The occurrence of the marked maximum in K_{AP} at pH 5.5 found in this work is in disagreement with the mechanism proposed for the action of ribonuclease by Herries, *et al.*[4]

A thermodynamic treatment of the temperature dependence of the binding constant is also difficult since the temperature dependence includes the temperature dependence of unknown ionization constants and of the pH-independent binding constants and equilibrium constants characterizing the various isomeric states of the enzyme and its complex with cytidine 3′-phosphate. A common procedure is to calculate values of the standard enthalpy and entropy changes, $\Delta H°$ and $\Delta S°$, respectively, by considering the variation of the binding constant with temperature at constant pH. The thermal parameters obtained with this procedure are given in Table II, and it can be seen that $\Delta H°$ varies from -4 to -16 kcal./mole and $\Delta S°$ from -37 to $+6$ e.u. Thermal parameters calculated by such a procedure cannot have a simple interpretation when the binding constants vary markedly with pH. Under certain conditions, namely when only variations of the ionization states of the free enzyme and cytidine 3′-phosphate are important, it can be shown that the best approximation to a pH-independent binding constant is the maximum value of K_{AP}. Accordingly, the thermodynamic parameters calculated with the maximum values of K_{AP} are included in Table II. Unfortunately the actual situation is almost certainly not as simple as this, so that the procedure of correlating maxima must be regarded as one of a group of not very good available alternatives.

Not many thermodynamic studies of substrate– or inhibitor–enzyme interactions have been carried out, so that it is not possible to make a comprehensive comparison of these results with those from other systems. However, the work of Myer and Schellman[15] certainly should be mentioned. They studied the binding of adenosine 5′-monophosphate (AMP) to ribonuclease and found rather weak binding of AMP to ribonuclease at two different sites on the enzyme. The binding constant was independent of pH in the pH range 6.5 to 8.5 and the average values of $\Delta H°$ and $\Delta S°$ were -4.2 kcal./mole and -2.0 e.u., respectively. Although AMP was reported to inhibit the hydrolysis of ribonucleic acid, the lack of pH dependence and the smallness of the binding constant would seem to indicate the interaction of AMP with ribonuclease is qualitatively different from that of cytidine 3′-phosphate with the enzyme.

Table II. Apparent Thermal Parameters at 25°

pH	$\Delta H°$, kcal./mole	$\Delta S°$, e.u.
5.0	−3.8	5.8
5.5	−4.7	3.9
6.0	−13.0	−25.6
6.5	−15.9	−37.0
7.0	−11.5	−24.0
Maximum of K_{AP}	−6.0	−0.4

Although we have emphasized the many difficulties involved in interpreting the measured binding constants the usefulness of having such data will be apparent in an accompanying paper[16] where the binding constants together with kinetic data lead to pH-independent enthalpies, entropies, and rate and equilibrium constants.

(15) Y. P. Myer and J. A. Schellman, *Biochim. Biophys. Acta*, **55**, 361 (1962).
(16) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4674 (1965).

Relaxation Spectra of Ribonuclease. II. Isomerization of Ribonuclease at Neutral pH Values[1]

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Ribonuclease isomerizes at 25° and at neutral pH values in the absence of substrates. The isomerization process, which has a single characteristic relaxation time that depends on a single ionizable group of pK = 6.1, is absent from subtilisin-modified ribonuclease and 1-carboxymethylhistidine-119-ribonuclease. This process is also absent from ribonuclease to which cytidine 2′-phosphate, cytidine 3′-phosphate, pyrophosphate, or cytidine are bound, but enzyme-bound sulfate has no effect on the isomerization. The specificity of these effects indicates that the group with a pK of 6.1, which is probably a histidine residue, is present in the region of the active site. The large D_2O effect and the magnitude of the rate constants that are found to be associated with isomerization suggest that a specific hydrogen bond is involved. Evidence is presented for the participation of a carboxylate anion in a hydrogen bond with histidine.

