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Equilibrium Measurements of the Binding of Cytidine 3′-Phosphate to Ribonuclease[1]

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Equilibrium studies of the binding of cytidine 3′-phosphate to ribonuclease have been made by use of ultraviolet difference spectra and a Sephadex dialysis technique. Measurements have been made at 25 and 7° in the pH range 5 to 7. The difference spectrum arising from the interaction of the enzyme and cytidine 3′-phosphate and the apparent binding constant vary markedly with pH and temperature. The observed pH and temperature dependence of the binding constants is consistent with the involvement of an imidazole side chain in the binding process. Apparent values of the standard enthalpy and entropy changes have been calculated at various pH values; these parameters depend on pH so that a meaningful physical interpretation of these results is not possible.

Introduction

The interaction of cytidine 3′-phosphate with ribonuclease has been subjected to a great deal of study with a variety of different methods. In particular, steady-state kinetics,[4,5] ultraviolet difference spectra,[6,7] and the temperature-jump method[8] have been employed. All of the data obtained thus far are consistent with the hypothesis that at sufficiently low concentrations of cytidine 3′-phosphate one molecule of cytidine phosphate binds to one molecule of ribonuclease at the active site.

Although the phenomenon of an ultraviolet difference spectrum accompanying binding of cytidine 3′-phosphate (and many of its analogs) to ribonuclease is well known,[6,7] a quantitative study of this phenomenon as a function of pH and temperature has not previously been reported. Apparent binding constants have been evaluated by use of the difference spectra and a Sephadex dialysis technique. The results obtained provide a useful comparison with those from kinetic studies[4,8] and also suggest some important aspects of the binding process. The limitations of this type of study for obtaining useful thermodynamic information are also indicated.

Experimental Section

The two different lots of lyophilized, phosphate-free ribonuclease A employed (Worthington) showed no detectable differences in their enzymatic activities and physical properties. The enzyme was assayed by the spectrophotometric method of Crook, *et al.*[9] Cytidine 3′-phosphate was prepared as previously described.[8] Enzyme and cytidine 3′-phosphate concentrations were determined spectrophotometrically.[8] All other chemicals used were standard reagent grade.

A stock buffer solution, pH 10, of 0.05 *M* Tris, 0.05 *M* sodium acetate, and 0.1 *M* $NaNO_3$ was used for all experiments. For each experiment the pH was lowered to the desired value with acetic acid; the

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(4) D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 127 (1962).
(5) M. Litt, *J. Biol. Chem.*, **236**, 1786 (1961).
(6) J. P. Hummel, D. A. Ver Ploeg, and C. A. Nelson, *ibid.*, **236**, 3168 (1961).
(7) A. P. Mathias, B. R. Rabin, and C. A. Ross, *Biochem. Biophys. Res. Commun.*, **3**, 625 (1960).
(8) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **86**, 3240 (1964).
(9) E. M. Crook, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, 74, 234 (1960).

resulting ionic strength was 0.20 M over the pH range investigated.

Sephadex, G-25 fine, in bead form, was purchased from Pharmacia Fine Chemicals Incorporated, Uppsala, Sweden. A slurry of 0.3 g. of gel in a total volume of 2 ml. of buffer was used in the dialysis experiment at pH 6 and 7. The detailed procedure for determining binding constants by this method has been previously reported.[10]

A Beckman DU spectrophotometer equipped with a photomultiplier attachment was employed for all spectral measurements. Difference spectra were measured directly by means of two thermostated cell compartments, each containing two cuvettes of 1-cm. path length, which were placed in series in the direction of the light path. Two cuvettes, placed in series, contained enzyme and cytidine 3′-phosphate, respectively; one of the two adjacent cuvettes contained the enzyme and cytidine-3′-phosphate, while the other cuvette contained only buffer. The temperature was held constant to ±0.1°. All measurements of the complete difference spectrum and of the enzyme and cytidine 3′-phosphate spectra were made at the same slit width (0.6 mm.) for all values of pH and temperature. The range of enzyme and cytidine 3′-phosphate concentrations employed at each pH and temperature is given in Table I.

