The Magnitude of Electrostatic Interactions in Inhibitor Binding and during Catalysis by Ribonuclease A†

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ABSTRACT: It is demonstrated that a model of nucleotide binding to ribonuclease A similar to that proposed by Hammes and coworkers (G. G. Hammes (1968), *Adv. Protein Chem. 23,* 1) is, at least, approximately applicable for both cyclic nucleotide substrates and mononucleotide inhibitors at pH values ≤6.5 and as a function of ionic strength. Calorimetric data on various inhibitors show that the binding reaction can be thermodynamically dissected into a contribution arising from van der Waal's interactions of the nucleoside moiety, characterized by a large negative enthalpy change, and a contribution arising from electrostatic interactions between the negatively charged phosphate group of the inhibitor and the positively charged protein fabric, characterized by a large positive unitary entropy change. Assuming a catalytic mechanism involving the formation of a dianionic pentacoordinated phosphate transition state intermediate, the magnitude of the effect of electrostatic interactions on the overall rate enhancement by the enzyme is estimated to be 2×10^2 to 10^6. It is suggested that this effect, along with substrate approximation effects, is sufficient to "explain" the catalytic behavior of the enzyme.

Ribonuclease A (RNase) is a structurally well defined enzyme (Richards and Wyckoff, 1971) upon which many investigations relating to its mechanism of action have been focused (Usher and Richardson, 1970; Usher et al., 1972; Witzel, 1963). Numerous studies have provided structural (Wyckoff et al., 1970), thermodynamic (Hammes et al., 1965), and kinetic (Herries et al., 1962; Hammes, 1968) information regarding the interaction of charged substrates and inhibitors with the enzyme. These investigations have clearly demonstrated the importance of electrostatic interactions between the anionic phosphate moiety of nucleotide inhibitors and substrates and the two histidine residues at the catalytic site of the enzyme. It has also been suggested (Witzel, 1963) that similar interactions with other positively charged groups of the enzyme, mainly lysine-41, may play a significant role in both thermodynamic stabilization of the complex and in the catalytic reaction. Most chemical mechanisms which have been proposed to describe the catalytic action of RNase include the formation of a dianionic pentacoordinated phosphate intermediate in the reaction scheme. The thermodynamic significance of such an intermediate is that it can potentially be stabilized by the positively charged environment of the catalytic site. In this communication the magnitude of such electrostatic stabilization and its possible importance in the catalytic reaction are estimated.

Experimental Section

Ribonuclease A (RNase) was purchased from Worthington Biochemical Corporation and used without further purification. Chromatographically pure cytidine, 2′-cytosine monophosphate (2′-CMP), and 3′-cytosine monophosphate (3′-CMP) were purchased from Boehringer Mannheim and used without further purification. The protein and inhibitor solutions were prepared and concentrations determined as previously described (Bolen et al., 1971; Flogel and Biltonen, 1975b). The calorimetric experiments and analysis of data were performed as described previously (Bolen et al., 1971; Flogel and Biltonen, 1975b).

Results

It has previously been demonstrated that the pH dependence of the binding of the cyclic nucleotide substrate, 2′,3′-cytosine monophosphate (2′,3′-CMP), can be phenomenologically described in terms of the ionization of two groups of the enzyme whose pK values increase upon binding (Herries et al., 1962). A model involving the coupled ionization of three ionizable groups was proposed for the binding of 3′-uridine monophosphate to RNase (Hammes and Walz, 1969). Calorimetric and potentiometric data related to the binding of 3′-cytosine monophosphate (3′-CMP) to RNase presented in the previous papers of this series (Flogel and Biltonen, 1975a,b) were analyzed in similar thermodynamic terms incorporating three histidine residues (12, 48, and 119) into the model. That model is summarized in terms of the apparent free energy change for binding, ΔG_{app}, as a function of pH in eq 1, where $\Delta G°$ is the

$$\Delta G_{app} = \Delta G° - RT\sum_{j=1}^{3} \ln\left(\frac{K_j' + (H^+)}{K_j + (H^+)}\right) - RT \ln (1 + (H^+)/K_T) \quad (1)$$

standard free energy change for the reaction in which the dianionic phosphate form of the inhibitor[1] binds to the pro-

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[1] There is little existing data which indicates that the charge of the ring nitrogen of the cytidine nucleotides (pK ~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.