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Introduction

In the first paper of this series[4] it was reported that a single measurable relaxation time, τ_1, occurs in temperature-jump experiments with ribonuclease at pH

(4) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **86**, 3240 (1964).

5 to 7.5 when only a colorimetric indicator is present. Three other relaxation times (τ_2, τ_3, τ_4) are observed in the presence of the substrate, cytidine 3′-phosphate.[4,5] The magnitude of one of these, τ_2, depends on the concentration of substrate and enzyme and is associated with the binding of substrate to enzyme. The participation in the binding process of an ionizable group of the free enzyme with a pK of 6.7 may be demonstrated both in temperature-jump experiments and with steady-state kinetics.[4-6] The two remaining relaxation times are independent of substrate and enzyme concentration. More than one ionizing group appears to be involved in the intramolecular relaxation process characterized by τ_3, and the presence of a large D_2O effect implicates a proton transfer.

In the present study ribonuclease is shown to undergo isomerization at neutral pH values in the absence of substrates. This isomerization, which is characterized by τ_1, does not occur when cytidine 2′-phosphate, cytidine 3′-phosphate, cytidine, or pyrophosphate are bound to ribonuclease. In contrast, sulfate ion, which is a weak competitive inhibitor of the enzymatic reaction, has no effect on this process. No such isomerization could be detected in subtilisin-modified ribonuclease (RNase-S)[7] or in carboxymethylhistidine-119-ribonuclease.[8]

Experimental Section

The temperature-jump apparatus, determination of enzyme concentration, and assay for enzymatic activity were as previously described.[4] Ribonuclease A, phosphate-free and salt-free (Worthington Lot No. RAF 6076), was also essentially free of aggregates.[9] Phosphate-free RNase-S was a gift of Dr. Frederic M. Richards. 1-Carboxymethylhistidine-119-ribonuclease was a gift of Drs. W. H. Stein and R. Heinrikson. Phosphate and phenol were removed from the sample by chromatography on IRC-50 followed by gel filtration on Sephadex G-25, and the product was lyophilized under conditions that minimize aggregation.[9,10] Cytidine 2′-phosphate was prepared from cytidine 2′,3′-phosphate by the method of Cohn[11] and rechromatographed to free it of the 3′ isomer. Phenol red (Fisher) was purified by reprecipitation.[12] The final product gave only a single spot in analysis by thin layer chromatography on silica gel G in three different solvent systems. Chlor phenol red (Fisher) and cytidine (Sigma) were used without further purification. The sample of cytidine was found to contain less than 0.014 equiv. of titratable impurities in the pH range of 6.2 to 8.7, and the pK was determined to be 4.2 at 25° in 0.1 M KNO_3.

All solutions for temperature-jump experiments were prepared with degassed, deionized distilled water. Measurements of pH were performed with a Radiometer TTTlb bitrator or Beckman Expandomatic pH meter. For experiments in which the pH profile was determined, stock solutions of KNO_3 and indicator were added to solid ribonuclease, the pH was adjusted with 1 N HCl, and the volume was adjusted to 10 ml. Absorbancy changes after the temperature jump were measured at 560 and 580 mμ with phenol red and chlorphenol red, respectively, as indicators. All measurements were performed with solutions containing 0.1 M KNO_3 and the respective concentrations of indicator and enzyme were 0.02 and 0.1 mM in most cases. The pD profiles were obtained in the manner described previously,[4] except that solutions of dry HCl in D_2O and NaOH in D_2O were used to adjust the pD. Difference spectra were measured at 25.0° using a well-thermostated cell holder with a Beckman spectrophotometer. Relaxation times were calculated from points taken from Polaroid photographs of oscilloscope traces with the aid of a magnifier with a comparator scale.

(5) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4674 (1965).
(6) D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 127 (1962).
(7) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 1029 (1960).
(8) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 2413 (1963).
(9) A. M. Crestfield, W. H. Stein, and S. Moore, *Arch. Biochim. Biophys. Suppl.*, **1**, 217 (1962).
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(11) W. E. Cohn, *J. Am. Chem. Soc.*, **72**, 2811 (1950).
(12) W. R. Orndorff and F. W. Sherwood, *ibid.*, **45**, 486 (1923).

