magnitude of two equilibrium constants in the nonmandatory coupling scheme. In addition the ionic strength dependence of $\Delta H°$(app) and $\Delta S°$(app) can be understood in a similar manner. On comparing $\Delta H°$(app) for the binding of 3′-CMP with ΔH_0°, the enthalpy change for the binding of 3′-CMP to the diprotonated protein, as shown in Figure 9, one finds that $\Delta H°$(app) is much more sensitive to ionic strength than ΔH_0° due to the contribution made to the former from the linked protonation of the histidines at the protein's binding site. Thus when the effect of ionic strength on both the pK_as of the histidines and the intrinsic Gibbs energy change for the binding of the ligand to the diprotonated protein are taken into account, the basis

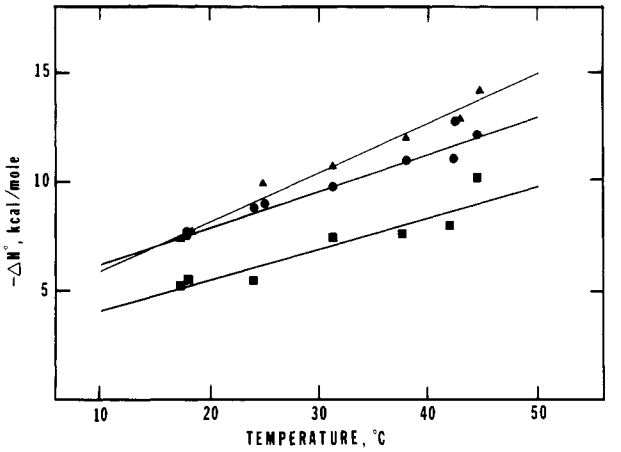


FIGURE 10: Temperature dependence of the apparent enthalpy change for the binding of 3′-CMP to RNase A at pH 5.0. Ionic strengths: 0.05 (▲), 0.2 (●), and 1.0 M (■).

for the apparent enthalpy–entropy compensation for the data at pH 5.5 as a function of ionic strength can be understood in terms of a nonmandatory coupling scheme.

Heat Capacity Changes for the Binding of 3′-CMP to Ribonuclease A. The temperature dependence (18–44 °C) of the apparent $\Delta H°$ for the binding of 3′-CMP to RNase A at pH 5.0 and at three different ionic strengths is shown in Figure 10. The plots appear linear, and the apparent ΔC_p° values are −225 ± 15, −175 ± 15, and −145 ± 25 cal/(mol·deg) at ionic strengths of 0.05, 0.2, and 1.0 M, respectively. Also an apparent ΔC_p° of −160 ± 20 cal/(mol·deg) was determined for the binding of 2′-CMP to RNase A at pH 5.0, ionic strength of 0.2 M.

A number of factors may contribute to the observed negative ΔC_p° values, as has been discussed by Sturtevant (1977) and Eftink & Biltonen (1980a). Under Theory we presented general models to illustrate that any two-state or multistate transition of the macromolecule (or ligand) can be the origin of a heat capacity change for binding. Of particular interest, in terms of our understanding of the functional aspects of proteins, is the possibility that a ligand-induced change in the conformation of the protein (be it two state or multistate) may give rise to a ΔC_p. The difficulty arises, however, in dissecting the contribution form a ligand-induced conformational change from other potential sources of a heat capacity change.

Here we will attempt to analyze the above ΔC_p° values for the binding of 3′-CMP to RNase A by first evaluating the contribution from these other potential sources. After this is done we will argue that any remaining portion of the ΔC_p° value is due to an induced change in the conformation of the protein. Among the other heat capacity change sources to be considered are the following two-state equilibria (which are specific examples of the general arguments of Scheme I): (1) coupled protonic equilibria, (2) coupled change in the state of aggregation of the protein, and (3) coupled change in the conformation of the ligand. In addition, a contribution from (4) changes in the solvation of the ligand and the protein binding site will be considered.