Table I. Range of Ribonuclease and Cytidine 3′-Phosphate Concentrations Employed

pH	Temp., °C.	[Enzyme], $M \times 10^5$	[3′-CMP], $M \times 10^4$
5.00	25	6.75–7.56	0.38–2.28
4.87	7.1	7.30–9.73	0.73–2.92
5.50	25	5.53–7.66	0.23–1.81
5.52	7.1	5.81–8.16	0.93–2.48
6.00	25	6.38–29.5	0.33–4.20
5.96	7.1	6.39–8.52	0.63–2.51
6.45	25	10.05	0.73–2.20
6.62	7.1	7.57–10.1	0.77–3.08
7.00	25	34.0	4.20
7.10	7.1	6.74–8.99	1.31–2.62

At 25° (±0.1°) all pH measurements were made on a Radiometer pH meter, while at 7.1° (±0.1°) all pH measurements were made on a Beckman Expandomatic pH meter. The error in the pH measurements is estimated to be ±0.05 pH unit.

Results and Treatment of Data

The difference spectrum between the enzyme–cytidine 3′-phosphate solution and free enzyme and free cytidine 3′-phosphate solutions was measured as a function of concentrations, pH, and temperature over the wave length region 256 to 293 mμ. In general, the largest differences were observed in the spectral region from 256 to 270 mμ; however, the shape of the difference spectrum is quite pH dependent. The observed absorbancy difference, Δa, is related to the concentration of the complex formed, (EP), by the equation

$$\Delta a = (\mathrm{EP})\Delta\epsilon \qquad (1)$$

where a 1-cm. path length has been assumed, and $\Delta\epsilon$ is the molar difference extinction coefficient, *i.e.*, $\epsilon_P + \epsilon_E - \epsilon_{EP}$. (These are the molar extinction coefficients of cytidine 3′-phosphate, enzyme, and the cytidine 3′-phosphate–enzyme complex, respectively.) The easiest and most direct way of obtaining $\Delta\epsilon$ is essentially to saturate the enzyme so that (EP) is known and $\Delta\epsilon$ can be calculated directly. The procedure was not possible since the total absorbancy of the solutions would become too large for determination of the difference spectrum. An additional relationship between the concentrations of enzyme, (E), cytidine 3′-phosphate, (P), and the complex at constant pH and temperature is given by the apparent equilibrium constant, K_{AP}

$$K_{AP} = \frac{(\mathrm{EP})}{(\mathrm{E})(\mathrm{P})} = \frac{(\mathrm{EP})}{[(\mathrm{E}_0) - (\mathrm{EP})][(\mathrm{P}_0) - (\mathrm{EP})]} \qquad (2)$$

where (E_0) and (P_0) are the total concentrations of ribonuclease and cytidine 3′-phosphate. Equations 1 and 2 can be solved simultaneously for K_{AP} and $\Delta\epsilon$ by measuring the difference spectra at a series of values of (E_0) and (P_0) at a given pH and temperature. Five to ten different combinations of (E_0) and (P_0) were used at each pH and temperature and a method of successive approximations was used to obtain $\Delta\epsilon$ and K_{AP}. In practice the wave length of maximum difference was used to determine K_{AP} and $\Delta\epsilon$; then $\Delta\epsilon$ was calculated for other wave lengths using the known value of (EP). The method of successive approximations used was to assume a value of $\Delta\epsilon$ and then calculate a value of K_{AP} for each combination of (E_0) and (P_0). The best value of $\Delta\epsilon$ at each pH was taken as that which minimized $\sum_i \Delta K_i/\delta K_i$, where the sum is taken over all combinations of (E_0) and (P_0); ΔK_i is the absolute value of the deviation of the calculated K_{AP} from the weighted average value of K_{AP} (see below), and δK_i is the maximum possible error in each K_i which was calculated by using a standard propagation of errors treatment on eq. 1 and 2. The best value of K_{AP} was taken as the weighted average, $\sum_i (K_i/\delta K_i)/\sum_i 1/\delta K_i$, of the calculated values of K_{AP} obtained by using the best value of $\Delta\epsilon$. This procedure does not have any strict statistical justification but is a simple way of allowing for the fact that the experimental error, and hence the reliability of a given measurement, depends on the magnitude of the measured value of Δa. The values of $\Delta\epsilon$ and K_{AP} obtained as a function of pH and temperature are given in Figures 1–3. The error in the values reported for K_{AP} is estimated to be about ±25%, while the error associated with the maximum values of $\Delta\epsilon$ (256 to 266 mμ) is about ±10%. At wave lengths where $\Delta\epsilon$ is smaller, the relative error naturally increases.

In order to obtain the molar extinction coefficient of the ribonuclease–cytidine 3′-phosphate complex from $\Delta\epsilon$, the extinction coefficients of the enzyme and cytidine 3′-phosphate must be known. The spectrum of the enzyme was found to be independent of pH in the range 5 to 7, while the spectrum of cytidine 3′-phosphate is quite dependent on pH over this same range. Plots of the molar extinction coefficient of cytidine 3′-phosphate at 7.1 and 25° as a function of wave length at various pH values are given in Figure 4.

