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# Calorimetric and Potentiometric Characterization of the Ionization Behavior of Ribonuclease A and Its Complex with 3′-Cytosine Monophosphate†

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ABSTRACT: The proton association behavior of ribonuclease A and its complex with 3′-cytosine monophosphate has been thermodynamically characterized in the pH range 4–8 at 25°, $\mu$ = 0.05. Calorimetric and potentiometric titration data have been used to estimate the apparent p$K$ values and enthalpy values for protonation of the four histidine residues of the protein, $\Delta H_p$. In the free enzyme the p$K$ values were deduced to be 5.0, 5.8, 6.6, and 6.7 and $\Delta H_p$ deduced to be −6.5, −6.5, −6.5, and −24 kcal/mol for residues 119, 12, 105, and 48, respectively. For the nucleotide–enzyme complex it was concluded that the apparent p$K$ values of residues 119, 12, and 48 increased to an average value of about 7.2, the $\Delta H_p$ values remaining constant for all histidine groups except 48. It was also concluded that only the dianionic phosphate form of the nucleotide inhibitor is bound to the enzyme in this pH range. These results are consistent with a thermodynamic model for the binding reaction in which inhibitor–enzyme association is coupled to the ionization of three imidazole residues (12, 119, and 48) and the interaction between the negative phosphate moiety of the inhibitor and the positively charged residues 12 and 119 is purely electrostatic. However, the "interaction" with residue 48 probably involves a conformational rearrangement of the macromolecule.

Many investigations have been directed toward the elucidation of the mechanism of the catalytic action of ribonuclease A (for a general review see Richards and Wyckoff, 1971; Usher and Richardson, 1970; Usher et al., 1972). These several studies have established that two histidine residues, 12 and 119, located at the active site are directly involved in the catalytic reaction, one acting as a proton acceptor and the other as a proton donor. In addition, it has been postulated that the reaction proceeds via a dianionic pentacoordinated phosphate intermediate (Witzel, 1963) which, in principle, can be thermodynamically stabilized by electrostatic interaction with the positively charged surface of the enzyme. It is thus apparent that a thermodynamic description of the ionization states of the enzyme and of specific substrate–protein interactions is vital to the development of a quantitative understanding of the enzymatic reaction.

Potentiometric titration data (Tanford and Hauenstein, 1956; Bull and Breeze, 1965) have provided a phenomenological description of the ionization behavior of the free enzyme. More recently, nuclear magnetic resonance (NMR) studies (Meadows et al., 1968; Ruterjans and Witzel, 1969;

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Roberts et al., 1969) have provided reliable estimates of the p$K$ values of the four histidine residues of the protein. In addition, spectrophotometric (Nelson and Hummel, 1961; Cathou et al., 1965), kinetic (Hammes, 1968; Herries et al., 1962), nuclear magnetic resonance (Meadows et al., 1969) and proton uptake (Hummel and Witzel, 1966) studies have provided information about the interaction of various charged substrates and inhibitors with the enzyme. However, the thermodynamic details of the various interactions, especially with regard to the influence of pH and ionic strength, have not been completely delineated. In order to do so it is necessary to define the thermodynamic changes associated with each of the equilibrium processes occurring under each set of environmental conditions (e.g., pH, ionic strength, and temperature).

In this first paper of a series the potentiometric and calorimetric titration behavior of the free enzyme and of its complex with 3′-cytosine monophosphate (3′-CMP) are reported. These data provide the necessary information to develop a complete thermodynamic description of the pH dependence of the nucleotide binding reaction which will be reported in the following paper (Flogel and Biltonen, 1975). In the third, and last, paper of this set (Flogel et al., 1975) the thermodynamic changes associated with the binding of several different inhibitors to RNase will be reported and compared. The latter results are used to estimate the magnitude of various specific interactions between the protein and ligand.

## Experimental Procedure

Ribonuclease A (lyophilized and phosphate free), purchased from Worthington Biochemical Corporation, Freehold, N.J., was used without further purification. The solutions were prepared as described by Bolen et al. (1971) and the protein concentrations determined by optical density measurement at 277.5 nm, assuming a molar extinction coefficient of 9800 $cm^{-1}$ $mol^{-1}$ at pH 7.6 (Sela and Anfinsen, 1957). The concentration of enzyme varied from 2 to 10 mg/ml. Chromatographically pure 3′-cytidine monophosphate was obtained from Boehringer Mannheim Corporation and used without further purification. All other chemicals were reagent grade. The ionic strength of all solutions was maintained at 0.05 with KCl.