Table I: Comparison of Calculated Thermodynamic Quantities for the Binding of 3′-CMP to Ribonuclease A.[a]

pH	$-\Delta G^\circ$ (kcal/mol)[b]	$-\Delta H^\circ$ (kcal/mol)[b]
4.0	10.6[c] (10.6)[d]	5.7[c] (5.7)[d]
4.5	11.0 (11.0)	8.0 (7.9)
5.0	10.7 (10.7)	7.3 (7.2)
5.5	10.7 (10.7)	8.5 (8.0)
6.0	10.5 (10.5)	7.7 (6.4)
6.5	11.2 (11.0)	9.8 (7.6)
Av[e]	10.8 ± 0.2 (10.8 ± 0.2)	7.8 ± 0.9 (7.1 ± 0.7)[d]

[a] Conditions: $T = 25°$, $\mu = 0.05$. [b] Standard state is unit mole fraction. [c] Quantities calculated according to eq 2 and 3 assuming pK_1 = 5.0 and pK_2 = 5.8 and $\Delta H_{p,1} = \Delta H_{p,2} = -6.5$ kcal/mol. [d] Quantities calculated using complete expression for ΔG_{app} and ΔH_{app} and parameters given in Table III of Flogel and Biltonen (1975b). [e] These average values differ slightly from those reported in the previous paper (ΔG° = 11.0 kcal/mol, ΔH° = −6.7 kcal/mol) because the latter values were averaged over the entire pH range of 4–9. In any case the differences are within the error estimates.

Table II: Standard Free Energy Changes, ΔG°, Associated with the Binding of Phosphate Ligands to Ribonuclease A.[a]

	$-\Delta G^\circ$ (kcal/mol)[b]			
pH	2′, 3′-CMP[c, d]	2′-CMP[c, e]	3′-CMP[c, e]	Orthophosphate[c, e]
4.0	6.5	10.3		
4.5	7.0	10.7	9.4	8.1
5.0	7.2	11.1	9.5	7.8
5.5		11.2	9.4	7.8
6.0	6.8	10.9	9.3	7.6
6.5	6.9	10.8	9.5	7.8
Av[f]	6.9 ± 0.2	10.8 ± 0.2	9.4 ± 0.1	7.8 ± 0.1

[a] Conditions: $\mu = 0.2$, $T = 25°$. [b] ΔG° refers to the standard free energy for dianion binding to the completely protonated enzyme calculated as indicated in the text. Standard state is unit mole fraction. [c] pK values used for the free enzyme were those reported by Meadows et al. (1969) under these salt conditions. The pK for the phosphate group of 2′- and 3′-CMP was assumed to be 6.1 (Flogel and Biltonen, 1975a). The pK of orthophosphate was assumed to be 6.7. [d] Based on the K_m values reported by Herries et al. (1962). [e] Based on the reported results of Anderson et al. (1968). [f] Deviations reported are the average deviations of the mean.

tein in which the three relevant histidine residues (j = 1–3) are protonated in both the free enzyme and complex. K_j and K_j' are the proton dissociation constants for the jth residue in the free enzyme and complex, respectively. K_I is the dissociation constant for the phosphate group of the inhibitor. (In this model it is assumed that only the dianionic phosphate form of the inhibitor binds to the protein, an assumption confirmed by recent nuclear magnetic resonance studies (Gorenstein and Wyrwicz, 1973; Haar et al., 1974) and thermodynamic arguments (Flogel and Biltonen, 1975a)).

The previous papers of this series and nuclear magnetic resonance studies (Meadows et al., 1969) have shown that all $K_j > K_j'$ and, at least for the binding of 2′-CMP and 3′-CMP, all $K_j' < 2 \times 10^{-7}\ M^{-1}$. In addition the pK of histidine-48 has been shown to be greater than 6.4 (Ruterjans and Witzel, 1969). Therefore, at pH values ≤6.5, eq 1 can be adequately approximated as

$$\Delta G_{app} \simeq \Delta G^\circ + RT \sum_{j=1}^{2} \ln (1 + K_j/(H^+)) - RT \ln (1 + (H^+)/K_I) \quad (2)$$

where the sum is now over the two terms representing the contribution of residues 12 and 119 of the enzyme. Similarly,

$$\Delta H_B \simeq \Delta H^\circ + \sum_{j=1}^{2} \frac{\Delta H_{p,j}}{K_j + (H^+)} \quad (3)$$