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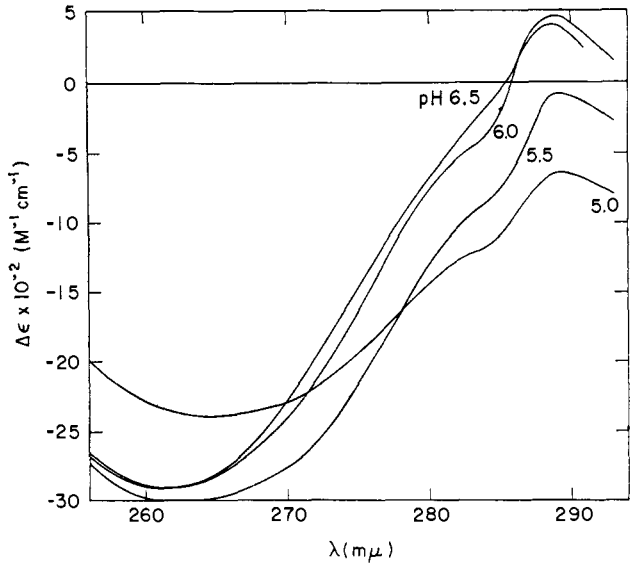

Figure 1. $\Delta\epsilon$ as a function of wave length and pH at 25°. The experimental conditions are specified in the text.

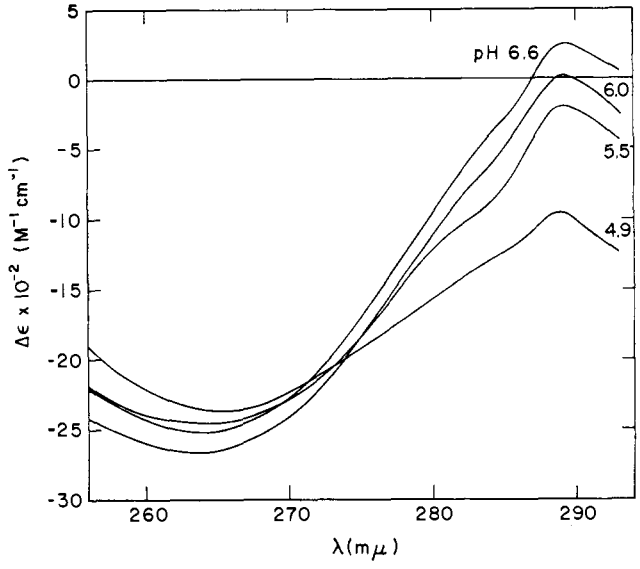

Figure 2. $\Delta\epsilon$ as a function of wave length and pH at 7.1°. The experimental conditions are specified in the text.

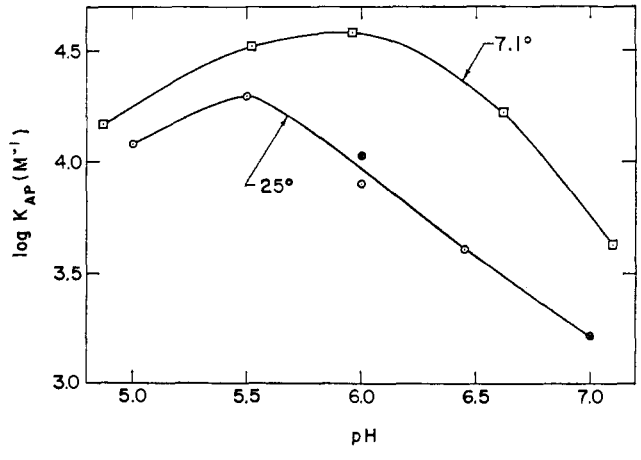

Figure 3. Log K_{AP} as a function of pH at 25 and 7.1°: K_{AP} at 25° as determined by difference spectra, ○; by Sephadex dialysis, ⊗; K_{AP} at 7.1° as determined by difference spectra, □.

