The pH Dependence of the Thermodynamics of the Interaction of 3′-Cytidine Monophosphate with Ribonuclease A†

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ABSTRACT: The apparent free energy (ΔG_{app}) and enthalpy changes (ΔH_B) associated with the interaction of 3′-cytosine monophosphate (3′-CMP) and ribonuclease A (RNase) are reported for the pH range 4–9, $T = 25°$, $\mu = 0.05$. The pH dependence of ΔG_{app} and ΔH_B has been interpreted in terms of coupled ionization of histidine residues 12, 48, and 119, assuming that only the dianionic form of the inhibitor is bound. The results of this analysis are consistent with the calorimetric and potentiometric titration results for the free enzyme and its 3′-CMP complex reported in the previous paper (M. Flogel and R. L. Biltonen ((1975), *Biochemistry,* preceding paper in this issue). This analysis allows the calculation of the thermodynamic quantities associated with hypothetical but clearly defined reactions (e.g., the reaction of the dianionic inhibitor with the completely protonated enzyme). It is concluded that the primary thermodynamic driving forces for the reaction are van der Waals interactions between the riboside moiety and the protein fabric and electrostatic interaction between the negatively charged phosphate group of the inhibitor and the positively charged histidine residues at the binding locus. It is also suggested that the binding reaction is weakly coupled (~0.5 kcal/mol) with a conformational change of the protein associated with protonation of residue 48. These results are consistent with the model originally proposed by G. G. Hammes ((1968), *Adv. Protein Chem. 23,* 1) and lend additional quantitative detail to the nature of the reaction.

A detailed thermodynamic study of the proton equilibria of ribonuclease A (RNase) and of its complex with 3′-cytosine monophosphate (3′-CMP) between pH 4 and 9 was reported in the previous paper (Flogel and Biltonen, 1975). The pH dependence of the apparent thermodynamic quantities (ΔG_{app}, ΔH_B, ΔS_{app}) of the inhibitor binding to the enzyme are reported in this paper. These two sets of results provide sufficient information to describe the processes which modulate the interaction of the enzyme with 3′-CMP under constant ionic and temperature conditions. The details of the thermodynamic mechanism can be described and the relative populations of the various protonation states of enzyme and enzyme–inhibitor complex estimated.

The results of this analysis are in essential agreement with those of Hammes and coworkers (Hammes, 1968), but provide more quantitative information about the molecular details of the interactions.

Experimental Section

Ribonuclease A was purchased from Worthington Biochemical Corporation and used without further purification. The protein solutions were prepared and concentrations determined as previously described (Bolen et al., 1971; Flogel and Biltonen, 1975). The concentration of protein in the reaction solution varied from 3 to 4×10^{-4} *M*.

Calorimetric measurements were made with an LKB flow microcalorimeter using a flow rate of 3 μl/sec. The apparent heat of reaction was determined by mixing solutions of RNase and 3′-CMP, both at the same pH, in the calorimeter. In the present studies complications in the analysis of the calorimetric data arise from the fact that the formation of the enzyme–inhibitor complex involves pH dependent proton absorption and release; thus, the heat measured at any particular pH includes contributions from the heat of protonation of the enzyme system and the heat of ionization of the effective buffering system, as well as the intrinsic heat of interaction. Normally, such complications can be reduced by using a high concentration of a single, well-defined buffer. At acid pH acetate can be used as the buffer since it exhibits no specific effects upon the binding reaction (Bolen et al., 1971). Unfortunately, it is not possible to use a buffer over the entire pH range of the current study because the usual candidate for a buffer near neutrality is phosphate which is well known to be a competitive inhibitor of RNase. Other buffers are inappropriate because of their large heats of ionization and unknown effects on RNase. For this reason the experiments were performed using only acetate as the principal salt and the measured heats corrected for the buffering contribution.

Results

Upon mixing solutions of the inhibitor and protein in the calorimeter, heat is evolved which is equal to the sum of the heats of dilution of the components and the apparent heat of reaction. The apparent heat of reaction per mole of protein, Q, was obtained by subtraction of the heats of dilution of the components which were measured separately.