Results and Treatment of Data

A simple mechanism for the relaxation effect, τ_1, observed with indicator–enzyme solutions, namely, reversible binding of the indicator and enzyme, can be

$$E + In \rightleftharpoons E\text{-}In \quad (1)$$

eliminated on the basis of several experimental findings. First, there was no detectable difference in the concentration of phenol red (0.1 mM) inside and outside of a dialysis sac containing 0.1 mM enzyme at pH 7.3 after equilibrium was reached. No significant leakage of enzyme through the sac occurred during the experiment. Hence, under conditions of pH and concentrations of reactants where a relaxation effect was clearly observable, there was no detectable amount of a ribonuclease–indicator complex. Second, there was no significant change in the visible spectra of (ribonuclease + phenol red) compared to phenol red alone at 0.1 mM reactants, 25°, and pH 5.9, 6.7, or 7.6. If phenol red were bound to ribonuclease, one would expect a perceptible difference spectrum. Third, addition of buffer to the enzyme–indicator solution decreased the amplitude of the relaxation process but did not alter the relaxation time. At 0.05 mM enzyme and phenol red, pH 7.2, the amplitude of the relaxation effect is decreased 70% by addition of assay buffer (Tris-acetate, pH 7.2) to a concentration of 1.0 mM, and the effect is no longer detectable at 10 mM buffer. If the relaxation effect were detected by a change in absorbance caused by binding of indicator to enzyme, then addition of buffer should have had no effect on amplitude. Finally, incorporation of 0.23 mM phenol red in the assay[6] for ribonuclease had no effect on the rate of the reaction. Therefore, phenol red does not compete with cytidine 2′,3′-phosphate for the active site of ribonuclease.

The above facts can be reconciled with a mechanism in which ribonuclease isomerizes relatively slowly to a configuration in which an ionizing group with a pK of 6.1 is exposed. (A somewhat analogous situation occurs in the relaxation effects observed with N,N-dimethylanthranilic acid[13] and *cis*-cyclopropanecarboxylic acids[14] where an intramolecular hydrogen

(13) M. Eigen and W. Kruse, *Z. Naturforsch.*, **18b**, 857 (1963).
(14) J. L. Haslam, E. M. Eyring, W. W. Epstein, G. A. Christiansen, and M. H. Miles, *J. Am. Chem. Soc.*, **87**, 1 (1965).

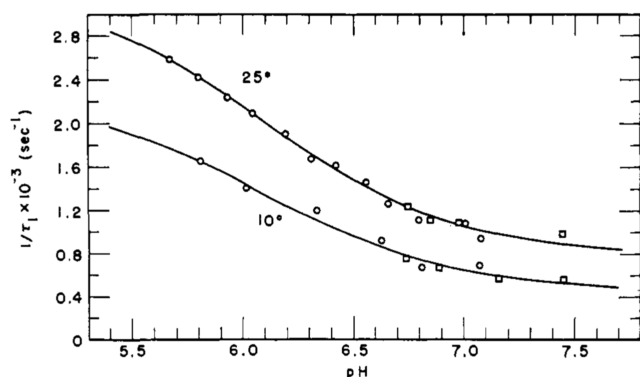
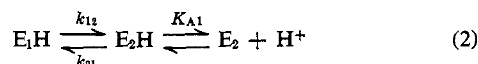


Figure 1. Dependence of $1/\tau_1$ on pH. The circles and squares represent average values of $1/\tau_1$ measured with chlor phenol red and phenol red, respectively. Most measurements at 25° and all measurements at 10° were at enzyme and indicator concentrations of 0.1 and 0.02 mM, respectively. Values of $1/\tau_1$ for enzyme (mM)/indicator (mM) mixtures of 0.05/0.05, 0.3/0.02, 0.05/0.02, and 0.1/0.04 did not differ significantly from those found at 0.1 mM/0.02 mM and are included in the averages. The solid lines are theoretical curves that were obtained by the least-squares treatment described in the text.