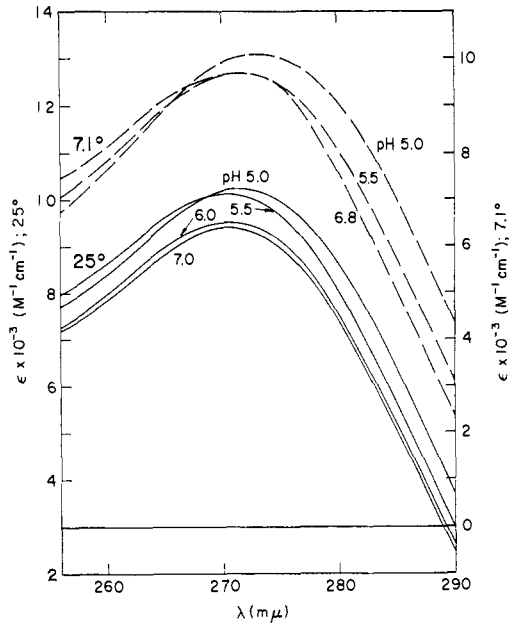

Figure 4. Absorption spectrum of cytidine 3′-phosphate as a function of pH at 25 and 7.1°. The experimental conditions are specified in the text.

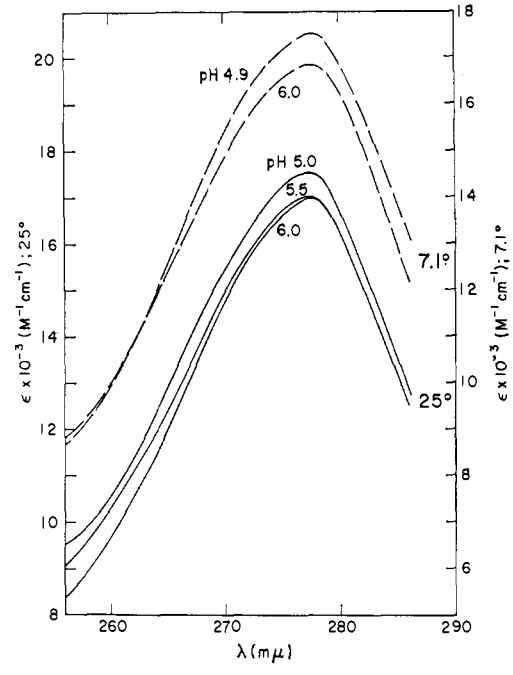

Figure 5. Absorption spectrum of the ribonuclease–cytidine 3′-phosphate complex as a function of pH at 25 and 7.1°. The experimental conditions are specified in the text.

Using the measured values of $\Delta\epsilon$, ϵ_P, and ϵ_E, the molar extinction coefficients of the complex can be readily calculated and are shown as functions of pH and wave length at 7.1 and 25° in Figure 5.

Extension of these measurements to other pH values was not possible since both K_{AP} and $\Delta\epsilon$ (in the region of maximal differences) decreases markedly at lower pH values, while K_{AP} decreases at higher pH values.

The binding constant was also measured at pH 6 and 7 at 25°, by use of a Sephadex dialysis technique. The procedure used for the calculation of the binding constant was previously described,[10] and the binding

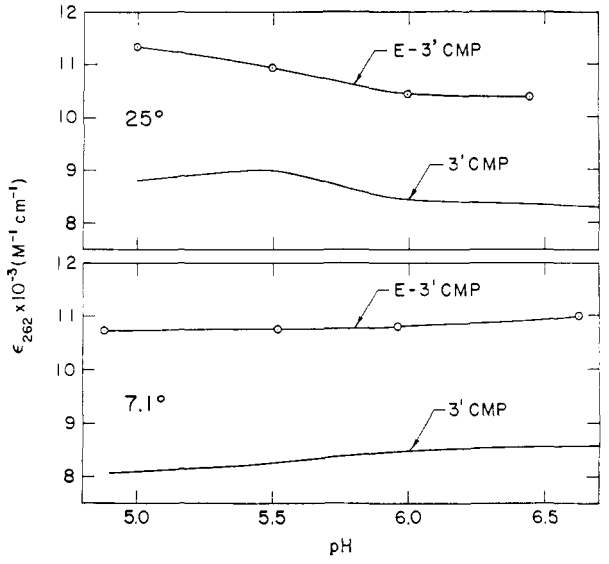

Figure 6. pH dependence of ϵ_{262} for cytidine 3′-phosphate and the cytidine 3′-phosphate–ribonuclease complex at 25 and 7.1°.

constants obtained from duplicate measurements at both pH values are included in Figure 3. The estimated error in these constants is ±20%.

