$a_H(k/K_N)$. This is similar to eq 2 which describes the rates of hydrolysis of Guo, dGuo, and dAdo. (A spontaneous or "water"-catalyzed reaction is not considered in these derivations.)

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Relaxation Spectra of Ribonuclease. VII. The Interaction of Ribonuclease with Uridine 2′, 3′-Cyclic Phosphate[1]

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Abstract: Stopped-flow temperature-jump studies were made of the interaction of ribonuclease A with uridine 2′,3′-cyclic phosphate in 0.2 *M* NaCl. A bimolecular reaction of enzyme and substrate was observed at pH 6.5, 15°, with a second-order rate constant of approximately $1 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ and a dissociation rate constant of $2 \times 10^4\ \text{sec}^{-1}$. A second relaxation process was observed at pH 6.0 and 6.5, 15°, which could be associated with an isomerization of the enzyme–substrate complex and the corresponding rate constants were determined. The isomerization of "free" ribonuclease was studied as a function of uridine 2′,3′-cyclic phosphate concentration at 25° in the pH range 5.5–7.5. The data suggest a mechanism where both isomeric forms of the enzyme bind the substrate, and an analogous isomerization can also occur with the enzyme–substrate complexes.

Earlier papers in this series dealt with the interaction of ribonuclease A with cytidine 3′-phosphate,[3,4] cytidine 2′,3′-cyclic phosphate,[5] cytidilyl-3′,5′-cytidine,[6] and uridine 3′-phosphate.[7] These studies have indicated that the mechanism of interaction of ribonuclease with all of the mentioned substrates involves an initial association between enzyme and substrate followed by an isomerization or conformational change of the enzyme–substrate complex. Three ionizable groups on the enzyme with approximate p*K* values of 5.4, 6, and 6.5 have been implicated in the binding mechanism and presumably the catalytic reaction. Moreover, the enzyme itself was found to undergo an isomerization at neutral pH values, with an ionizable group on the enzyme with a p*K* of approximately 6 being involved in the conformational change.[8] The results of these kinetic investigations have been correlated with the known three-dimensional structure of ribonuclease,[9,10] and a mechanism for the action of the enzyme can be postulated in molecular terms.[7]

The work reported here is concerned with stopped-flow temperature-jump studies of the interaction of ribonuclease A with uridine 2′,3′-cyclic phosphate. A relaxation effect was observed at pH 6.5, 15°, which is related to the initial association of enzyme and substrate; a second relaxation process is seen at pH 6 and 6.5, 15°, which corresponds to an isomerization of the enzyme–substrate complex. The isomerization of the "free" enzyme[8] was studied as a function of substrate concentration at pH 5.5–7.5, and the pH dependence of the enzyme isomerization at high substrate concentrations indicates that both isomeric forms of the enzyme bind the substrate. Moreover, the enzyme–substrate complexes themselves undergo an isomerization similar to that of the free enzyme.

Experimental Section

Bovine pancreatic ribonuclease A (phosphate free, lyophilized) was purchased from Worthington Biochemical Corp. and was used without further purification. Its concentration was determined spectrophotometrically as described previously.[11] Uridine 2′,3′-cyclic phosphate was prepared as previously described[11] and all other reagents were of analytical grade.

A combined stopped-flow temperature-jump apparatus[12,13] was used. All solutions used in the temperature-jump experiments were 0.2 *M* in NaCl, and were prepared from freshly boiled deionized distilled water. The pH changes accompanying the relaxation processes were observed using 2×10^{-5} *M* pH indicators. Methyl red (pH range 4.4–6.2), chlorophenol red (pH range 5.2–6.6) and phenol red (pH range 6.6–8) were used and the absorbance changes were observed at 520, 573, and 558 mμ, respectively. The pH of the solutions was adjusted with NaOH and HCl using a Radiometer Model 26 pH meter.

Steady-state experiments were done in 0.1 *M* Tris acetate–0.1 *N* NaCl buffers at 15.0 ± 0.1° using a Cary 14 spectrophotometer. The experimental procedure was as previously described[11] except that the time allowed for temperature equilibration was 10 min, mixing was by inversion, and the absorbance change was recorded using a 0–0.1 absorbancy unit slidewire.