In the present experiments Q is a complex function of the intrinsic heat of binding, enthalpy changes associated with proton release or absorption and the enthalpy of ionization of all buffering species as described in the Appendix. The true heat of interaction between 3′-CMP and RNase, in-

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[1] The definitions and terms used, and the derivation of appropriate equations, are based upon the assumption that the stoichiometry of the reaction is 1:1. This assumption has been thermodynamically established for the 3′-CMP–RNase system (Bolen et al., 1971).

cluding the heat associated with proton release and absorption, is

$$Q_t = Q/\left(1 - \frac{\Delta N_t}{\Delta H_B} Q_{Buf}\right) \quad (1)$$

where ΔN_t is the number of protons absorbed and ΔH_B the true heat of the interaction per mole of complex formed. Q_{Buf} is the heat per mole of proton associated with the buffering action of the system. Assuming that the stoichiometry of the reaction between 3′-CMP and RNase is 1:1[1]

$$\frac{1}{Q_t} = \frac{1}{\Delta H_B} + \frac{1}{\Delta H_B \cdot K_B}[I_f] \quad (2)$$

where K_B is the association constant for the reaction and $[I_f]$ is the free concentration of ligand. It is shown in the Appendix that

$$[I_f] = [I_t] - \frac{Q[M_t]}{(\Delta H_B + \Delta N_t Q_{Buf})} \quad (3)$$

where $[I_t]$ and $[M_t]$ are the *total* inhibitor and protein concentrations, respectively.

The best values of ΔH_B and K_B for the binding reaction at each pH were determined by least-squares analysis using eq 2 in the following way: the value of ΔN_t was assumed to be that which was experimentally determined as described in the previous paper (Flogel and Biltonen, 1975). Q_{Buf} was calculated at each concentration of protein, inhibitor, and buffer as described in the Appendix. An initial estimate of ΔH_B was made and Q_t and $[I_f]$ were calculated. Q_t as a function of $[I_f]$ was then analyzed in the usual fashion to obtain an estimate of K_B and a new estimate of ΔH_B. This process was repeated with each new value of ΔH_B until a convergent solution was obtained.

Although the correction factors in eq 1 and 3 are complicated, the analysis is relatively straightforward. For example, at pH 5.4, $\Delta N_t \simeq 0$ and the buffering correction factor $\simeq 0$. At pH values below 5.5 the dominant buffer was acetate, and a constant buffering correction term was applicable. This term was, in fact, small because the enthalpy of ionization for acetate is almost zero. At pH values from pH 5.5 to 7.0 the buffering correction factor became more significant, since at low 3′-CMP concentration the primary buffer was the protein, but at high 3′-CMP concentration the primary buffer was the phosphate group of the inhibitor. At pH values higher than 7 the protein dominated the buffering effect at all inhibitor concentrations.

The effect of the buffering correction terms is demonstrated in Figure 1A and B for data obtained at pH 7.0 and 9.0. Curve I is the "best fit" curve calculated assuming no buffering effects; curve II was calculated taking into account the buffering effects; and curve III is the true binding isotherm if the heat of ionization of the buffer is zero. The data in Figure 1A demonstrate that at pH 7.0 the changing buffering system affects both the shape and magnitude of the apparent binding isotherm. At pH 9.0, Figure 1B, the protein is the primary buffer at all inhibitor concentrations and the effect is primarily a reduction in the magnitude but not the shape of the experimental isotherm.

In Table I values of the apparent free energy, $\Delta G_{app} = -RT \ln K_B$, and enthalpy of binding, ΔH_B, of 3′-CMP to ribonuclease are tabulated for several pH values. The first set of values corresponds to those obtained without taking into account the heat of buffering of the system; the second set corresponds to corrected estimates as just described. It can be seen that the primary effect of buffering is to alter the magnitude of the enthalpy change (0–4.6 kcal/mol); relatively little change is seen in the free energy of binding (<0.4 kcal/mol). It should be noted that the buffering effect is most pronounced at high pH where protein buffering dominates. At lower pH, where acetate and 3′-CMP phosphate buffering dominate, the magnitude of the buffering effect is smaller because the heats of ionization of acetate and phosphate are quite small.

Table I: Apparent Thermodynamic Quantities for the Binding of 3′-Cytosine Monophosphate to Ribonuclease A.[a]

pH	$-\Delta G^{*b}$	$-\Delta G_{app}{}^b$	$-\Delta H^{*b}$	$-\Delta H_B{}^b$
4.0	5.2	5.2	6.4	6.4
4.5	6.2	6.2	9.8	9.8
5.0	6.2	6.3	11.6	11.5
5.5	6.2	6.2	15.3	15.6
6.0	5.6	5.6	16.6	17.6
6.5	5.0	5.4	18.8	21.5
7.0	4.7	4.9	14.1	18.7
7.5	4.6	4.4	11.2	14.5
8.0	4.5	4.3	9.1	11.5
8.5	3.9	3.7	7.2	9.3
9.0	3.2	3.4	7.2	10.0

[a] Conditions: $\mu = 0.05$, $T = 25°$. [b] Units: kcal/mol, standard state = 1 mol/l. ΔG^* and ΔH^* are thermodynamic estimates ignoring buffering effects; ΔG_{app} and ΔH_B are estimates corrected for buffering as described in the text.