Potentiometric titrations were carried out with a Radiometer PHM4b pH meter. The titrant, 0.1 $N$ HCl, was added to 3 ml of RNase solution by means of a microburet in portions of 0.001–0.002 ml. Proton uptake and proton release titration was performed by mixing equal volumes of RNase and 3′-CMP solutions, both at identical pH and ionic strength, and titrating the resulting solution back to the initial pH with either 0.01 $N$ HCl or 0.01 $N$ NaOH. All titrations were performed under nitrogen atmosphere at 25°.

Calorimetric titrations were performed with an LKB-flow microcalorimeter. Protein and HCl solutions of identical ionic strength were pumped into the reaction vessel of the calorimetric unit at a flow rate of approximately 0.003 ml/sec and the resulting heat of mixing measured. The pH of the resulting solution was measured with a Sargent-Welch DR pH meter in a flow vessel. The enthalpy changes associated with protonation of the enzyme (or enzyme–inhibitor complex) were obtained by subtracting the heats of dilution of HCl and the protein from the measured heat of mixing. The microcalorimeter was calibrated electrically as previously described (Zimmer and Biltonen, 1972).

## Results

*Titration of Ribonuclease A.* The heat evolved upon mixing a solution of RNase (pH ~8.5) with HCl of varying concentration was measured calorimetrically. The pH of the resulting solution was measured using a flow electrode assembly. These experimental results, corrected for the heats of dilution of the components and adjusted such that $\Delta H_t = 0$ at pH 8.0, are summarized in Figure 1. $\Delta H_t$ at any pH is the molar enthalpy change associated with titrating RNase A from pH 8.0 to that pH. Assuming that the protonation sites of the protein are independent,[1] $\Delta H_t$ at each particular pH value is related to the sum of the heats of protonation of the individual ionizing groups in the following way:

$$\Delta H_t = \sum_j \delta n_j \Delta H_{p,j} = \sum_j \left\{ \frac{[H^+]}{K_j + [H^+]} - \frac{[H^+]_0}{K_j + [H^+]_0} \right\} \Delta H_{p,j} \quad (1)$$

where $\delta n_j$ is the number of protons absorbed by group $j$ between the initial hydrogen ion concentration $[H^+]_0$ ($10^{-8}$ $M$), and the final hydrogen ion concentration $[H^+]$. $K_j$ is the apparent dissociation constant and $\Delta H_{p,j}$ is the enthalpy change associated with protonation of group $j$.

Although it is, in principle, possible to deduce the appropriate set of $K$ and $\Delta H_p$ values from the calorimetric results presented in Figure 1, the precision of the current data do not justify such an exercise. However, a detailed comparison of the calorimetric data with potentiometric titration data provides a useful basis upon which a thermodynamic interpretation can be developed.

It can be noted that $\Delta H_t$ becomes essentially independent of the pH below pH 5 (also see Figure 4) where the carboxyl groups begin to dominate the titration behavior. Thus it is reasonable to assume that $\Delta H_p \simeq 0$ for the carboxyl groups[2] and that $\Delta H_t$ is primarily the result of protonation of the four histidine residues and any amino groups incompletely ionized by pH 8.0. In Figure 2 $\Delta H_t$ vs. the number of protons absorbed per mole of protein, $N_E$, is plotted. $N_E$ at any pH, determined by potentiometric titration, is the number of protons absorbed between that pH and pH 8.0, our reference state. The slope at any point in Figure 2 is an apparent average value for heat of protonation of the groups titrating in that pH region. It can be seen that this average value is $\simeq -14$ kcal/mol for the first two protons (pH 8–6.5) and $\simeq -6.5$ kcal/mol for the next 2–3 protons (pH 6.5–5.0) absorbed. The latter value of $-6.5$ kcal/mol is approximately that which would be expected for a normal imidazole residue. Since Ruterjans and Witzel (1969) have shown that the p$K$ values of histidines 12 and 119 at low salt are between 5 and 6, a value of $\Delta H_p = -6.5$ kcal/mol has been assigned to these two groups. This average value is

[1] The assumption of independent sites is not strictly correct because of obvious electrostatic effects and possible coupling, via hydrogen bonding, of the ionization of the histidine residues (Ruterjans and Witzel, 1969). However, a more complex analysis of our data is not warranted. Furthermore, a strictly phenomenological characterization of the difference in titration behavior between the free enzyme and the nucleotide complex is sufficient to thermodynamically describe the pH dependence of the binding reaction (Wymans, 1948).

[2] At low pH the slope of the $\Delta H_t$ vs. pH curve changes sign suggesting that protonation of the carboxyl groups is slightly endothermic. In any case, a small heat of protonation of the carboxyl groups will have negligible effect upon our estimates of the $\Delta H_p$ for the histidine residues.