bond is broken relatively slowly, and then protolysis may follow.) In this mechanism, binding of indicator to enzyme is not required. A mechanism consistent with the observed results is



$$E_1H \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E_2H \overset{K_{A1}}{\rightleftharpoons} E_2 + H^+ \quad (2)$$

The oscilloscope trace observed with the relaxation effect (ref. 4, Figure 1, top) is in accord with a very fast initial drop in the pH of the solution, a fast equilibration between E_2 and E_2H, and a slower relaxation of the equilibrium between the two isomers, E_1H and E_2H, with a concomitant net *uptake* of protons. As protons are bound to enzyme the concentration of the dissociated form of indicator increases, and the absorbancy of the solution increases. If the equilibration between E_2H and E_2 is rapid compared to that between E_1H and E_2H, the expression for the slow relaxation time can be easily derived as follows.[4,15]

When the deviations of concentrations from equilibrium are small, the approach of the deviation of $[E_1H]$, $\delta[E_1H]$, to equilibrium is given by

$$-d(\delta[E_1H])/dt = k_{12}\delta[E_1H] - k_{21}\delta[E_2H] \quad (3)$$

the equation for conservation of mass may be written as

$$\delta[E_1H] = -\delta[E_2H]/(1 + \delta[E_2]/\delta[E_2H]) \quad (4)$$

and differentiation of the expression for the constant K_{A1} yields

$$K_{A1}\delta[E_2H] = [H^+]\delta[E_2] + [E_2]\delta[H^+] \quad (5)$$

Since the enzyme–indicator solution is slightly buffered by enzyme during the relaxation process, $\delta[H^+] \cong 0$, and

$$\delta[E_2]/\delta[E_2H] = K_{A1}/[H^+] \quad (6)$$

Substitution of eq. 4 and 6 into (3) leads to the desired expression.

(15) M. Eigen and L. de Maeyer in "Technique of Organic Chemistry," Vol. VIII, Part II, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963, p. 895.

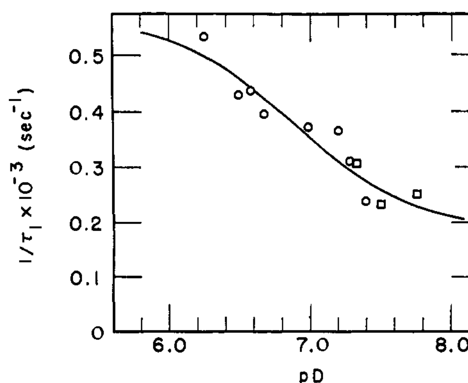


Figure 2. Dependence of $1/\tau_1$ on pD at 25°. The circles and squares represent average values of $1/\tau_1$ measured with chlor phenol red and phenol red, respectively. The solid lines are theoretical curves that were obtained by the least-squares treatment described in the text.

$$-d(\delta[E_1H])/dt = \left\{k_{12} + \frac{k_{21}}{1 + K_{A1}/[H^+]}\right\}\delta[E_1H] \quad (7)$$

$$1/\tau_1 = k_{12} + \frac{k_{21}}{1 + K_{A1}/[H^+]} \quad (8)$$

Figure 1 is a graph of $1/\tau_1$ *vs.* pH at 25 and 10°. The theoretical curves were obtained in the following way. Values of k_{12} and k_{21} were obtained by a least-squares analysis of eq. 8, where K_{A1} was assumed to have various constant values, and the independent variable was $1/(1 + K_{A1}/[H^+])$. The root-mean-square error in $1/\tau_1$ at pH = pK_{A1} was plotted against the assumed value for pK_{A1}. In both cases, minima were observed at pK_{A1} = 6.1. The best values for k_{12} and k_{21}, together with their standard errors when the variation in K_{A1} is neglected, are listed in Table I.