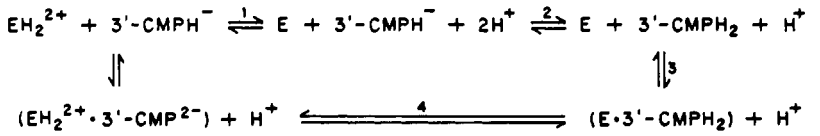
The contribution due to the linked proton dissociation of His residues 12 and 119 at pH 5.0 can be evaluated in terms of Schemes I and III and eq 4. (Actually only the second term on the right hand side of eq 4 needs to be considered in this evaluation since the $\gamma_1 K$ term will be small at pH 5.0.) As listed in Table I, the ΔC_p contributions due to these linked protonic equilibria range from −88 cal/(mol·deg) at μ = 0.05 M to −15 cal/(mol·deg) at μ = 1.0 M. These contributions are actually relatively small in magnitude at this pH. The

Table I: Apparent Heat Capacity Changes for the Binding of 3′-CMP to RNase A and Estimated Contributions to These Values

	cal/(mol·deg) at		
	$\mu = 0.05$	$\mu = 0.2$	$\mu = 1.0$
1. ΔC_p°(app) for 3′-CMP binding	−225 ± 15	−175 ± 15	−145 ± 25
2. estimated ΔC_p° contributions[a]			
a. protonic equilibria: coupled protonation of His-12 and -119 on ligand binding at pH 5.0[b]	−88	−41	−15
b. induced syn → anti transition of the nucleotide[c,d]	−5	−5	−5
c. removal of ligand from water[c]	50	50	50
d. removal of water from the protein's binding site[c]	−25	−25	−25
sum of the above contributions	−68	−21	+5
3. remaining ΔC_p° contribution attributable to an induced protein conformational change	−157	−154	−150

[a] See text for further explanation. [b] Values calculated by using the relationship $\Delta C_p = -\sum_{i=1}^{2}[\Delta H_i^{\circ 2}[H^+]K_i/[RT^2([H]^+ + K_i)^2]] \approx -2\Delta H^{\circ 2}[H^+]\bar{K}/[RT^2([H^+] + \bar{K})^2]$ where $\bar{K}$ is the average acid dissociation constant for His-12 and -119, equal to 4×10^{-6} M, 1×10^{-5} M, and 3.2×10^{-7} M at ionic strength 0.05, 0.2, and 1.0 M, respectively, and ΔH° is the heat of proton ionization of these residues, taken to be 6.5 kcal/mol at each ionic strength. A contribution from the coupled deprotonation of the 4-amino group of the cytosine ring was also considered. The maximum contribution from this source is calculated to be only about −1 cal/(mol·deg) and is thus deemed negligible. [c] The value for the ΔC_p° contribution is assumed to be the same at each ionic strength. [d] As discussed in the text, a negligible contribution of about −1 cal/(mol·deg) may result from an induced imino → amino tautomerization of the cytosine ring upon binding.

Scheme IV



magnitude of the contribution is greatest at low ionic strength due to the fact that the pK_a values of the histidines are closer to pH 5.0, the experimental pH. After this protonic equilibria contribution is subtracted, the remaining ΔC_p° values are approximately the same at each ionic strength, about −130 cal/(mol·deg).

The binding of 3′-CMP to RNase A does not appear to depend on the state of aggregation of the protein. Although aggregated forms of RNase A do exist, no concentration dependence of the ΔH°(app) for 3′-CMP binding has been observed (Bolen et al., 1971). Thus, we include in Table I no contribution from this source to the apparent ΔC_p°.

The ligand 3′-CMP can exist in both a syn and anti conformation with respect to the relative orientation of the pyrimidine and ribose ring due to rotation about the glycosidic bond (Haschemeyer & Rich, 1967). 3′-CMP appears to bind to the protein in the anti conformation (Richards & Wyckoff, 1971). In solution the Gibbs energy change and enthalpy change for the syn → anti transition of 3′-CMP have been determined by NMR studies to be −0.6 and −2.9 kcal/mol, respectively (Lavallee & Coulter, 1973). From these values (and an analogue of eq 8) one can calculate a ΔC_p° contribution of ~−5 cal/(mol·deg) due to this induced change in the conformation of the ligand.

Also the cytosine ring will exist to a very small extent in the imino tautomeric state. If we assume that only the amino state binds, the shift in this tautomeric equilibrium will contribute only ~−1 cal/(mol·deg) to the total ΔC_p°(app). [The equilibrium constant and ΔH° for the imino → amino tautomerization are taken from Lee et al. (1972).]

The transfer of the ligand from water to the binding site of the protein and the removal of water from the binding site may contribute to the apparent ΔC_p° due to the release of electrostricted water or "hydrophobic" water (Edsall & Wyman, 1958; Sturtevant, 1977). For the present system we can estimate these contributions by first considering the transfer of the ligand from an aqueous to a nonaqueous environment and then considering the removal of water from the protein's binding pocket.