where ΔH° is the intrinsic heat of binding and $\Delta H_{p,j}$ is the heat of protonation of the jth residue. The heat associated with ionization of the phosphate group is 0 ± 300 cal/mol and its contribution is not explicitly taken into account in eq 3. The advantage of this approximate representation is that the first term related to the pH dependence of ΔG_{app} and ΔH_B is simply related to the ionization parameters of two residues in the free enzyme which have been well defined by nuclear magnetic resonance (NMR) experiments (Meadows et al., 1969; Ruterjans and Witzel, 1969) at a number of salt concentrations, and as such, are identical for all nucleotide binding reactions. The second pH dependent term in ΔG_{app} is only a function of the pK of any particular inhibitor. Thus ΔG° and ΔH° values can be calculated for the binding of nucleotide inhibitors to RNase without any knowledge regarding the pK values of the histidine residues in the enzyme–inhibitor complex.

The validity of the above described model for the binding of 3′-CMP to RNase at $\mu = 0.05$ has been established in the previous paper (Flogel and Biltonen, 1975b). The accuracy of the approximation given in eq 2 and 3 for ΔG° and ΔH° is demonstrated in Table I where these quantities have been calculated for 3′-CMP using both the complete and approximate mathematical representations from results obtained at pH values from 4 to 6.5 (Flogel and Biltonen, 1975b). As can be seen, the deviations in ΔG° between the two methods of calculations for a given pH value are less than the average deviation of their mean values over this pH range. Significant deviations in ΔH° are only observed at pH values >6.0. Insofar as only ΔG° values for different ligands will generally be compared in this paper, these results demonstrate the validity of the approximate representation over the pH range of 4–6.5. It is to be noted that the standard state for the quantities listed in Table I and subsequent tables is unit mole fraction.[2]

The general validity of this model for the binding of several ligands to RNase at 0.2 M salt as well as the use of the approximate equation for ΔG° is demonstrated in Table II. Under these salt conditions the pK values of the protein are different than used in the analysis of the results for 3′-CMP at 0.05 M salt. However, the appropriate pK values have previously been determined by nuclear magnetic resonance experiments (Meadows et al., 1968) and the calculations were performed using these estimates. Excellent self-consistency of the calculated values of ΔG° is obtained over the pH range 4–6.5 for all four ligands.

In an effort to thermodynamically dissect the various interactions involved in ligand binding to RNase, the thermodynamics of binding of several ionic ligands have been determined calorimetrically at 25°, $\mu = 0.05$ as previously described (Flogel and Biltonen, 1975b). These results are tab-

[2] The unitary free energy and entropy change are those thermodynamic quantities calculated assuming a standard state of unit mole fraction. The quantities differ by −2.4 kcal/mol and +8 cal/(mol deg) from those based on a standard state of 1 m/l. and correct for a mixing contribution.

Table III: Thermodynamic Quantities Associated with the Binding of Various Ligands to Ribonuclease at 25°, $\mu = 0.05$.[a]

Ligand	$-\Delta G_{app}$[b,c]	$-\Delta H_B$[b]	$-\Delta G°$[b,c]	$-\Delta H°$[b,c]	$\Delta S°$[c] (cal/(mol deg))
Cytidine (pH 6.5)	5.1	6.1	5.1	6.1	−3
Phosphate[d] (pH 6.5)	6.8	12.2	10.5	0.3	36
Phosphate (pH 5.5)	6.6	8.2	9.4	−0.1	31
3′-CMP[e] (pH 4–6.5)			10.8	7.1	12
2′-CMP (pH 6.5)	8.6	18.3	12.0	6.6	18
2′-CMP (pH 5.5)	9.6	13.1	11.7	6.0	19

[a] The values listed in the first two columns are the experimental quantities from which the values in the last three columns have been calculated according to eq 2 and 3 as described in the text. These latter values correspond to the reaction in which the fully ionized inhibitor binds to the fully protonated enzyme. [b] Units are kcal/mol. [c] The standard state is unit mole fraction. [d] See text regarding the interpretation of the calculated free energy change from this experiment. [e] The calculated thermodynamic quantities are the average values obtained over the pH range 4–6.5 using the complete expression defining the pH dependence of the apparent thermodynamic quantities.

ulated in Table III. The first set of results, ΔG_{app} and ΔH_B are the apparent thermodynamic quantities obtained, and the second set, $\Delta G°$, $\Delta H°$, and $\Delta S°$, calculated as described in the preceding text and the footnote to the table, refer to the binding of the dianionic inhibitor to the protonated enzyme.