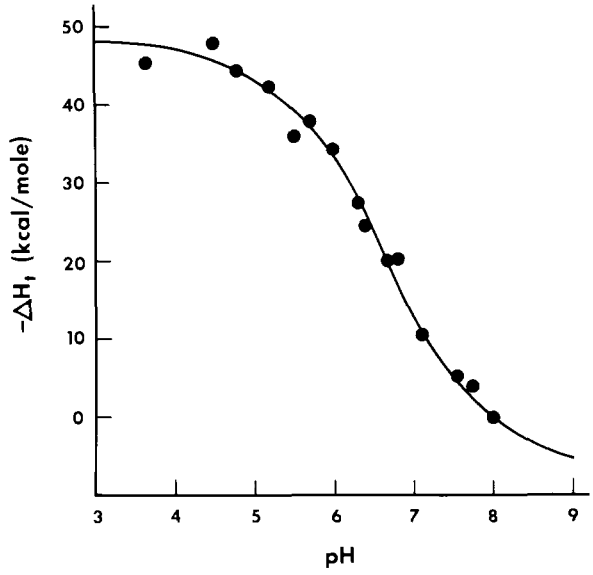


FIGURE 1: Enthalpy titration curve for ribonuclease A, $\mu = 0.05$, $T = 25°$. $\Delta H_t$ has been set equal to zero at pH 8.0. The solid line was calculated assuming p$K$ and $\Delta H_p$ values given in Tables I and II.

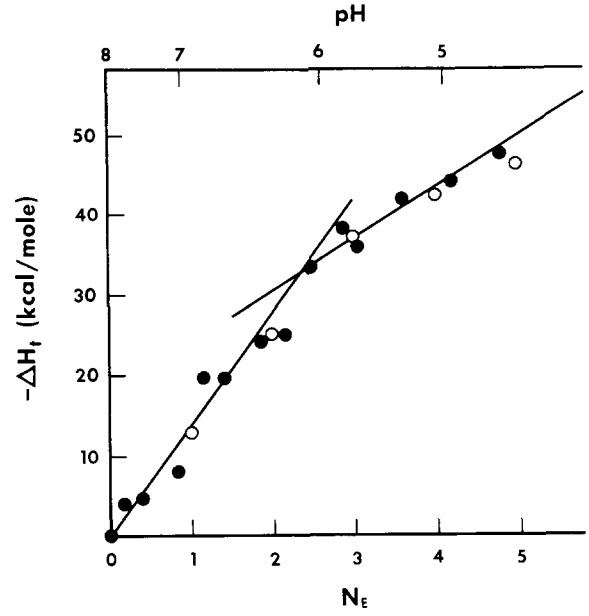


FIGURE 2: Enthalpy, $\Delta H_t$, vs. proton uptake, $N_E$, curve for ribonuclease A from pH 8.0 to pH 4.5, $\mu = 0.05$, $T = 25°$. $\Delta H_t$ and $N_E$ have been seen equal to zero at pH 8.0. The solid lines have slopes of 14 kcal/mol ($N_E = 0$–2) and 6.5 kcal/mol ($N_E = 2$–5) but have no other direct theoretical significance. See text for details.

in good agreement with −4.0 and −6.3 kcal/mol deduced from the temperature dependence of the p$K$ values of these two groups in 0.2 $M$ salt (Roberts et al., 1969).

Interpretation of the titration region between pH 8 and 6 is somewhat more difficult because contributions from protonation of the amino groups may be significant. Since in the vicinity of pH 7.0 $d\Delta H_t/dN_E = -14$ kcal/mol it can be concluded, however, that at least one of the groups titrating in this region exhibits an abnormally large $\Delta H_p$. It is most likely that this residue is histidine-48 since $\Delta H_p$ for histidine-105 has been estimated to be −8.2 kcal/mol (Roberts et al., 1969) and since it is unlikely that amino group titration is making any major contribution to $\Delta H_t$ at pH 7 or below.

Our potentiometric titration data between pH 8 and 4 were analyzed in terms of contributions from four independent histidine residues, a single "effective" amino group,[3] and the carboxyl groups. It was assumed that the contributions of carboxyl groups were as described by Tanford and Hauenstein (1956) using an electrostatic interaction parameter, $\omega = 0.09$. Initial estimates for the apparent p$K$ values of the histidine residues were taken to be those deduced by Ruterjans and Witzel (1969) from NMR experiments at a salt concentration <0.1 $M$. The titration curve was then computer simulated allowing the p$K$ values of the four histidine groups to vary ±0.3 about these initial guesses and allowing the p$K$ value of the single "effective" amino group to vary from 7.5 to 8.5. The "best fit" [4] p$K$ values were found to be 5.0, 5.8, 6.6, and 6.7 for the histidine residues and an "effective" p$K \sim 8.0$ for the amino group.