The above treatment of the data assumes that only one molecule of cytidine 3′-phosphate binds to each molecule of ribonuclease. The fact that all of the data reported here are consistent with this assumption is strong evidence in favor of this hypothesis. Furthermore, at a given pH and temperature, $\Delta\epsilon$ is independent of the concentrations of ribonuclease and cytidine 3′-phosphate, thus excluding the possibility that two or more binding sites associated with different difference spectra are involved. Previous evidence in favor of this hypothesis was the fact that cytidine 3′-phosphate is a competitive inhibitor of the hydrolysis of cytidine 2′,3′-phosphate[4] and the fact that cytidine 2′-phosphate binds to ribonuclease with a 1:1 stoichiometry.[11]

Discussion

A detailed molecular interpretation of the difference spectra is clearly not possible. However, several points are worth noting. The general form of the spectra is in good agreement with the results of previous workers.[6] The largest differences occur in the spectral region 256 to 270 mμ. Since the extinction coefficient of cytidine 3′-phosphate is much larger (about a factor of two at 256 mμ) than that of ribonuclease in this spectral range, the nucleotide chromophore is probably the major cause of the difference spectra. A similar conclusion was reached by Hummel, *et al.*,[6] through an examination of difference spectra caused by the interaction of several different nucleotides with ribonuclease. The very small bumps consistently observed around 280–286 mμ may be due to the perturbation of tyrosine residues on the enzyme; such phenomena have been observed with other nucleotides.[6] The variation of the shape and magnitude of the difference spectra with pH and temperature indicates that these parameters must be carefully controlled. In particular, $\Delta\epsilon$ must be determined independently for each set of conditions if a binding constant is to be obtained. (Unfortunately such a procedure is often not adopted.)

The pH dependence of the spectrum of the cytidine 3′-phosphate complex is marked in the spectral region 256 to 270 mμ where the magnitude of the difference spectrum is largest. A plot of the extinction coefficient of the complex at 262 mμ *vs.* pH is shown in Figure 6. Included is a similar plot for cytidine 3′-phosphate. The curves for the complex and cytidine 3′-phosphate are quite similar at the same temperature except that at 25° the extinction coefficient of cytidine 3′-phosphate decreases at lower pH, whereas that for the complex tends to reach a plateau. Since this decrease is presumably due to protonation of the ring nitrogen, it can be tentatively concluded that the ring nitrogen of the cytidine 3′-phosphate in the complex is probably not protonated in the same pH region as that of free cytidine 3′-phosphate. The increase in absorption at 262 mμ below pH 6 is probably due to protonation of the secondary phosphate group, and these results strongly suggest that this group is protonated and deprotonated in roughly the same pH region as free cytidine 3′-phosphate. At 7.1° the absorbancies at 262 mμ of both cytidine 3′-phosphate and its complex with ribonuclease are rather insensitive to pH over the pH range investigated.

The pH profiles of the binding constant at 25 and 7.1° are given in Figure 3. A meaningful interpretation of the observed pH dependence of a binding constant cannot be made, in general, unless a considerable amount of additional information is available. This is readily seen if the binding constant is written as

$$K_{AP} = \frac{\sum_i (EP_i)}{\sum_j (E_j) \sum_k (P_k)} \qquad (3)$$

where the sums extend over all possible species. The pH dependence of K_{AP} then involves the ionization states of three different entities; only the various ionization states of cytidine 3′-phosphate can be determined without ambiguity. At both temperatures the curve goes through a maximum although the shape of the curve changes with temperature and the maximum is shifted from pH 5.5 to 6 as the temperature changes from 25 to 7.1°. The fact that the binding constant is very sensitive to pH in the pH range 5 to 7 suggests that imidazole side chains may be involved in the binding process. The pK of imidazole would increase about 0.4 unit if the temperature were changed from 25 to 7.1°[12]; this is consistent with the observed shift in the pH profile of K_{AP}. Also it should be remarked that 1-carboxymethylhistidine-119-ribonuclease does not bind cytidine 3′- (and 2′-) phosphate.[13,14] Values of the Michaelis constant for cytidine 3′-phosphate, which have been measured by Herries, *et al.*,[4] are in quantitative agreement (within experimental error) with the measured binding constants at 25°, indicating the Michaelis constant can be considered as a true equilibrium constant. However, a value of the Michaelis constant is not reported at pH 5.5 or 6.0.

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

(12) R. B. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1964, p. 65.

(13) R. E. Cathou, unpublished results of equilibrium dialysis experiments.

(14) S. T. Yang and J. P. Hummel, *J. Biol. Chem.*, 239, 3775 (1964).

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.

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

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