Analysis of Results. Any thermodynamic mechanism proposed for the binding of 3′-CMP to RNase must be quantitatively reconcilable with the present results plus those reported in the previous paper (Flogel and Biltonen, 1975). The assumptions of the simplest such scheme are that (1) 1:1 stoichiometry between inhibitor and protein exists (see Bolen et al., 1971); (2) inhibitor binding and proton binding to histidine residues 12, 48, and 119 (j = 1,2,3) are thermodynamically coupled; (3) all proton binding sites in the free enzyme and complex are independent; and (4) only the dianionic phosphate form of the inhibitor is bound to the enzyme in the pH region of 4–9.[2] The relevant thermodynamic relationships for this model are given in eq 4–9.

$$\Delta G_{app} = \Delta G_0^\circ - RT\sum_{j=1}^{3} \ln\frac{(1 + H^+/K'_j)}{(1 + H^+/K_j)} + RT \ln (1 + H^+/K_I) \quad (4)$$

$$\Delta H_B = \Delta H_0^\circ + \sum_{j=1}^{3} (f'_j \Delta H'_{p,j} - f_j \Delta H_{p,j}) \quad (5)$$

$$\Delta N_E = \Delta N_t + H^+/(K_I + H^+) = \sum_{j=1}^{3} (f'_j - f_j) \quad (6)$$

ΔN_t, ΔH_B, and ΔG_{app} have been defined in the previous section. ΔG_0° and ΔH_0° refer to the standard free energy and enthalpy changes associated with the binding of the dianionic inhibitor to the enzyme in which the relevant histidine residues are unprotonated (i.e., high pH). $f'_j = [H^+]/(K'_j + [H^+])$ and $f_j = [H^+]/(K_j + [H^+])$ are the fractional degree of proton association, with K'_j and K_j the dissocia-

[2] There are little existing data which indicate that the charge of the ring nitrogen of the cytidine nucleotides ($pK \sim 4.3$) has any influence on the binding or catalytic reactions of ribonuclease A. In fact, Anderson et al. (1968) report that the pK_a for this functional group in the enzyme complex corresponds to the pK_a for the free nucleotide. Therefore, it is assumed that only the ionization state of the phosphate group is relevant to the discussion at hand and any reference made to monoanionic or dianionic forms of the ligand refer to the charge state of the phosphate group.

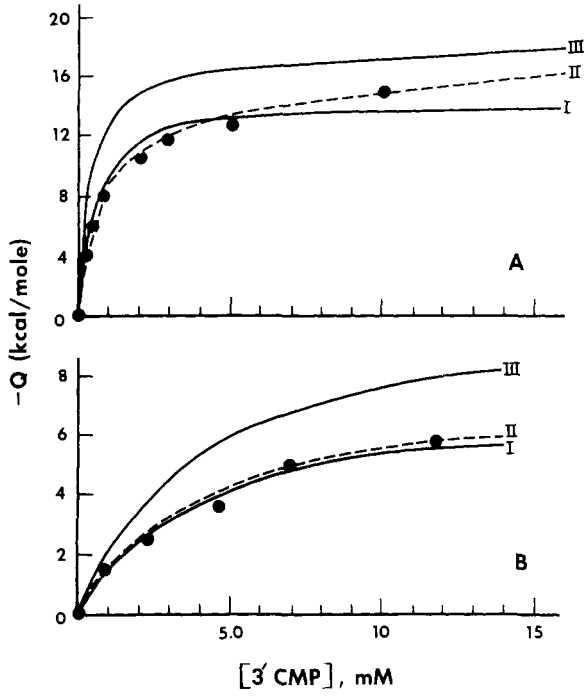

FIGURE 1: Heat of reaction, Q, vs. total 3'-CMP concentration at pH 7 (A) and pH 9 (B). Temperature = 25°, and μ = 0.05. Curves I, II, and III were calculated as described in the text.