The calorimetric titration curve for RNase (Figure 1) was analyzed according to eq 1 assuming that $\Delta H_p = -6.5$ kcal/mol for the groups with p$K$ = 5.0, 5.8, and 6.6 and assuming $\Delta H_p = -11$ kcal/mol[5] for the amino group, p$K$ = 8.0. $\Delta H_p$ for the group with p$K$ = 6.7 was allowed to vary until a good fit to the data was achieved. The best fit value was found to be −24 kcal/mol. The solid curve in Figure 1 was calculated using these estimates for the various $\Delta H_p$ and p$K$ values.

Insofar as the exact value of the "effective" p$K$ for the amino group could influence the estimated value of $\Delta H_p$ for the histidine group with p$K$ = 6.7, additional simulations were performed assuming different values between 7.5 and 8.5 for the p$K$ of the amino group. In all cases a good representation of the data was achieved but the variation in the estimated value of $\Delta H_p$ for the p$K$ = 6.7 group was only from −22 to −27 kcal/mol.

*Titration of the 3′-CMP–RNase Complex.* Once the ionization properties of the histidine residues of free enzyme were satisfactorily defined, calorimetric and potentiometric titrations of the RNase–3′-CMP complex were carried out to determine the appropriate p$K$ and $\Delta H_p$ values for the ionizable groups of the complex. The concentration of 3′-CMP used was such that it provided essential saturation of the enzyme over the entire pH range studied (Flogel and Biltonen, 1975). The ionization behavior of the RNase–3′-CMP complex was determined by mixing solutions of RNase and 3′-CMP, both at the same pH and back-titrating the resulting mixture to the initial pH. The number of proton equivalents absorbed per mole of enzyme, $\Delta N_t$, was calculated from the amount of titrant added. These results are presented as the solid circles in Figure 3A.

[3] Although several amino groups may be actually contributing to the titration behavior of the protein below pH 8.0 their contribution is probably dominated by the α-amino group of lysine-1. Therefore the data have been treated assuming that the amino group contribution arises totally from a single residue.

[4] The best fit was selected primarily upon the basis of a minimum in the RMS deviation between the observed and calculated values of $\Delta H_t$ or $\Delta N_t$. In some cases the set of parameters chosen as "best fit" was not that which gave a minimum in the RMS deviation but was selected to minimize systematic deviations in any region of the curve. In all cases, however, the RMS deviation ≤1 kcal/mol or 0.1 protons/mol.

[5] A nominal value of −11kcal/mol was assumed for the heat of protonation of the "effective" amino group. This assignment was based upon the facts that $\Delta H_p$ for protonation of the ε-amino group of lysine is −11.0 kcal/mol (Gruen et al., 1959) and $\Delta H_p$ for the protonation of that α-amino group of glycylglycine is −10.6 kcal/mol (Brunetti et al., 1968).

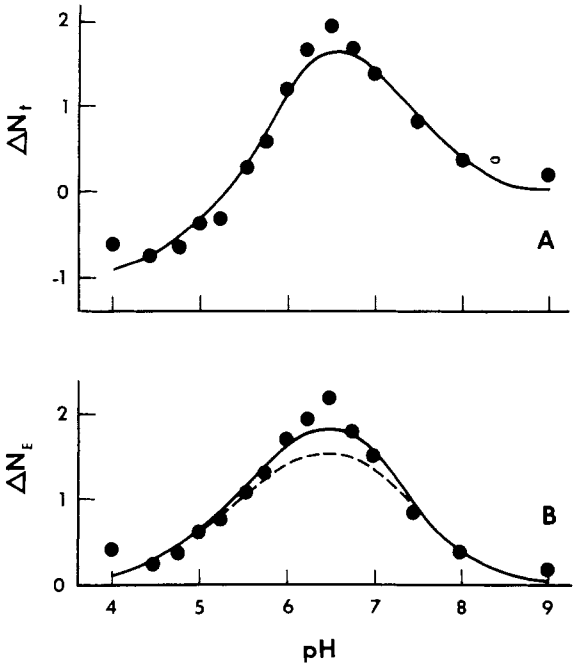


FIGURE 3: Proton uptake curve for formation of the 3′-CMP–ribonuclease A complex, $\mu = 0.05$, $T = 25°$. $\Delta N_t$ (A) is the experimental data and the solid line calculated assuming p$K$, p$K'$, $\Delta H_p$, and $\Delta H_p'$ values given in Table I and assuming p$K_I = 6.1$ and that only the dianionic form of the inhibitor binds to the enzyme. $\Delta N_E$ (B) is the experimental data corrected for ionization of the phosphate group of the inhibitor. The solid line was calculated assuming the ionization parameters given in Table I. The broken line was calculated assuming only two ionizable groups are involved in binding with p$K$ values of 5.0 and 5.8 and p$K'$ values of 7.4 each.