Discussion

Interpretation of $\Delta G°$ and $\Delta H°$. The intrinsic thermodynamic quantities ($\Delta G°$, $\Delta H°$, and $\Delta S°$) given in Table III provide a basis for the interpretation of the details of the molecular interactions. First consider the binding of the nucleoside, cytidine, to the enzyme. No change in proton binding upon cytidine binding at pH 6.5 was observed (see also Hummel and Witzel, 1966) which indicates that the ligand does not significantly interact with the histidine residues of the enzyme. Thus the thermodynamic changes associated with cytidine binding are pH independent, and the $\Delta G_{app} = \Delta G°$. It is seen in Table III that the thermodynamic driving force for binding of the nucleoside is a favorable enthalpy change compensated by a small loss in entropy. Such a thermodynamic description would be expected for an association reaction primarily involving strong van der Waal's interactions, including hydrogen bonding.

The lack of a positive entropy change for cytidine binding indicates that hydrophobic interactions are not a significant factor in this interaction. This conclusion is consistent with the thermodynamic results of Alvarez and Biltonen (1973) and Scruggs et al. (1972) for the transfer of nucleic acids from water to organic solvents. It is also significant that $\Delta G°$ and $\Delta H°$ for cytidine are very similar to these same quantities for the binding of the dianionic form of 3′-CMP to the *unprotonated* protein ($\Delta G_0° = -5.8$ kcal/mol and $\Delta H_0° = -8.7$ kcal/mol; Flogel and Biltonen, 1975b).

The results in Table III show that the binding of the orthophosphate dianion to RNase is entropically favored. It is to be noted that the approximate equations (eq 2 and 3) may not apply to the binding of orthophosphate at pH 6.5, $\mu = 0.05$ because this ligand may not shift the pK values for His-119 and -12 to sufficiently large values (i.e., pK values in the complex may be < 7.0. See Meadows et al. (1969)). In any case the calculated values for $\Delta G°$ and $\Delta H°$ at this pH still demonstrate that the reaction is entropically driven. This is to be expected since ionic association in an aqueous medium is usually dominated by a positive entropy change (Kauzmann, 1959). It is interesting to note that the thermodynamics of interaction of the phosphate dianion with the positively charged enzyme at 0.05 M salt is similar to proton association to diphosphoric acid ($\Delta G° = -9.4$ kcal/mol and $\Delta S° = 32$ cal/(mol deg)).

Comparison of the $\Delta G°$ and $\Delta H°$ values for binding of 2′- and 3′-CMP with that of cytidine indicates that the enhanced affinity of the nucleotides is primarily the result of a positive entropy contribution to the free energy change; $\Delta H° = 6.6 \pm 0.5$ kcal/mol for cytidine, 3′-CMP and 2′-CMP under the conditions explored in the experiments cited in Table III.[3] These results indicate that the thermodynamics of binding of the nucleotides to RNase can be separated into distinct contributions: the interaction of the nucleoside moiety characterized by a large negative enthalpy change and the interaction of the phosphate group, characterized by a large positive entropy change. The enhanced affinity of the nucleotides, relative to cytidine is, most likely, the result of the attractive electrostatic contribution to the overall free energy change. It thus appears that binding of nucleotides to RNase is the combined result of "structural specificity", as topologically defined by the enzyme binding site for the nucleoside moiety, and "thermodynamic selectivity" which is quantitatively defined by interaction of the negatively charged phosphate group with the positively charged environment of the enzyme.

Insofar as $\Delta H°$ for the binding of the nucleoside or nucleotides appears to be invariant, within experimental error, differences in $\Delta G°$ between different ligands are a quantitative index of the magnitude of the electrostatic interaction between the negatively charged phosphate group of the ligand and the positively charged protein fabric. This conclusion will be used to estimate the extent of the electrostatic interaction for specific ligands for which equilibrium binding data are available, but for which estimates of $\Delta H°$ are unavailable.

Estimation of Electrostatic Stabilization in Ligand Binding to RNase. The intrinsic free energies of binding of several ligands to RNase, $\Delta G°$, are tabulated in Table IV. The results tabulated were calculated as described using data presented in the literature, except for the new results presented in this paper. The results have been grouped according to the type of nucleic acid base and to the ionic strength under which the experiments were made. Several generalizations based upon these results can be made.