Concerning the transfer of the ligand out of water, it has been demonstrated by Alvarez & Biltonen (1973) that the transfer of a pyrimidine ring (thymine) from water to ethanol is characterized by ΔC_p equal to zero. Since thymine is expected to be more hydrophobic than the other pyrimidine rings, we can argue that changes in heat capacity due to the desolvation of the cytosine ring of 3′-CMP will be negligible.

The desolvation of the phosphate group of 3′-CMP (release of electrostricted water) must also be considered. To do this, consider the model shown in Scheme IV in which the binding process is divided into the following steps: (1 and 2) neutralization of both the phosphate group of 3′-CMP (in water) and the two histidines of the free protein, (3) the association of the neutral ligand to the neutral protein (i.e., the transfer of the ligand to the protein's binding site without the desolvation of charged species), and (4) the transfer of two protons from the phosphate of the bound 3′-CMP to the histidine residues. If we assume that there is no heat capacity change associated with the latter two steps of this scheme, we can arrive at an estimate for the contribution of the ΔC_p° due to desolvation of the ligand and the histidine residues at the protein's binding site (steps 1 and 2 of the scheme) from knowledge of the ΔC_p° for the primary acid dissociation of the phosphate of 3′-CMP [assuming the value for phosphoric acid, $\Delta C_p^\circ = -50$ cal/(mol·deg) (Bates & Acree, 1943)] and for the dissociation of the two histidine residues [twice the value for imidazole for which $\Delta C_p^\circ \approx 0$ (CRC Handbook of Biochemistry and Molecular Biology)]. From these values we calculate a ΔC_p° contribution of 50 cal/(mol·deg) for the desolvation of the charged groups. That the desolvation of 3′-CMP makes a minimal or positive contribution to the observed ΔC_p° is also consistent with the data of Bahr et al. (1965) which shows no curvature for a plot of log (solubility) of 3′-CMP vs. $1/T$ (i.e., one can argue that the dissolution of 3′-CMP into water is similar to the dissociation of 3′-CMP from RNase A).

Concerning the removal of water from the protein's binding site, X-ray crystallographic studies by Richards, Wyckoff, and co-workers (1971) on RNase S reveal a few water molecules at the active site region. More may exist within the specificity pocket but may not be localized enough for X-ray analysis. The heat capacity of water molecules hydrating proteins has been found to be higher than that of bulk water. From the study by Yang & Rupley (1979) of the specific heat capacity

of lysozyme as a function of the degree of hydration, one can estimate that water bound to a protein has a heat capacity that is approximately 2.5 cal/(mol·deg) higher than that of bulk water. This is estimated from the difference between the apparent specific heat capacity of fully hydrated lysozyme and that of the dry protein (and dividing by 305, the approximate number of water molecules bound per mole of hydrated protein). If we assume that 10 water molecules are displaced from the RNase's binding site by the riboside portion of the ligand, a ΔC_p° of approximately −25 kcal/(mol·deg) would result.

Upon summing the contributions made by all the above-mentioned sources, we are left with a ΔC_p° value of about −150 cal/(mol·deg) which can then be attributed to a ligand-induced change in the conformation of the protein. Much experimental evidence for the existence of some type of ligand-induced change in the conformation of RNase A has been presented. Marcus and co-workers (1968) found that the susceptibility of RNase A to proteolysis is greatly reduced by the presence of 2′- and 3′-CMP. Other studies have focused on rearrangements in the positioning of the histidine residues at the binding site (Richards & Wyckoff, 1971) and rearrangements in the so-called "hinge" region of the protein that includes His-48 and Tyr-25 (Markley, 1975).

It is our opinion that the ligand-induced conformational change involves a subtle readjustment or tightening of the conformation of the protein such as that described by the multistate model presented under Theory. The linearity of Figure 10 argues against the conformational change being a two-state refolding transition (i.e., compare to Figure 5). A study of particular note, in comparison with our heat capacity change measurements, is that by Martin, Petsko, and co-workers (1978). They have found that the temperature factor of the crystal structure of RNase A decreases throughout the molecule upon the binding of 3′-CMP. Rosa & Richards (1982), on the other hand, have found evidence using the technique of medium resolution hydrogen exchange that suggests that the binding of 2′-CMP to RNase S induces a tightening primarily of the C-terminus region of the protein.

In a separate paper, we will present evidence that the binding of a substrate to RNase A also is characterized by a negative ΔC_p° of similar magnitude, thus suggesting that an induced conformational change is of importance in the functioning of this enzyme (Eftink & Biltonen, 1983).