Table I. Rate Constants for Isomerization of Ribonuclease

Conditions	No. of observations	k_{12}, sec.$^{-1}$	k_{21}, sec.$^{-1}$	pK_{A1}
H_2O, 25°	142	780 ± 21	2468 ± 97	6.1
H_2O, 10°	79	446 ± 13	1823 ± 64	6.1
D_2O, 25°	69	183 ± 6	387 ± 27	6.9

The actual accuracy of the rate constants is estimated to be not better than ±15%, and the pK values are probably accurate to ±0.1. The amplitude of the relaxation effect vanishes below pH 5.6, as the buffering capacity of ribonuclease increases and the relative change in the hydrogen ion concentration due to the perturbation of the equilibrium decreases. The fact that τ_1 is independent of the molar ratio of enzyme to indicator and is independent of their absolute concentrations over the range studied is further evidence against any direct enzyme–indicator interaction in this relaxation process. The values of k_{H_2O}/k_{D_2O} for k_{12} and k_{21} were 4.3 and 6.4, respectively, and the value of K_{H_2O}/K_{D_2O} was 5.0 for K_{A1} (*cf.* Table I and Figure 2). The activation parameters for k_{12} and k_{21} at 25° are, respectively, $\Delta G°*$, 13.5 and 12.8 kcal./mole; $\Delta H°*$, 5.7 and 2.8 kcal./mole; and $\Delta S°*$, −26 and −33 e.u. The thermodynamic parameters for the isomeri-

zation at 25° are $\Delta G° = 0.7$ kcal./mole, $\Delta H° = 2.9$ kcal./mole, and $\Delta S° = 7$ e.u.

A solution of 0.1 mM RNase-S containing 0.02 mM chlor phenol red and 0.1 mM KNO_3 was examined for relaxation effects in the time range 0.1 to 10 msec., and in the pH range 6.0 to 7.2. There was no indication of any relaxation process under these conditions, and the enzyme solution was fully active at the end of the experiment. However, the relaxation process corresponding to τ_3 appeared upon subsequent addition of solid cytidine 3′-phosphate to a concentration of 0.2 mM. The value of τ_3 at pH 6.0 for RNase-S, 2430 sec.$^{-1}$, agrees with that found for RNase-A under the same conditions (2210 sec.$^{-1}$).[4] Further addition of cytidine 3′-phosphate did not alter the observed relaxation time.

When a solution of 0.1 mM 1-carboxymethylhistidine-119-ribonuclease was examined in a similar manner, no relaxation process that corresponded to τ_1 could be detected in the pH range 5.5 to 6.8.

Although the amplitude of the relaxation process, as recorded in oscilloscope traces, is not necessarily a linear function of concentration of buffers or inhibitors, it is at least a measure of the relative influence of buffers and inhibitors on the process. The results given in Table II show that cytidine 2′-phosphate, a very effective inhibitor of ribonuclease activity, specifically interferes with isomerization, whereas sulfate has no effect at concentrations that strongly inhibit enzyme activity. The sulfate binding site(s)[16,17] of ribonuclease is therefore not directly involved in the isomerization. The results for inorganic phosphate are ambiguous since the inhibition constants are larger than those of the cytidine phosphates[16]; consequently, the buffering effect of phosphate at the relatively high concentrations that are required for binding would be expected to be sufficient to cause the observed decrease in amplitude. Even the effect of pyrophosphate on the isomerization might be explained in this way. However, both phosphate and pyrophosphate, when bound to ribonuclease, cause the appearance of a new relaxation effect that is similar to the effect associated with the isomerization of the enzyme–substrate complex,[4] τ_3, but the former substances have a shorter value of τ_3 at low pH. Thus, the relaxation time for the enzyme–pyrophosphate complex at 25° is about 180 μsec. in the pH range 5 to 6 and in the range of inhibitor concentration of 0.4 to 0.8 mM. In contrast, the relaxation time associated with the enzyme–cytidine 2′-phosphate complex is longer than that associated with the enzyme–cytidine 3′-phosphate complex.[4,5] Cytidine, at a concentration of 10 mM, and in the pH range 5.3 to 6.9, causes the complete disappearance of the relaxation effect associated with τ_1. Since the values of K_I are relatively large (14 mM at pH 6.5 (Table II) and 25 mM at pH 7.6[18]), this result cannot be attributed to competitive inhibition alone, unless K_I is actually larger than the true dissociation constant of the enzyme–inhibitor complex. There is probably sufficient buffering capacity in the sample below pH 7 to contribute partially to the disappearance of the τ_1 effect, but not enough to obscure the effect of cytidine acting as a competitive inhibitor.