(1) For any series of ligands possessing the same nucleic acid base, $\Delta G°$, at constant ionic strength, becomes progressively more negative in the order: nucleoside, 2′,3′-cy-

[3] The actual $\Delta H°$ for the binding of 2′-CMP may be less negative than for 3′-CMP by 1–2 kcal/mol. The currently available data suggest this to be the case, but are insufficient in quantity to establish it to be so. Since the electrostatic interaction for the 2′ derivative is greater than for the 3′ nucleotide this is not surprising; enhanced electrostatic interaction could, in fact, be achieved at the expense of reduced van der Waals "contact". If this is the case, our estimates of the electrostatic interaction for 2′-CMP using $\Delta G°$ values are *lower* estimates for such interactions and it would be more appropriate to compare $\Delta S°$ values. In any case this possibility does not reduce the validity of the arguments regarding the relative magnitude of electrostatic interactions.

Table IV: Summary of the Free Energy Changes Associated with the Binding of Ligands to Ribonuclease.

Ligand	$-\Delta G^{\circ}$ [a,b]	$-\Delta G_{elec}$ [a,c]	$-\Delta G_{His}$ [a,d]
Phosphate (μ = 0.05)[g]	9.4	9.4	
Phosphate (μ = 0.2)[h]	7.8 ± 0.1	7.8	
Cytidine (μ = 0.05)[g]	5.1	0.0	
3'-CMP (μ = 0.05)[g]	10.8 ± 0.1	5.7	4.7[e]
2'-CMP (μ = 0.05)[g]	11.9 ± 0.2	6.8	
2',3'-CMP (μ = 0.2)[h]	6.9 ± 0.2	1.8	
3'-CMP (μ = 0.2)[h]	9.4 ± 0.1	4.3	4.7[f]
2'-CMP (μ = 0.2)[h]	10.8 ± 0.1	5.7	5.4[f]
Uridine (μ = 0.03)[i]	5.0	0.0	
2' 3'-UMP (μ = 0.2)[h]	7.2 ± 0.3	2.2	
3'-UMP (μ = 0.2)[h]	9.3 ± 0.1	4.3	
2'-UMP (μ = 0.2)[h]	10.9	5.9	

[a] Units are kcal/mol assuming a standard state of unit mole fraction. All calculations refer to 25°. The ionic strength is given in parentheses following the ligand name. [b] Values which have an error estimate are averaged results obtained at two or more pH values. The error listed is the average deviation of the mean. For phosphate (μ = 0.05) a single value was calculated from the results at pH 5.5 because the approximate representation used in these calculations may not apply at the higher pH values for this ligand. See text for details. [c] ΔG°_{elec} was calculated as described in the text. For orthophosphate it was assumed that $\Delta G^{\circ} = \Delta G^{\circ}_{elec}$. For cytidine and uridine it was assumed that $\Delta G^{\circ}_{elec} = 0$. [d] ΔG_{His} is the electrostatic interaction between the phosphate dianion and histidine residues 12 and 119 calculated as described in the text. [e] Calculated using pK values estimated by calorimetric and potentiometric titration experiments (Flogel and Biltonen, 1974a). [f] Calculated using pK values estimated by NMR experiments (Meadows et al., 1969). [g] Calculated from results reported in this paper. [h] Calculated from results reported by Anderson et al. (1968). [i] Calculated from results reported by Ukita et al. (1961) as summarized in Richards and Wyckoff (1971). The original data were obtained at pH 7.6, 37°, and ionic strength = 0.03. Assuming that the binding reaction was independent of pH and ionic strength, in analogy to cytidine binding, ΔG° at 25° was calculated assuming an enthalpy of binding = −6.1 kcal/mol.

clic nucleotide, 3'-nucleotide, 2'-nucleotide. This increase in affinity is the result of improved electrostatic interactions and is an example of thermodynamic selectivity mentioned above.

(2) It appears that at constant ionic strength ΔG° is very similar for any pair of cytosine or uracil derivatives having the phosphate group in the same position. Thus thermodynamic discrimination between "specific" ligands (i.e., those derived from cytosine or uracil) is primarily based on the phosphate moiety as manifested in the magnitude of the electrostatic interactions.