Conclusions

Enthalpy–entropy compensation and heat capacity changes for protein–ligand interactions can be a manifestation of the fact that sensitive transitions (for which ${}_1\Delta H^\circ \neq 0$) are perturbed during the binding process. Since proteins are known to exist in different structural states and different states of protonation or aggregation (i.e., flucutations occur in the state of proteins), and since these different states are expected to have different affinities for ligand, induced changes in the state of a protein should be characteristic of protein–ligand interactions.

Previous discussions of the molecular basis for both enthalpy–entropy compensation and heat capacity changes for protein–ligand interactions have focused on the participation of water in such reactions (Lumry & Rajender, 1970; Sturtevant, 1977).[3] Arguments have been made that the perturbation, release, or shift in the state of water upon the binding of ligand to a protein is the primary source of compensating enthalpy and entropy changes (with a "T_c" of 250–315 K being diagnostic of the involvement of water) and of heat capacity changes. In our treatment we have discussed in general terms the shift in equilibria of the protein as being the source of the compensation, which can appear linear with a slope near the experimental temperature, and heat capacity changes. We recognize that water may be involved in a number of ways (as solvent, as proton source, as water of hydration, etc.) in the transition between states of the protein. For example, a ligand-induced change in the volume of the protein or a change in the degree of exposure of amino acid side chains may certainly be coupled to a perturbation (whatever the molecular details) in the state of surrounding water molecules. It must be emphasized that such a relaxation of water molecules must be triggered by a ligand-induced change in the state of the protein. The response of the solvent can thus be considered as a part of the change in the thermodynamic state of the protein.

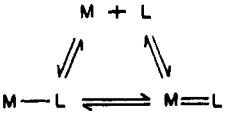
The release upon binding of hydration water surrounding the ligand or the displacement of water from the binding site may also contribute to the thermodynamic changes, particularly the ΔC_p°, of the binding process. The release of such water molecules can be considered a process distinct from a change in the state of the protein. One can attempt, as in the above study with RNase A, to estimate the heat capacity change contribution due to the dehydration of the ligand from measurements of the ΔC_p for the transfer of the ligand (or an analogue of the ligand) from water to some nonaqueous solvent such as ethanol (Alvarez & Biltonen, 1973; Halsey & Biltonen, 1975). Estimation of the ΔC_p° contribution due to the displacement of binding site water molecules is difficult. One can assume that such water molecules have a heat capacity similar to other water molecules that hydrate a protein, which is approximately 2.5 cal/(mol·deg) higher than for bulk water (Yang & Rupley, 1979). The transfer of each water molecule from the binding site to the bulk phase could thus lead to a decrease in heat capacity of 2.5 cal/(mol·deg).

The model and simulations presented under Theory illustrate that enthalpy–entropy compensation will be observed for the binding of a ligand to a protein when conditions are varied in such a fashion that the equilibrium distribution of states of the unliganded protein is shifted. This shift in the state of the free protein will be accomplished most readily by changes in pH (i.e., when ${}_1K$ is related to an acid dissociation of a group on the protein). It should be noted that hairpin curves such as in Figure 1 have been observed in studies in which the thermodynamic parameters were measured over a broad pH range. At times it may seem that the pH dependence of the thermodynamic parameters for the binding of a ligand to a protein cannot be understood in terms of the coupled dissociation of a small number of ionizing groups. However, it is our opinion that it is necessary whenever possible to analyze data in terms of the involvement of a small number of groups. Such an analysis can provide insight concerning the molecular nature of the binding process and allows one to separate protonic equilibria from other aspects of the binding process.

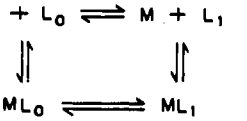
An analysis of the pH dependence of the binding of 3′-CMP to RNase A has been successfully performed by Flogel & Biltonen (1975b). On the basis of our understanding of the coupled protonic equilibria for this system, the apparent enthalpy–entropy compensation for 3′-CMP binding as pH is varied can be explained in terms of coupled proton binding processes. Also our understanding of the pH dependence of 3′-CMP binding has allowed us to dissect the contribution of

[3] It has also been pointed out by a number of workers (Exner, 1964; Leffler, 1955; Krug et al., 1976) that enthalpy–entropy compensation can arise due to the high correlation between ΔH°(app) and ΔS°(app) determined from van't Hoff analyses.

Scheme V



Scheme VI



coupled protonic equilibria from the apparent heat capacity change for ligand binding.