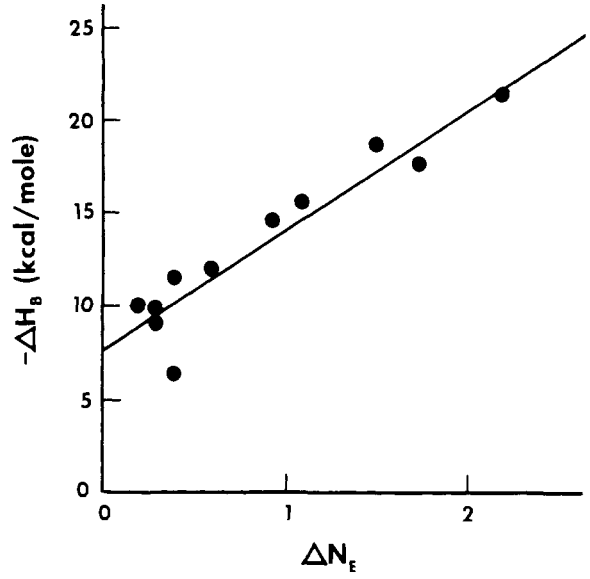

FIGURE 2: The estimated heat of binding of 3'-CMP to RNase vs. the net proton uptake by the protein at 25°, μ = 0.05, pH 4-9. ΔH_B was determined as described in the text. ΔN_E is the measured proton uptake corrected for proton release by the phosphate group of 3'-CMP upon binding. The solid curve is the least-squares line through the points. See text for details.

tion constants and $\Delta H'_{p,j}$ and $\Delta H_{p,j}$ the enthalpies of protonation, of the jth histidine residues of the complex and free enzyme, respectively. Since ΔH_p for the phosphate group is 0 ± 300 cal/mol its contribution to eq 5 has not been explicitly included. ΔN_E, the net proton uptake by the protein, is the observed proton uptake corrected for proton release by the phosphate group of the inhibitor with proton dissociation constant K_I (see eq 2 and Figure 3B in Flogel and Biltonen, 1975).

It will become apparent later (see also Flogel et al., 1975) that the thermodynamic relations for the hypothetical reaction of the binding of the dianionic phosphate inhibitor to the enzyme in which all relevant histidine residues are protonated are those in which we are most interested. These relations, $\Delta G°$, $\Delta H°$, and $\Delta S°$, are given below in terms of previously defined quantities:

$$\Delta G° = \Delta G_0° - RT\sum_{j=1}^{3} \ln (K_j/K'_j) \quad (7)$$

$$\Delta H° = \Delta H_0° + \sum_{j=1}^{3} (\Delta H_{p,j}' - \Delta H_{p,j}) \quad (8)$$

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (9)$$

In Figure 2, ΔH_B is plotted vs. proton uptake by the protein, ΔN_E. The solid line in Figure 2 is the least-squares line with a slope = −6.4 kcal/mol and intercept = −7.8 kcal/mol. These results are to be interpreted as follows: the average ΔH for proton absorption upon formation of the enzyme-inhibitor complex is −6.4 kcal/mol, and the average ΔH for binding in the *absence* of proton absorption by the protein is −7.8 kcal/mol. This interpretation is consistent with the experimental results presented in the preceding paper (Flogel and Biltonen, 1975) in which it was concluded that ΔH_p for histidine residues 12 and 119 was −6.5 kcal/mol in *both* the free enzyme and complex and that complex formation resulted in a shift in the pK of these two residues. Although the ionization behavior of residue 48 quantitatively influences both proton uptake and ΔH_B its effect is apparently small. This is consistent with the results presented in the previous paper (Flogel and Biltonen, 1975) which indicated that the shift in pK of His-48 upon complex formation was only about 0.4.

ΔH_B is plotted vs. pH in Figure 3 and has been analyzed according to eq 5 assuming that only histidine residues 12, 48, and 119 are thermodynamically coupled to inhibitor binding. Initial estimates for the various K_j and K'_j and $\Delta H_{p,j}$ were taken from the previous paper (Flogel and Biltonen, 1975). ΔH_p for residues 12 and 119 was restricted to −6.5 kcal/mol in both the free enzyme and enzyme-inhibitor complex, but values for the other parameters were allowed to vary within small limits of the initial estimates and the curve simulated by computer calculation to find the "best fit".[3] The solid curve in Figure 3 was calculated using the parameters given in the legend to the figure.