Table II. Effect of Buffer and Inhibitors on the Amplitude of the Relaxation Process at 25°

Buffer or inhibitor (mM)	pH	% decrease in amplitude	K_I, mM[a] pH 5.6	K_I, mM[a] pH 7.3
Sodium sulfate (100)	6.7	0	6.0	35
Tris-acetate (1)	7.2	70	..	
Cytidine (10)	6.5	100	b	
Inorganic phosphate (0.1)	6.0	50	5.0	20
Inorganic pyrophosphate (0.1)	6.3	50	0.065	2
Cytidine 3′-phosphate (0.051)	6.0	26	c	
Cytidine 3′-phosphate (0.102)	6.0	83	c	
Cytidine 2′-phosphate (0.017)	6.1	14	0.0045	0.3
Cytidine 2′-phosphate (0.035)	6.1	44	0.0045	0.3

[a] See ref. 16. [b] K_I at pH 6.5, 25°, and 0.1 M ionic strength was found to be 14 mM by a pH-stat assay with cytidine 2′,3′-phosphate that was similar to that of Vithayathil and Richards.[7] [c] The apparent binding constant for cytidine 3′-phosphate at pH 6.0, 25°, and 0.1 M ionic strength is 0.12 mM: G. G. Hammes and P. R. Schimmel, *J. Am. Chem. Soc.*, **87**, 4665 (1965).

Discussion

The relaxation process that occurs with ribonuclease in the presence of a colorimetric indicator is an isomerization rather than a direct interaction of indicator with enzyme. The precise nature of this isomerization has yet to be determined. The large deuterium isotope effect for k_{12} and k_{21}, which is also evident in the isomerization associated with τ_3, demonstrates that a proton is involved in the process, and a likely possibility is that the isomerization may be completely represented by the making and breaking of a hydrogen bond. However, substitution of deuterium for hydrogen might, for instance, have a more general effect on equilibria for configurational changes prior to a rate-determining configurational transition. (In other words, the structure of the enzyme may not be the same in H_2O and D_2O.) The process in question clearly must involve an *intra*molecular proton transfer rather than an *inter*molecular one, since the magnitude of the relaxation time is independent of the presence of added buffer. It is not clear at present whether there is any necessary chemical relationship between the processes associated with τ_1 and τ_3. The pH profile of $1/\tau_1$ is in a direction opposite to that of $1/\tau_3$, and only one ionizing group is involved subsequent to the former isomerization, but the deuterium isotope effects, k_{H_2O}/k_{D_2O}, for the two processes are quite similar. The pK of 6.1 probably represents a histidine residue of the enzyme.[19] This value is lower than normal, a result to be expected if it were situated in a net positive cluster of the type proposed by Saroff and co-workers.[17,20] However, a rather disturbing fact is that $\Delta H°$ for the ionization of E_2H is approximately zero. Values of $\Delta H°$ for histidine residues lie in the neighborhood of 8 kcal./mole[21]; for a 15° decrease in temperature an increase of about 0.3 pH unit in pK_{A1}

(16) C. A. Nelson, J. P. Hummel, C. A. Swenson, and L. Friedman, *J. Biol. Chem.*, 237, 1575 (1962).

(17) H. A. Saroff and W. A. Carroll, *ibid.*, 237, 3384 (1962).