Results and Treatment of Data

The steady-state kinetic parameters for the ribonuclease-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate to uridine 3′-phosphate at 15°, pH 6.0 and 6.5,

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Table I. Kinetic Parameters and Rate Constants for the Ribonuclease-Catalyzed Hydrolysis of Uridine 2′,3′-Cyclic Phosphate[a]

pH	k_S (sec^{-1})	$10^3 K_S$ (M)	$10^{-7} k_6$ (M^{-1} sec^{-1})	$10^{-4} k_{-6}$ (sec^{-1})	$10^{-4} k_5$ (sec^{-1})	$10^{-4} k_{-5}$ (sec^{-1})
6	1.58	0.80			2.8	1.8
6.5	1.92	1.46	1.1	2.1	0.9	2.6

[a] 15°: k_S and K_S values were obtained in 0.1 M Tris acetate–0.1 M NaCl buffers; k_6, k_{-6}, k_5, and k_{-5} were obtained in 2×10^{-5} M chlorophenol red–0.2 M NaCl.

are given in Table I. They were calculated by a weighted least-squares analysis of 13 substrate concentrations (0.577×10^{-3} M–2.10×10^{-3} M) at pH 6, and 9 substrate concentrations (0.454×10^{-3} M–2.72×10^{-3} M) at pH 6.5 (*cf.* ref 11). The estimated error in each kinetic parameter is ±15%.

Relaxation effects occurring only in the presence of both ribonuclease and uridine 2′,3′-cyclic phosphate were observed at 15°, pH 6 and 6.5, using initial enzyme concentrations of about 5×10^{-4} M. The 7.5° temperature perturbation was applied 7 msec after mixing of the enzyme and substrate solutions; after 7 msec the reaction had proceeded to less than 2% completion. A time delay of 40 μsec was applied after the temperature jump before recording the oscilloscope trace of the relaxation effect. A minimum of four oscilloscope traces were taken for each data point at pH 6 and 6.5.

At pH 6 a single relaxation effect was observed (other than the relaxation process also observed with the free enzyme). The concentration dependence of the relaxation time is shown in Figure 1. The relaxation time is concentration dependent at low-substrate concentrations and becomes concentration independent at high-substrate concentrations. This is characteristic of a mechanism where the interconversion of the enzyme–substrate complex between different states can become rate limiting. A suitable mechanism is

$$E + S \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} ES^1 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} ES^2 \qquad (1)$$

where E, S, ES^1, and ES^2 are enzyme, substrate, and enzyme–substrate complexes, respectively. The subscripts of the rate constants and relaxation times in this paper were chosen to agree with the numbering used in previous papers. If the bimolecular process is much faster than the second step in the mechanism

$$1/\tau_5 = k_{-5} + \frac{k_5}{1 + k_{-6}/(k_6[(\bar{E}) + (\bar{S})])} \qquad (2)$$

where $(\bar{E})$ and $(\bar{S})$ are the equilibrium concentrations of the enzyme and substrate, respectively. The equilibrium concentrations were calculated from the Michaelis constant, with the assumption that the latter is a true equilibrium constant. This is a reasonable assumption since the reciprocal relaxation times observed are about three orders of magnitude greater than the maximum turnover number of the enzyme–cyclic nucleotide complex (*cf.* ref 5). (It has also been assumed that negligible amounts of the product and enzyme–product complexes have been formed when the relaxation effects were observed since less than 2% of the substrate has reacted.) Equation 2 can be rearranged to give

$$(1/\tau_5 - k_{-5})^{-1} = 1/k_5 + k_{-6}/(k_5 k_6[(\bar{E}) + (\bar{S})]) \qquad (3)$$