ΔG_{app} is plotted vs. pH in Figure 4A. In Figure 4B ΔG_{app} adjusted for the ionization of the phosphate group of the inhibitor is plotted vs. pH. The solid curves in Figure 4A and 4B were calculated according to eq 4, using the pK values given previously (Flogel and Biltonen, 1975) as initial estimates and allowing these quantities to vary within small limits. The values of the parameters used to calculate ΔG_{app} are summarized in the legend to the figure. It is to be noted that the shape of the ΔG_{app} curve is more sensitive to the *exact* pK values used in the calculation than the ΔH_B curve. A "best fit" was found for pK'_j values of 6.7 and 7.3 in simulating the ΔG_{app} curve. A difference in the pK_j values for these residues is consistent with the NMR results of Meadows et al. (1969) who found that pK_{119} = 7.4 and pK_{12} = 8.0 for the 3'-CMP–RNase complex in 0.2 M salt.

[3]The best fit was selected as minimum in the RMS deviation between the experimental and calculated values of ΔH_B and ΔG_{app}. The respective deviations are noted in Table III.

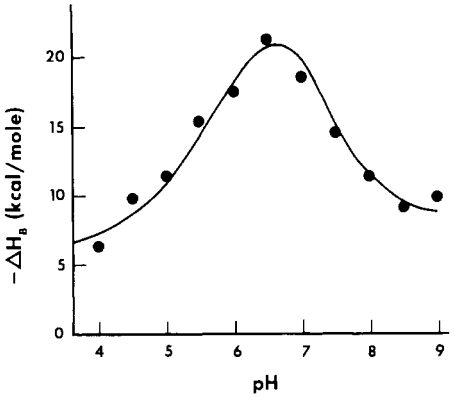

FIGURE 3: The apparent heat of binding, ΔH_{app} vs. pH; $T = 25°$, $\mu = 0.05$. The solid curve was calculated assuming $pK_1 = 5.0$, $pK_2 = 5.8$, $pK_3 = 6.7$, $pK_1' = pK_2' = pK_3' = 7.1$; $\Delta H_1 = \Delta H_2 = \Delta H_1' = \Delta H_2' = -6.5$ kcal/mol, $\Delta H_3 = -24$ kcal/mol, $\Delta H_3' = -22$ kcal/mol, and $\Delta H_0° = -8.7$ kcal/mol. See text for details.

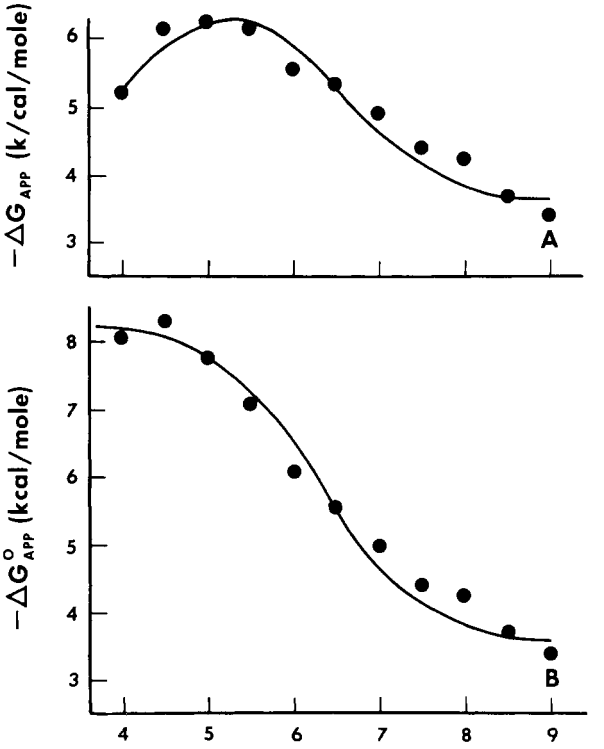

FIGURE 4: The apparent free energy change of binding, ΔG_{app}, 3′-CMP to RNase under conditions listed in Figure 3 vs. pH (A). ΔG_{app} corrected for ionization of the phosphate group of 3′-CMP is presented in B. The solid curves in A and B were calculated assuming the pK_i values given in the legend to Figure 3 and $pK_1' = 6.7$, $pK_2' = 7.3$, $pK_3' = 7.0$, $pK_I = 6.1$ and $\Delta G_0° = -3.4$ kcal/mol. See text for details.

Discussion

The Quantitative Validity of the Model. In this and the previous paper calorimetric and potentiometric data have been presented which allow the development of a self-consistent thermodynamic model for the binding of 3′-CMP to RNase over the pH range 4–9, $T = 25°$, $\mu = 0.05$. The basic features of the model are that inhibitor binding is thermodynamically coupled to ionization of histidine residues 12, 48, and 119 and that only the dianionic form of the inhibitor is bound. These features are totally consistent with conclusions regarding the reaction previously deduced by Hammes (1968), Meadows et al. (1969), Gorenstein and Wyrwicz (1973), and Haar et al. (1974).