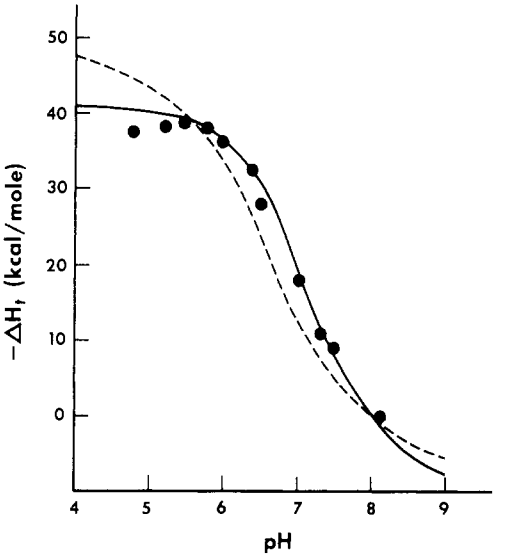


FIGURE 4: Enthalpy titration curve for the 3′-CMP–ribonuclease A complex, $\mu = 0.05$, $T = 25°$. The solid line was calculated for the complex assuming p$K'$ and $\Delta H_p'$ values given in Tables I and II. The broken line is the calculated enthalpy titration curve for the free enzyme as given in Figure 1.

Above pH 5.4 protons are absorbed upon binding of the inhibitor to the enzyme; below pH 5.4 protons are released. Since the ionization state of 3′-CMP presumably changes upon binding to the enzyme, analysis of the data must take into account ionization of the inhibitor. Assuming that only the dianionic phosphate form of the inhibitor[6] binds to the enzyme, an assumption whose validity will be established later, means that the proton releasing process at low pH is due to the phosphate moiety of inhibitor. The solid circles in Figure 3B are the results in Figure 3A corrected for proton release by the phosphate group of the inhibitor, and thus represent the actual proton absorption by the enzyme.

The relationship between $\Delta N_t$ and the ionization constants of the free enzyme and the complex is

$$\Delta N_t = \sum_j \left( \frac{[H^+]}{K_j' + [H^+]} \right) - \sum_j \left( \frac{[H^+]}{K_j + [H^+]} \right) - \frac{[H^+]}{K_I + [H^+]} \quad (2)$$

where $\Delta N_t$ is the experimentally determined proton uptake, $K_j$ and $K_j'$ are the dissociation constants of the ionizable groups of the free enzyme and the enzyme–inhibitor complex, and $K_I$ the dissociation constant for the phosphate group of 3′-CMP (p$K_I$ was found to be 6.12 in 0.05 $M$ KCl). Initial estimates for the $K_j$ values were assumed to be those obtained as outlined in the previous section. Estimates of the various $K_j'$ were then obtained by computer simulation of the $\Delta N_t$ vs. pH curve. The broken curve in Figure 3B was obtained by assuming that the p$K$ values of only two histidine residues, namely 12 and 119, increased to 7.4 upon complex formation. This curve qualitatively fits the data. However, if it is assumed that the p$K$ values of three histidine residues increased to about 7.3 upon complex formation the fit is improved. The solid curves in Figure 3A and B have been calculated assuming p$K$ values of the histidine residues as tabulated in Table I for the free enzyme and the complex.

Calorimetric titration of the 3′-CMP–RNase complex was carried out by mixing a solution of protein and excess nucleotide with solutions of varying concentration of HCl. The heat evolved was corrected for the heats of dilution of the components and the heat associated with titration of the excess nucleotide. The latter correction was not significant until the final pH was less than 5 since the heat of protonation of the phosphate group, p$K$ = 6.1, of the nucleotide is equal to 0 ± 300 cal/mol. Below pH 5, protonation of the ring nitrogen of the inhibitor makes a significant contribution to the measured heat. These results for the calorimetric titration of the inhibitor–protein complex, adjusted such that $\Delta H_t = 0$ at pH 8.1, are presented in Figure 4.

The solid curve in Figure 4 was calculated according to eq 1 assuming that the ionization characteristics of the "effective" amino group were the same as those deduced for the free enzyme (an "effective" p$K$ = 8, $\Delta H_p = -11$ kcal/mol) and that only $\Delta H_p$ for the residue 48 changed upon complex formation. The "best fit" [4] curve was found for the p$K$ and $\Delta H_p$ values tabulated in Tables I and II.