(3) Increasing ionic strength reduces the affinity of the dianionic phosphate ligands approximately equally. The difference between ΔG° values at μ = 0.05 and μ = 0.2 M is 1.5 kcal/mol for orthophosphate, 1.4 kcal/mol for 3'-CMP, and 1.1 kcal/mol for 2'-CMP. Ionic strength does not appear to effect ΔG° for cytidine (M. Flogel, unpublished observations), which suggests that the observed effect of ionic strength on ΔG° for the dianionic compounds is the result of reduced electrostatic interactions. However, this ionic strength effect does not appear to be simply a charge shielding phenomena, as will be discussed later.

The results in Table IV can also be used to estimate the magnitude of the free energy changes associated with electrostatic interactions upon ligand binding to RNase. First, the unitary free energy changes associated with the binding of orthophosphate would appear to be purely electrostatic. As such, ΔG° for phosphate binding at μ = 0.05 represents an upper limit of this interaction of −9.4 kcal/mol for a dianionic ligand. The electrostatic interaction of the phosphate group of a nucleotide would be expected to be significantly less than this, however, because of tethering on the protein surface by the nucleotide moiety.

An estimate of the magnitude of the electrostatic stabilization provided by the phosphate group of any nucleotide can be obtained by comparison of ΔG° for nucleotide binding with that for the nucleoside. Using the results tabulated in Table IV it is found that this free energy contribution

$$\Delta G^{\circ}_{elec} = \Delta G^{\circ}(\text{nucleotide}) - \Delta G^{\circ}(\text{nucleoside}) \quad (4)$$

varies from −1.8 kcal/mol for 2',3'-CMP at μ = 0.2 to −6.8 kcal/mol for 2'-CMP at μ = 0.05. These estimates, also summarized for various ligands in Table IV, probably represent lower limits, however, because of a potentially greater loss in configurational entropy in the nucleotide than in the nucleoside upon binding.

ΔG°_{elec} includes contributions from interactions of the phosphate group with the two histidine residues (12 and 119) at the binding site in addition to interaction with other charge groups on the protein surface, including lysine-41. The contribution of the electrostatic interaction with the two histidine residues, ΔG°_{His}, can be estimated from the shift in pK values of the two histidine residues of the enzyme in the presence of the nucleotide. That is, the free energy of interaction between the phosphate group and the histidine residues is

$$\Delta G^{\circ}_{His} = 2.303RT\sum_{j}(pK_j - pK_j') \quad (5)$$

where pK and pK' are the pK values of a particular histidine residue on the free enzyme and complex, respectively.

ΔG°_{His} can only be estimated for 3'-CMP at μ = 0.05 using the results of Flogel and Biltonen (1975a,b) and for 3'-CMP and 2'-CMP at μ = 0.2 using the pK values for the free enzyme and nucleotide complex determined by Meadows et al. (1969) using NMR.[4] These estimates are tabulated in column III of Table IV. We see first that ΔG°_{His} accounts for the majority of ΔG°_{elec}, as might be expected since residues 12 and 119 are in closest proximity to the phosphate group of the inhibitors. Second, it appears that the increased electrostatic stabilization of the 2'-nucleotide over the 3'-nucleotide is mostly, but not totally, the result of enhanced electrostatic interactions between the phosphate group and histidine residues 12 and 119. It also appears, on the basis of these limited results, that ΔG°_{His} is not a very strong function of the ionic strength; although ΔG°_{elec} increases by 1.4 kcal/mol for 3'-CMP when the ionic strength is increased from 0.05 to 0.2, ΔG°_{His} is unchanged. This last, tentative, conclusion suggests that ionic strength effects on nucleotide binding affinity is not simply a charge-shielding effect. In fact, the manifestation of ionic strength effects may be coupled with the conformational change associated with protonation of histidine-48 (Flogel et al., 1973). Further study is, of course, necessary to clarify the situation.

Electrostatic Interactions during Enzyme Catalysis. Usher and coworkers have demonstrated that conversion of 2',3'-nucleotides to the 3'-product by RNase most probably

[4] Although apparent pK values for the histidine residues in the presence of orthophosphate have been reported (Cohen et al., 1973; Meadows et al., 1969), these values cannot be used to accurately estimate ΔG° (His) since the enzyme was not completely saturated under the conditions of the experiments. Nevertheless, a lower limit of ΔG° (His) of approximately −6 kcal/mol can be calculated based on those results.