It is to be noted that the present results at $\mu = 0.05$ are quantitatively different from the thermodynamic quantities reported for the binding of 3′-CMP to RNase at $\mu = 0.2$ (Anderson et al., 1968). The principal reason for this difference is a strong ionic strength dependence of the thermodynamic quantities for the reaction (Bolen et al., 1971). For example, it was previously reported that at pH 5.4, ΔG_{app} and ΔH_B change by 1.1 and 7 kcal/mol, respectively, when the ionic strength is increased from 0.05 to 0.50 *M*. The variance between the reported results and those of Anderson et al. (1968) are qualitatively those which would be expected due to the differences in ionic strength used in the two studies.

The statement regarding the uniqueness of the derived set of parameters made in the previous paper (Flogel and Biltonen, 1975)) applies as well in this case. The test of uniqueness of our representation rests upon the fact that essentially a single set of parameters relating to the ionization behavior of RNase and its complex with 3′-CMP is adequate to quantitatively describe the potentiometric and calorimetric titration data of both protein species as well as the pH dependence of the apparent free energy and enthalpy changes associated with binding.

In order to quantitatively interpret these results it has been assumed that the ionization behavior of these three histidine residues is independent. Insofar as the ionization of the histidine residues will be thermodynamically coupled, as for example by repulsive electrostatic interactions, this assumption is not strictly correct. This is of minor importance, however, if ΔG_{app}, ΔH_B, and ΔN_t are only to be represented accurately as a function of pH. For example, the correct representation of ΔG_{app} is

$$\Delta G_{app} = \Delta G° + RT \ln \int \Delta N_t \, d(\ln (H^+))$$

regardless of the exact details of the model (Wymans, 1948).

In Table II the average best fit[3] values for the thermodynamic parameters related to ionization of the histidine residues and to the intrinsic binding quantities are summarized. These parameters can be used to correct the ΔG_{app} and ΔH_B values to $\Delta G°$ and $\Delta H°$, the standard free energy and enthalpy changes for the hypothetic reaction in which the dianionic inhibitor binds to the protein in which all histidine residues are protonated (eq 7–9). In Table III are summarized the estimates of $\Delta G°$ *and* $\Delta H°$ derived from the ΔG_{app} and ΔH_B values at different pH values. The good agreement between these estimates indicates the internal self-consistency of the model, as well as the degree of accuracy of these phenomenological parameters. On this basis we conclude that our model is quantitatively accurate.

Interpretation of the Parameters. The quantities $\Delta G_0°$, $\Delta H_0°$, and $\Delta S_0°$ refer to the thermodynamic changes associated with the interaction of the dianionic form of 3′-CMP with RNase in which the histidine residues are all unprotonated. The principle driving force for the reaction is the negative enthalpy change; the unitary entropy change ~ -10 cal/(mol deg).[4] Since both the classical hydrophobic

[4] The unitary entropy change is simply that thermodynamic quantity calculated assuming a standard state of unit mole fraction. It is 8 cal/deg greater than that based on a standard state of 1 mol/l. and corrects for a mixing contribution to the latter quantity.

Table II: Average Values of the Best Fit Thermodynamic Parameters Describing the Reaction of 3′-CMP with RNase, pH 4–9.[a]

	Free Enzyme	3′-CMP Complex
pK_{12}[b]	5.8	7.4[c]
ΔH_{12}	−6.5 kcal/mol	−6.5 kcal/mol
pK_{119}[b]	5.0	6.8[c]
ΔH_{119}	−6.5 kcal/mol	−6.5 kcal/mol
pK_{48}[b]	6.7	7.1
ΔH_{48}	−24 kcal/mol	−22 kcal/mol
ΔG_0° = −3.4 kcal/mol		ΔH_0° = −8.7 kcal/mol

[a] Conditions: $T = 25°$, $\mu = 0.05$. [b] The assignment of pK values to specific histidine residues was based upon the NMR results of Meadows et al. (1968) and Ruterjans and Witzel (1969). [c] In assigning different pK values to these two groups in the complex it was assumed that their average pK = 7.1 ± 0.3. See text for details.