## Discussion

*Errors in Measurement and Analysis of the Data.* The accuracy of the calorimetric and potentiometric measurements over the experimental pH range are on the order of 1 kcal/mol and 0.1 proton/mol, respectively. However, the

[6] There is little existing data which indicate that the charge of the ring nitrogen of the cytidine nucleotides (p$K \sim 4.3$) has any influence on the binding or catalytic reactions of ribonuclease A. In fact, Anderson et al. (1968) report that the p$K_a$ for this functional group in the enzyme complex corresponds to the p$K_a$ 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.

Table I: Apparent p$K$ Values for the Histidine Residues of Ribonuclease A and Its Complex with 3′-CMP.[a]

| | $pK_{12}$ | $pK_{48}$ | $pK_{105}$ | $pK_{119}$ |
|---|---|---|---|---|
| | Ribonuclease A | | | |
| Potentiometric titration | 5.8 | 6.7 | 6.6 | 5.0 |
| Calorimetric titration | 5.8 | 6.7 | 6.6 | 5.0 |
| Difference titration | 5.8 | 6.6 | | 5.0 |
| | 3′-CMP–Ribonuclease Complex | | | |
| Calorimetric titration | 7.1 | 7.1 | 6.6 | 7.1 |
| Difference titration | 7.3 | 7.2 | | 7.3 |

[a] Conditions: $T = 25°$, $\mu = 0.05$.

Table II: Apparent Thermodynamic Quantities for Histidine Ionization of Ribonuclease A and Its Complex with 3′-CMP.[a]

| Residue | p$K$[b] | $-\Delta G°$ (kcal/mol) | $-\Delta H°$ (kcal/mol) | $\Delta S°$ (kcal/mol) |
|---|---|---|---|---|
| | Ribonuclease A | | | |
| 12 | 5.8 | 7.9 | 6.5 | 5 |
| 48 | 6.7 | 9.1 | 24 | −50 |
| 105 | 6.6 | 9.0 | 6.5 | 8 |
| 119 | 5.0 | 6.8 | 6.5 | 1 |
| | 3′-CMP–Ribonuclease Complex | | | |
| 12 | 7.2 | 9.8 | 6.5 | 11 |
| 48 | 7.1 | 9.6 | 20 | −35 |
| 105 | 6.6 | 9.0 | 6.5 | 8 |
| 119 | 7.2 | 9.8 | 6.5 | 11 |

[a] Conditions: $T = 25°$, $\mu = 0.05$. Standard state is 1 mol/l. [b] The listed p$K$ values are average values derived from the results summarized in Table I.

error in estimating particular $\Delta H_p$ and p$K$ values is substantially greater than this. Also note that the assignment of p$K$ values to specific histidine residues is based on NMR experiments by Ruterjans and Witzel (1969) under low salt conditions.

The errors in the p$K$ and $\Delta H_p$ values for residues 12 and 119 for the free enzyme are estimated to be approximately ±0.2 and ±2 cal/mol, respectively. However, we are unable, within terms of our data, to clearly distinguish between residues 48 and 105 with respect to either the enthalpy of protonation or the associated p$K$ value. For example, although it is clear that their average $\Delta H_p \simeq -14$ kcal/mol apportionment of the total heat is equivocal. Equally consistent with our reported analysis would be that the $\Delta H_p$ for each residue is equal to −15 kcal/mol. Our reported analysis (Tables I and II) was based upon the *assumption* that residue 105 is normal with respect to both p$K$ and $\Delta H_p$.

Analysis of the enzyme–inhibitor complex data is even more difficult because the apparent p$K$ values for all four histidine residues are similar and it is impossible to clearly distinguish between them. Although we can identify the existence of three histidine residues with an average p$K$ of about 7.1 and one with a somewhat lower p$K$ we are unable to make any clear assignments. The assignment in Table II has been made assuming the p$K$ and $\Delta H_p$ of His-105 are unaffected by the presence of inhibitor. Because of the close similarity of p$K$ values of all four histidine residues the assignment of exact $\Delta H_p$ values is impossible. 3′-CMP binding experiments as a function of pH show, however, that the $\Delta H_p$ values for His-12 and -119 are essentially unchanged upon complex formation (Flogel and Biltonen, 1975) and because His-105 does not appear to be influenced by inhibitor binding (Meadows et al., 1969) the unusually large $\Delta H_p$ was again assigned totally to His-48. It should be noted that the p$K$ values of residues 12 and 119 in the complex have been assumed to be equal. This is probably incorrect (Meadows et al., 1969; Flogel and Biltonen, 1975) and thermodynamic quantities listed for these two residues are, in fact, only average values for the two. For example, an average p$K$ = 7.1 is essentially equivalent to the two residues possessing p$K$ values of 6.8 and 7.4, respectively.