As illustrated by the studies of the ionic strength dependence of the thermodynamics of the association of 3′-CMP to RNase A, enthalpy–entropy compensation observed as a result of a change in ionic strength can be due, at least partly, to salt-induced changes in the pK_a of groups. Compensation found in studies with a congener series of ligands or with a group of similar proteins may also be due to differences in the pK_a of the series' members. We stress, therefore, that understanding the pH dependence of the thermodynamics of the binding of a ligand to a protein is of upmost importance.

Multisubunit proteins which show cooperative ligand binding are systems for which enthalpy–entropy compensation and heat capacity changes are expected to be commonly observed. Allosteric effects have been explained in terms of a two-state model, and the apparent enthalpy, entropy, and heat capacity changes should include a contribution due to the ligand-induced shift in the state of the protein. Niekamp et al. (1977) have determined the thermodynamic parameters for binding of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase at several temperatures and pH values. Cooperative binding of NAD^+ is observed at pH 8.5, particularly at higher temperatures. The apparent enthalpy change for NAD^+ binding varies drastically with temperature [ΔC_p° = −550 to −750 cal/(mol·deg)] and pH. As we discussed elsewhere (Eftink & Biltonen, 1980a), the cooperativity and thermodynamic parameters, including the large ΔC_p, for this system can be explained in terms of a simple two-state model similar to Scheme I for which ${}_1\Delta H$ = +100 kcal/mol and ${}_1K$ = 1 at 10 °C (for pH 8.5 data).

Another interesting study involving cooperative binding is that by Subramanian and co-workers (1978) of the heterotropic interactions in the binding of ligands to glutamate dehydrogenase. They found negligible ΔC_p° values for the formation of binary complexes between the enzyme and either NADPH or glutamate. However, a very large and negative ΔC_p° [$\sim$ −500 cal/(mol·deg)] was found for the formation of the ternary complex. Although the molecular basis for the linked binding of these two ligands to the enzyme is unknown, the large ΔC_p° value strongly suggests that a transition or isomerization of the protein (or the ligands; see below) is induced by the binding process. In additional studies with this system, Fisher et al. (1981) have found the enthalpy change and heat capacity change for the binding of NADPH to depend on temperature in a manner such as that shown in Figure 5. From this they concluded that the binding of coenzyme induces a shift in the preexisting equilibrium between two macroscopic states of protein.

In our discussions we have emphasized the existence of different states of the protein, but different states of the ligand may also exist. A related consideration is that a ligand may possess two or more lobes (i.e., the adenine and nicotinamide moieties of NAD^+) that are capable of interacting with complementary binding pockets on a protein. On binding, both of the lobes may be tightly bound to the protein, or one may be bound tightly with the other lobe remaining free to rotate. For such a system the binding process would be represented by Scheme V (where M—L and M=L are complexes in which one and both lobes, respectively, of the ligand interact with the protein) or, if two distinct conformations of the ligand exist, by Scheme VI. The fact that various protein–ligand complexes (M=L and M—L or ML_0 and ML_1) can form will give rise to a heat capacity change on ligand binding. If the relative population of the M=L and M—L complexes depends for some reason on pH, ionic strength, solvent composition, or the chemical nature of a ligand (in a congener series), enthalpy–entropy compensation might also be observed.

Many of the important functional properties of proteins are due to the existence of sensitive equilibria in a protein (or a ligand) that are easily perturbed by a binding process. The induced fit and rack models for enzyme catalysis employ the perturbation of the state of an enzyme upon substrate binding (Lumry, 1959; Koshland, 1970; Jencks, 1975). Also, as discussed above, the existence of sensitive equilibria in a protein can be the molecular basis for linked binding phenomena (Wyman, 1964). Enthalpy–entropy compensation and heat capacity changes are an experimental manifestation of the responsiveness of proteins to the binding of ligands. The proper understanding of such thermodynamic information hopefully can lead to a better understanding of the functioning of proteins.

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Over the past decade, Dr. Rufus Lumry has been largely responsible for promoting an interest in the molecular basis for enthalpy–entropy compensation associated with protein reactions. Recently at the second Biophysical Discussion (Airlie, VA, May 1980), Dr. Lumry, along with Drs. C. Jolicoeur and E. Battistel, presented some ideas concerning the molecular basis for such compensation that are along the general lines of the models presented here. While the above presented models were arrived at independently, we would like to acknowledge the input of Dr. Lumry in motivating us in this direction.

Registry No. RNase A, 9001-99-4; 3′-CMP, 84-52-6.

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