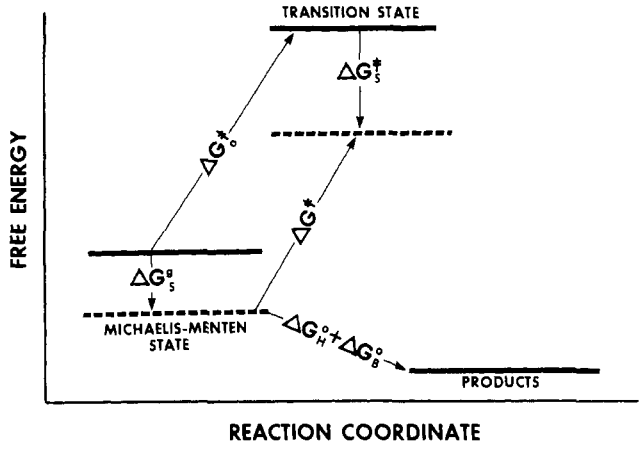

FIGURE 1: A schematic representation of the free energy profile along the reaction coordinate for the ribonuclease-catalyzed hydrolysis of cyclic mononucleotides. The solid lines represent the free energies of the enzyme–substrate Michaelis–Menten state and transition state which would exist in the absence of electrostatic stabilization. The broken line represents the free energies of these states in the presence of electrostatic interactions. $\Delta G_0^{\ddagger}$ and $\Delta G^{\ddagger}$ are the activation free energies in these two cases, respectively. The magnitude of electrostatic stabilization is labeled as ΔG_s^g for the Michaelis–Menten state and $\Delta G_s^{\ddagger}$ in the presumed dianionic transition state. ΔG_H° and ΔG_B° are the standard free energy changes for the hydrolysis reaction and product binding to the enzyme, respectively.

proceeds via an "in-line" mechanism in which one histidine residue acts as a proton acceptor and the other as a proton donor (Usher and Richardson, 1970). Such a mechanism is entirely consistent with kinetic data as well as X-ray crystallographic and nuclear magnetic resonance information regarding the structure of RNase and its nucleotide complexes (Richards and Wyckoff, 1971). Assuming such a mechanism and that a dianionic pentacoordinated phosphate intermediate exists in the transition state, the electrostatic effects on rate enhancement, as provided by interactions with His-12, His-119, and the remainder of the positively charged protein fabric, can now be estimated.

In Figure 1 a schematic diagram of the free energy profile along the reaction coordinate from the Michaelis–Menten complex to product is provided. The solid lines represent the free energies of the enzyme–substrate Michaelis–Menten state and the transition state which would exist in the absence of electrostatic stabilization. Under such conditions the activation free energy, labeled $\Delta G_0^{\ddagger}$, is related to the rate of the chemical reaction proceeding on the enzyme surface as catalyzed by the histidine residues without electrostatic stabilization making any contribution. (Note that while change in the charge distribution may occur along the reaction pathway, this does not influence the interpretation of $\Delta G_0^{\ddagger}$ in this hypothetical situation.)

In the actual reaction, however, both the Michaelis–Menten and transition states can be stabilized by electrostatic interaction. The free energies of these states in this case are represented by the broken lines. Chemical catalysis presumably only occurs when one of these histidine residues is protonated and the other unprotonated (Herries et al., 1962; del Rosario and Hammes, 1970). Thus the "active" Michaelis–Menten state can be stabilized by electrostatic interaction between a monoanionic phosphate and a single positively charged histidine. The magnitude of this interaction, ΔG_s^g, has been estimated to be equal to $\Delta G^{\circ}_{His}/4$ = 1.2–1.4 kcal/mol, where ΔG°_{His} is equal to the electrostatic interaction between a dianionic phosphate group and two positively charged histidine residues.

In the formation of the pentacoordinate transition state complex two thermodynamically significant events occur: the dianionic substrate intermediate is formed and the previously uncharged histidine residue becomes protonated. Thus the electrostatic interaction in the transition state is similar to that during binding a dianionic nucleotide inhibitor to the ionized protein. Thus the transition state is stabilized by an amount $\Delta G_s^{\ddagger} = \Delta G^{\circ}_{elec}$. Although it is difficult to estimate the electrostatic stabilization in the transition state, a lower limit of 4.3 kcal/mol can be established using 3′-CMP, μ = 0.2, as the appropriate analog. If 2′-CMP, μ = 0.05, is the appropriate analog the electrostatic stabilization afforded the hypothetical transition state complex is estimated to be 6.8 kcal/mol. Thus the net free energy of stabilization, $\Delta G_s^{\ddagger} - \Delta G_s^g$, is about 3–5.5 kcal/mol using the cytosine nucleotides as transition state analogs. Insofar as each 1.36 kcal/mol of net stabilization is the equivalent of a tenfold rate enhancement, it is found that electrostatic stabilization can provide a rate enhancement of about 5×10^2 to 10^4.