Lysine-41 is an important residue involved in the binding of nucleotides to RNase. In the preceding analysis and discussion it has not, however, been explicitly taken into account for several reasons. First, for this residue to seriously influence the data reported herein it is necessary that its p$K$ < 8.5 in either or both the free enzyme and the nucleotide–enzyme complex. There is little existing evidence suggesting this to be the case; the "effective" amino group with p$K$ ~ 8.0 is most likely the $\alpha$-amino group of lysine-1. Second, unless the p$K$ of the "effective amino" group is seriously shifted ($\Delta pK > 0.3$) upon complex formation its influence upon the proton uptake curve (Figure 3) and deduced thermodynamic model for nucleotide binding (Flogel and Biltonen, 1975) will be negligible. In the third paper of this set (Flogel et al., 1975) it is shown that the majority of the electrostatic interaction between the ligand and the protein occurs with the two histidine residues at the defined binding site. Third, inclusion of such a shift in the p$K$ of an amino group would require a more complex model to describe the binding reaction as a function of pH.

Although we cannot exclude small perturbations of the p$K$ of lysine-41 upon complex formation it does not appear that this amino acid residue is an important feature of the overall thermodynamic model presented in this and the following papers. It is not to be concluded that this residue is insignificant in the binding of nucleotides to RNase, but rather it does not profoundly influence the phenomenological characteristics of the binding reaction over the pH range 4–9.

It is to be emphasized that the sets of parameters tabulated in Tables I and II are not to be considered unique in the sense that no other set could equally well represent the results for a particular type of experiment. For example, the potentiometric titration behavior of histidine groups on the free enzyme is equally well described by assuming an intrinsic p$K$ = 6.5 for all four groups and allowing for electrostatic interaction. However, NMR studies clearly show that such a model is incorrect. For this reason the analysis of our results began by using published NMR results as a point of origin from which to search for a self-consistent set of parameters which adequately described the potentiometric, calorimetric, and difference titration results. The test of uniqueness of our representation thus rests upon the fact that a single set of such parameters was found.

*Thermodynamic Identification of the Proton Releasing Group.* NMR studies (Ruterjans and Witzel, 1969) have clearly established that the p$K$ values of the four histidine residues in the RNase-3′-CMP complex are greater than 6.4. Therefore, the ionizable group which is releasing a proton upon complex formation as seen in the differential titra-

tion data (below pH 5.5) must be either the phosphate group of the inhibitor or a carboxyl group (or groups) of the enzyme. Assuming that only one such group is involved, its p$K$ can be estimated in the following way. The proton uptake function $\Delta N_t$ (eq 2), can be written as

$$\Delta N_t = \Delta N(\text{His}) + \frac{[H^+]}{K_c^* + [H^+]} - \frac{[H^+]}{K_f^* + [H^+]} \quad (3)$$

where $\Delta N$(His) is proton uptake due only to the histidine residues of the protein, and $K_c^*$ and $K_f^*$ are the dissociation values of the proton releasing group in the complex and free form, respectively. Using the estimated p$K$ values for the histidine residues in the free enzyme and letting all p$K' \geq$ 6.4 bounds can be established on $\Delta N$(His) at pH 5.4 where $\Delta N_t = 0 \pm 0.1$ such that $1.1 \geq \Delta N(\text{His}) \geq 0.7$. These bounds establish a lower limit on p$K_f^* \geq 5.8$. Since $\Delta N_t = -0.7$ at pH 4.0 an upper limit on p$K_c \leq 3.7$ can also be set. Since a p$K \geq 5.8$ is very large for a carboxyl group and approximately equal to the measured p$K$ for the phosphate group of 3′-CMP we conclude that the proton releasing group involved in the interaction of 3′-CMP with RNase is the phosphate of the inhibitor, and that above pH 4.0, 3′-CMP is predominantly in the dianionic form when bound to the enzyme.

The above conclusion is, however, contradictory to the model of Hummel and Witzel (1966) which assumed that only the monoanionic form of the phosphate bound to RNase in the pH range 4–7. While their model provided a reasonable representation of their results, we have rejected it as a viable alternative for several reasons. First, given that the binding locus of the enzyme is positively charged, it is thermodynamically unreasonable to expect that the p$K$ of a negatively charged ligand will be reduced upon binding. Second, their conclusion that the fact the pH position of zero proton uptake appeared to be invariant with the p$K$ of the phosphate group of the ligand implied the proton releasing group is associated with the protein is not valid. Simple calculation shows that the p$K$ of this releasing group $\geq$5.8 (cf. p 21). If this is so the point of zero proton uptake which is $\ll$5.8 is virtually insensitive to that p$K$. For example, the point of zero proton uptake will change 0.05 pH unit if the p$K$ of the releasing group changes from p$K$ = 6 to p$K = \infty$. Third, the development of any model to represent our results would necessarily be more complex if we assumed that only the monoanion bound. Furthermore, the recent phosphorus NMR results of Gorenstein and Wyrwicz (1973) and Haar et al. (1974) clearly show that the predominant form of the phosphate group is dianionic when bound to RNase. For these reasons we conclude that the only dianionic phosphate form of the ligand binds to RNase in the pH range of 4–9.