Table III: Estimates of the Standard Free Energy and Enthalpy Change for the Binding of the Dianionic Form of 3′-CMP to Fully Protonated RNase at 25°, μ = 0.05.[a]

pH	$-\Delta G^\circ$ (kcal/mol)	$-\Delta H^\circ$ (kcal/mol)
4.0	8.2	5.7
4.5	8.6	7.9
5.0	8.3	7.2
5.5	8.3	8.0
6.0	8.1	6.4
6.5	8.6	7.6
7.0	9.0	5.5
7.5	9.1	5.6
8.0	9.3	6.5
8.5	8.7	6.2
9.0	8.6	7.6
Av	8.6 ± 0.1[b]	6.7 ± 0.3[b]
	ΔS° = 6 ± 1 (cal/(mol deg))	

[a] ΔG° and ΔH° calculated according to eq 7 and 8 assuming an independent proton binding site model and parameters listed in Table II. Standard state is 1 mol/l. [b] Average values calculated using all the results. Errors listed are standard errors of the mean value.

effect and attractive electrostatic interactions in aqueous solution are dominated by positive entropy changes, it can be concluded that such interactions are not of primary significance in the reaction. This conclusion is consistent with the studies of Alvarez and Biltonen (1973) and Scruggs et al. (1972) who concluded that nucleic acid bases do not exhibit any significant hydrophobic character and with the fact that the histidine residues at the binding site are uncharged. It therefore appears that the thermodynamic driving force for protein–inhibitor association at alkaline pH is attractive van der Waal's interaction.

The thermodynamic quantities for association of the dianionic form of 3′-CMP to RNase in which the histidine residues are fully protonated are given in Table III. The enthalpy change was found to be −6.7 kcal/mol and the unitary entropy estimated to be +14 cal/(mol deg).[4] It is seen that the entropy change favorably influences this binding reaction. The favorable entropy change in this case is most certainly the result of attractive electrostatic interactions between the ionized histidine residues and the inhibitor phosphate.

The magnitude of the electrostatic interaction between the nucleotide inhibitor and the charged histidine residues at the binding site (12 and 119) is reflected in the shift of their pK values upon complex formation. Because the enthalpy of ionization of these histidine residues is unchanged upon nucleotide binding, this interaction is purely entropic in nature; electrostatic interactions are expected to be primarily entropic since $-T\Delta S_{elec} = 1.37\Delta G_{elec}$ in water (Kauzmann, 1959). The magnitude of the electrostatic contribution for 3′-CMP binding to RNase can be estimated from

$$\Delta G_{elec} = \sum_{j=1}^{3} 2.303RT(pK_j - pK'_j)$$

and is found to be −5.1 kcal/mol. Thus a significant fraction of the favorable free energy change for binding at acid pH arises from the electrostatic interactions at the binding site.

The work of Hammes and coworkers (French and Hammes, 1965; Hammes, 1968) indicated that a conformational change in both the protein and the inhibitor–protein complex is associated with protonation of His-48. Our calorimetric and potentiometric titration data reported in the preceding paper are consistent with this conclusion. Most important, however, is the observation that inhibitor binding is *not* strongly coupled with the conformational change. This conclusion follows from the fact that the shift in pK of residue 48 upon complex formation is 0.4 ± 0.1, which is equivalent to an "interaction energy" of 560 ± 140 cal/mol. Consistent with this conclusion is the fact that the pH dependence of the "conformational" relaxation time of the protein is almost identical in both the presence and absence of inhibitors and substrates (French and Hammes, 1965; del Rosario and Hammes, 1969, 1970).

In the next paper (Flogel et al., 1975) use will be made of this phenomenological model to estimate the thermodynamic changes associated with the binding of several inhibitors to RNase under hypothetical conditions in which the inhibitor is in its dianionic form and the histidine residues completely protonated. Since inhibitor binding is only weakly coupled to the conformational change of the protein comparison of the thermodynamics of the binding of different ligands will provide details of specific interactions with the protein and their relationship to the overall rate of catalysis by the enzyme.

Appendix: Analysis of Calorimetric Data for an Interacting System in a Mixed Buffer

1. General. Consider a buffered system in which a macromolecule, M, interacts with a ligand, X, and concomitant proton release or absorption occurs. Assuming a 1:1 stoichiometry, the overall reaction scheme can be written as

$$B + M + X \longrightarrow MX + \Delta N_t H^+ + B \longrightarrow MX + \Delta N_t BH^+$$

where B represents the buffer, and ΔN_t is the number of proton equivalents released per mole of complex formed. The measured heat per unit volume for the reaction is

$$\Delta Q = [MX]\Delta H_B + [MX]\Delta N_t Q_{Buf} \qquad (A\text{-}1)$$

where ΔH_B is the molar heat of ligand binding in the absence of any buffering and Q_{Buf} is the heat (per mole of proton) associated with the buffering action. Defining $Q = \Delta Q/[M_t]$ and $Q_t = ([MX]/[M_t])\Delta H_B$ where $[M_t]$ = total macromolecule concentration it follows that

$$Q = Q_t\left(1 + \frac{\Delta N_t}{\Delta H_B} Q_{Buf}\right) \qquad (A\text{-}2)$$