However, the total rate enhancement achieved by electrostatic stabilization of the dianionic intermediate may be greater than the above estimate if movement of the phosphate group, relative to the charge centers on the protein molecule, occurs upon formation of the dianionic intermediate as has been suggested by model building studies of Richards and Wyckoff (1971). This added effect, which could result from such movement toward lysine-41, for example, can also be estimated. Using ΔG° for orthophosphate as the best estimate for the maximal electrostatic interaction, the maximum change in ΔG°_{elec} occurring upon conversion of 2′,3′-CMP to a dianionic form is estimated to be ΔG° (phosphate) $- \Delta G_s^g \cong 8.0$ kcal/mol. Thus it is potentially possible to achieve a reduction in "the activation" free energy of 8.0 kcal/mol through electrostatic effects if appropriate translocation of the phosphate group relative to the charge surface of the enzyme occurs during catalysis. Such a possibility, which may include possible conformational rearrangement of the enzyme, appears to be quite likely since Hammes and coworkers (Hammes et al., 1965) have clearly demonstrated the existence of a conformational rearrangement in RNase upon both substrate and inhibitor binding. Thus the maximum contribution of electrostatic interactions as it effects the catalytic rate is equivalent to a rate enhancement of approximately 10^6. Of course, that part of the electrostatic stabilization which requires molecular rearrangement must be compensated by the free energy changes associated with the conformation change thus reducing the net effect. The actual rate enhancement will depend upon the geometrical details of the electrostatic potential energy surface described by all charge groups on the protein and substrate. Nevertheless, the lower limit on the rate enhancement requires no structural change so that the net rate enhancement which can be afforded by the electrostatic stabilization forces is between 5×10^2 to 10^6.

These calculations establish that the net electrostatic stabilization of the dianionic transition state intermediate can potentially provide −8 kcal/mol for the rate enhancement of the RNase catalyzed hydrolysis of 2′,3′-ribonucleotides with a resulting maximum rate increase of approximately 10^6. Since the total catalytic rate enhancement by RNase is on the order of 10^8 (Richards and Wyckoff, 1971), it appears that electrostatic stabilization of the transition state intermediate can provide a major contribution toward such

enhancement. It is thus concluded that electrostatic stabilization of a pentacoordinate dianionic transition state intermediate in the hydrolysis of cyclic nucleotides by ribonuclease A is very likely to be an important part of the overall catalytic reaction scheme and that this stabilization plus approximation of the substrate to the catalytic moieties of the enzyme is sufficient to "explain" the hydrolytic rate enhancement by the enzyme.

Concluding Remarks

The quantitative thermodynamic details of mononucleotide binding to RNase have been presented in this paper. In particular, the magnitude of specific electrostatic interactions has been estimated and the participation of such interactions in "explaining" the catalytic behavior of the enzyme has been discussed. It should be emphasized that estimation of the specific thermodynamic interactions required a correct phenomenological model for the binding reaction so that thermodynamic quantities for a precisely defined reaction could be calculated. The validity of the assumed model, as well as calculation of specific thermodynamic quantities, relied upon nuclear magnetic resonance studies, kinetic experiments, and other equilibrium studies, in addition to the calorimetric results reported in this series of papers. The calculation of the influence of electrostatic stabilization on the enzymatic rate enhancement was additionally dependent upon knowledge of the reaction mechanism, particularly with respect to charge redistribution during the chemical processes. The conclusions reached appear to be consistent with all available thermodynamic, kinetic, and structural information relating to the enzymatic action of ribonuclease A. The current hypothesis is that electrostatic interactions are a significant part of the catalytic reaction. Since it appears that ionic strength apparently influences the magnitude of such interactions, detailed thermodynamic and kinetic studies as a function of ionic strength should serve as a valid test of the hypothesis. Such studies are currently underway and will be reported in the future.

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