This conclusion does not exclude the possibility of monoanionic binding, but only means that it binds much more weakly than the dianionic form. It should therefore be possible to describe the thermodynamics of interaction of 3′-CMP, as well as other nucleotide inhibitors, with RNase over this pH range assuming that only the dianionic form of the ligand is bound.

*Histidine Ionization.* The calorimetric and potentiometric titration results are dominated by two facts. (1) There exist two imidazole residues in the free enzyme which possess unusually low proton association constants but which dramatically increase upon complex formation. The heats of protonation of these groups, however, are normal and not appreciably affected by the presence of the inhibitor. This conclusion is in total agreement with previous NMR studies. (2) There is at least one imidazole group, presumably that of histidine-48, which is characterized by a normal free energy of protonation (related to a p$K$ of about 6.7–7.2) but which shows an unusually high enthalpy ($-24 \pm 3$ kcal/mol) and entropy change ($-50 \pm 10$ cal/(mol deg)) associated with the protonation process. The ionization characteristics of this group do not appear to be as significantly affected by complex formation as those of residues 12 and 119. These results are consistent with previous studies (Cathou and Hammes, 1965; Ruterjans and Witzel, 1969; Haar et al., 1974).

The two histidine residues which have the low p$K$ values of 5.0 and 5.8 in 0.05 $M$ salt are certainly residues 119 and 12, respectively. This has been well established by nuclear magnetic resonance experiments (Ruterjans and Witzel, 1969; Meadows et al., 1968; King and Bradbury, 1971). The two histidines in the binding site are located relatively close to positively charged residue(s). Insofar as electrostatic interactions in an aqueous environment are entropically dominated (Kauzmann, 1959), the fact that the heat of protonation of residues 12 and 119 is "normal" is consistent with the conclusion that electrostatic interaction is responsible for the suppression of their p$K$ values.

The increase in these p$K$ values upon complex formation is expected because of the presence of the negatively charged phosphate group of the inhibitor. The magnitude of the shift in p$K$ values of these histidine residues upon inhibitor binding indicates that the electrostatic interaction with the phosphate group may provide about 5 kcal/mol of favorable free energy (Flogel and Biltonen, 1975) which is the dominant driving force for complex formation. This result demonstrates the thermodynamic importance of the phosphate group in complex formation and will be discussed in more detail in a later paper.

Our results have shown that there exists at least one histidine residue which has an unusual heat of ionization. It is impossible from our data to determine whether this unusual behavior is to be attributed to one or both histidine-105 and -48. However, other studies strongly suggest that this unusual behavior is to be found primarily in residue 48. X-Ray analysis (Richards and Wyckoff, 1971) of the crystal structure shows a fairly high degree of solvent accessibility for residue 105. NMR studies (Ruterjans and Witzel, 1969; Meadows et al., 1969; Roberts et al., 1969) have established that the chemical shift, p$K$, and $\Delta H_p$ of histidine-105 are similar to those for a normal imidazole and that these properties are not significantly affected by either complex formation or ionic strength.

On the other hand, X-ray studies (Wyckhoff et al.,1970) have shown that residue 48 is "buried" in the enzyme and not readily accessible to the solvent. NMR experiments (Ruterjans and Witzel, 1969) have shown that the chemical shift of histidine-48 is different than that for a normal histidine residue and that the chemical shift and the line width are very strong functions of ionic strength. King and Bradbury (1971) have also demonstrated that the chemical shift of the C-2 carbon of residue 48 undergoes a "sudden" change in the pH region near its p$K$. Cathou and Hammes (1965) have concluded that a single histidine residue in RNase with a p$K \simeq$ 6.7–6.8 possesses a very large heat of protonation (10–20 kcal/mol). This residue is apparently not located at the binding site but is implicated in a conformational change in both the enzyme and its complex. They concluded that the most likely candidate for this residue

was histidine-48. On the basis of this information we conclude that all of the unusual heat of protonation of the histidine residues is associated with histidine-48 and is probably a manifestation of a conformation rearrangement of the macromolecule.

We have also found that ionic strength profoundly affects the heat of protonation of this histidine residue (Flogel et al., 1973). Since the influence of salt on the ionization behavior of all of the residues and the binding of the nucleotide inhibitors will be coupled to some extent, detailed analysis of the pH and ionic strength dependence of binding of 3′-CMP with RNase should provide further insight into the problem.

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