However

$$Q_t = Q/\left(1 + \frac{\Delta N_t}{\Delta H_B} Q_{Buf}\right) = \frac{K_B[I_f]}{1 + K_B[I_f]} \Delta H_B \quad \text{(A-3)}$$

where K_B is the ligand–macromolecule association constant and $[I_f]$, the free ligand concentration in the mixture, is

$$[I_f] = [I_t] - \frac{Q_t}{\Delta H_B}[M_t] = [I_t] - \frac{Q[M_t]}{(\Delta H_B + \Delta N_t Q_{Buf})} \quad \text{(A-4)}$$

$[I_t]$ = total ligand concentration. Rearrangement of eq A-3 leads to

$$\frac{1}{Q_t} = \frac{1}{\Delta H_B} + \frac{1}{\Delta H_B K_B [I_f]} \quad \text{(A-5)}$$

Thus K_B and ΔH_B can be solved by an interative least-squares analysis given that ΔN_t and Q_{Buf} are known.

2. Definition of the Buffering Effect. The role of the buffer is to maintain the pH of the sytem essentially constant during the reaction. This buffering action will not influence the actual degree of ligand binding but will affect the magnitude of the heat change measured upon mixing ligand and macromolecule as shown in the previous section. The net effect of the buffering action on the measured heat change will be a function of the relative buffering capacity of each specie in solution. In order to correct for this effect it is necessary to consider in detail the basis of the buffering action.

The specific buffering capacity of a component j in the system is defined as

$$\beta_j = (\partial N_j/\partial \mathrm{pH})C_j$$

where C_j is the molar concentration of that component and N_j is the number of proton equivalents absorbed by that component at a given pH. The total buffering capacity of the system $\beta = \Sigma_j \beta_j$ and $b_j = \beta_j/\beta$ is the relative buffering capacity of component j. In other words, for each mole of protons buffered by the system a fraction b_j will be buffered by component j.

Similarly, the heat associated with this buffering action, Q_{Buf}, is $\Sigma_j \delta H_j b_j$ per mole of proton buffered where $\delta H_j = (\partial H_j/\partial N_j)$, the heat of protonation (per mole of proton) of component j at a given pH. It thus follows that

$$Q_{Buf} = \sum_j \delta H_j b_j = \frac{1}{\beta} \sum_j \left(\frac{\partial H_j}{\partial N_j}\right)\left(\frac{\partial N_j}{\partial \mathrm{pH}}\right) C_j = \frac{1}{\beta} \sum_j \left(\frac{\partial H_j}{\partial \mathrm{pH}}\right) C_j \quad \text{(A-6)}$$

Therefore, if the buffering characteristics of each component of the system are known, Q_{Buf} can be readily calculated.

3. Calculation Procedure. C_j for all components is assumed known; $(\partial H_j/\partial \mathrm{pH})$ and β_j for each component can be calculated from the ionization characteristics of each species and from the slope of the calorimetric titration curve; and ΔN_t can be determined at each pH by differential titration (see Flogel and Biltonen, 1975). Thus $\Delta N_t Q_{Buf}$ can be calculated for each mixture of buffering components. An initial estimate of ΔH_B is then made to calculate Q_t and $[I_f]$ for each data pair using eq A-3 and A-4 and this set of values analyzed by a weighted linear least-squares procedure according to eq A-5 to obtain new estimates for K_B and ΔH_B. The new value of ΔH_B is then used to recalculate a new set of data pairs which are again analyzed in the same manner. This iterative cycle is continued until a convergent solution is obtained.

Although this iterative procedure appears to be complex it is straightforward and can be very accurate if ΔN_t and Q_{Buf} are well defined and care is taken in the experimental design. If $Q_{Buf} \ll \Delta H_B$ the correction of Q to Q_t is small. This can be accomplished by using a relatively high concentration of one buffer whose heat of protonation is small (e.g. acetate). Also, if conditions can be established in which $[M_t] \leq 1/K_B$ then $[I_f] \simeq [I_t]$ and the correction of $[I_t]$ to $[I_f]$ is minor. The results of such a procedure for analyzing calorimetric data for the RNase–3′-CMP are given in the Results section.

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