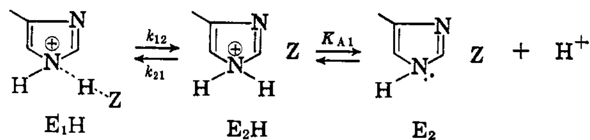
(18) T. Ukita, K. Waku, M. Irie, and O. Hoshino, *J. Biochem.* (Tokyo), 50, 405 (1961).

(19) C. Tanford and I. J. Brink have unpublished evidence for a very slight variation of optical rotatory power with pH; the pK associated with this change is about 6.0.

(20) G. I. Loeb and H. A. Saroff, *Biochemistry*, **3**, 1819 (1964).

(21) A. H. M. Kirby and A. Neuberger, *Biochem. J.*, **32**, 1146 (1938).

would be expected. The experimental error in the calculated constants might obscure such an effect. An alternative but somewhat less likely model that would not be expected to involve a change in pK with temperature[22] would be the dissociation of a carboxyl group from a net negative cluster. The isomerization might be represented by the making and breaking of a hydrogen bond between a histidine residue of ribonuclease and some other group of a net positive cluster of basic and acidic groups.



The group Z cannot be a histidine residue of the free enzyme because it would be observed in the pH profile of $1/\tau_1$. It cannot be a lysine or arginine residue because the proton in question would remain with these groups, so that the sudden lowering of pH that is observed in the temperature-jump experiments would be followed by a net *loss* of protons from enzyme. Most likely, Z is a carboxylate anion of a glutamate or aspartate residue. Loeb and Saroff[20] have suggested that the iodoacetate-reactive histidine residues[8] of ribonuclease are present in a net positive cluster involving two carboxylate anions, perhaps aspartate-121 and -14. Since it appears that carboxymethylation of histidine-119 interferes with isomerization, either this residue, or its three-dimensional neighbor histidine-12, are more likely to be the groups involved in isomerization than are the other two histidine residues at positions 48 and 105.

Both histidines at positions 12 and 119 are considered to be intimately involved in nucleotide and anion binding.[16,23] The fact that cytidine 2′-phosphate and cytidine 3′-phosphate specifically inhibit the isomerization of native ribonuclease supports the above conjecture that the group with a pK of 6.1 is one of these residues. The other histidine residue is probably the group with a pK of 6.7 that has already been detected in temperature-jump experiments and with steady-state kinetics.[4–6] The fact that sulfate is without effect on the isomerization process, but is a weak competitive inhibitor of enzyme activity and is also an inhibitor of histidine-specific carboxymethylation, can be explained if only one of the two abnormally reactive histidines (that with a pK of 6.7) is associated with sulfate binding. Both histidine residues would then be involved in the binding of the cytidine phosphates and possibly of phosphate compounds in general. In most cases histidine-119 is carboxyalkylated at pH 5.5 more readily than histidine-12, and the simplest explanation for these results is that histidine-119 has a lower pK than histidine-12.[23,24] Tentatively, the isomerization site may be assigned to histidine-119 and the sulfate binding site to histidine-12.

Whereas carboxymethylhistidine-119-ribonuclease appears to have much the same configuration as the native molecule,[25] subtilisin-modified ribonuclease may be partially unfolded[26,27] in the pH range of maximum activity. Binding of S-peptide to S-protein is increased by substrate. Iodoacetate reacts with RNase-S, but not S-protein, at pH 6 to form predominantly the 1-carboxymethylhistidine-119 derivative.[27] Since τ_3, but not τ_1, is detected in RNase-S, the substrate presumably forces RNase-S into a configuration in which the isomerization associated with τ_3 can occur.

Evidence for the participation of the isomerization of native ribonuclease in the mechanism of action of the enzyme is presented in an accompanying paper.[5]

Acknowledgment. The authors are grateful to Dr. F. M. Richards and Dr. W. H. Stein for supplying them with samples of RNase-S and 1-carboxymethylhistidine-119-ribonuclease and to Dr. C. Tanford for giving them access to his unpublished work.

(22) R. B. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1964, p. 65.
(23) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2421 (1963).
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