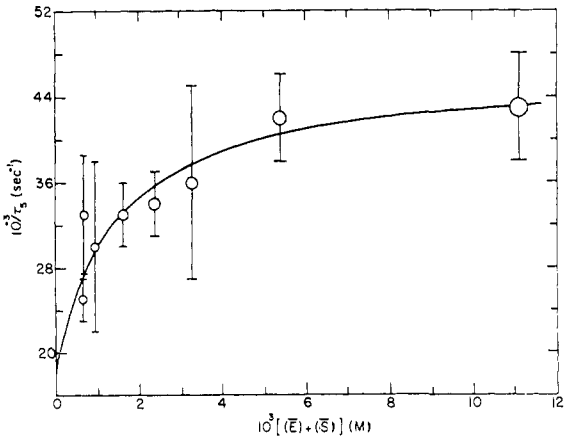


Figure 1. Variation of $1/\tau_5$ with $[(\bar{E}) + (\bar{S})]$ at pH 6.0, 15°. The solid line was calculated from eq 2 using $k_5 = 2.8 \times 10^4$ sec^{-1}, $k_{-5} = 1.8 \times 10^4$ sec^{-1}, and $k_{-6}/k_6 = 1.4 \times 10^{-3}$ M.

Values of k_{-5} were chosen by trial and error until a good fit of the data to eq 3 was obtained. The following parameters were found to describe the data: $k_5 = 2.8 \times 10^4$ sec^{-1}, $k_{-5} = 1.8 \times 10^4$ sec^{-1}, $k_{-6}/k_6 = 1.4 \times 10^{-3}$ M; the concentration dependence of the relaxation time calculated from these parameters is given by the solid line in Figure 1. These constants give an overall dissociation constant $[k_{-6}/k_6(1 + k_5/k_{-5})]$ of 0.56×10^{-3} M which is in reasonable agreement with the observed Michaelis constant, 0.80×10^{-3} M.

Two relaxation effects could be observed for the ribonuclease–uridine 2′,3′-cyclic phosphate reaction at 15°, pH 6.5. The same time delays after mixing and temperature jump were applied as at pH 6. A relaxation process at low substrate concentrations was observed whose reciprocal relaxation time varied linearly with the sum of the equilibrium concentrations of enzyme and substrate. At higher concentrations of substrate, where at least 50% of the enzyme is saturated with substrate, a relaxation effect was observed which was essentially concentration independent. These results can be interpreted according to the mechanism of eq 1, which is characterized by two relaxation times. If the first step is assumed to equilibrate much faster than the second step, then the two relaxation times are given by eq 2 and 4

$$1/\tau_6 = k_6[(\bar{E}) + (\bar{S})] + k_{-6} \qquad (4)$$

Equation 4 predicts that a plot of $1/\tau_6$ *vs.* $(\bar{E}) + (S)$ should be a straight line with a slope of k_6 and an intercept of k_{-6}. The concentration dependence of the fastest relaxation time conforms to eq 4 and gives values of k_6 and k_{-6} of 1.1×10^7 M^{-1} sec^{-1}, and 2.1×10^4 sec^{-1}, respectively. If the Michaelis constant is assumed to be identical with the reciprocal of the overall binding constant and the concentration-independent value of $1/\tau_5$ is taken to be equal to $k_5 + k_{-5}$ (see eq 2), then $k_{-5} = 2.6 \times 10^4$ sec^{-1} and $k_5 = 0.9 \times 10^4$ sec^{-1}. The theoretical curves for the two relaxation times plotted according to eq 2 and 4 with the above parameters are shown in solid lines in Figure 2.

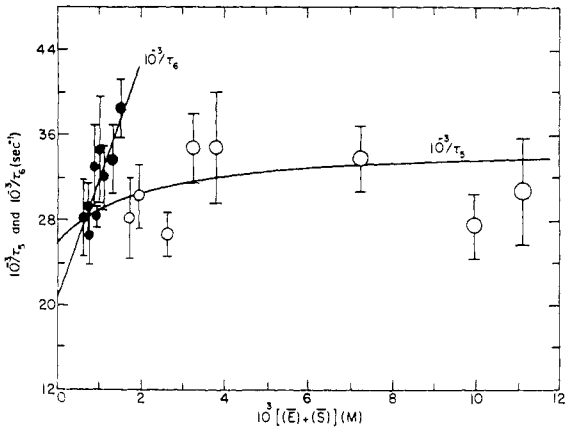


Figure 2. Variation of $1/\tau_5$ and $1/\tau_6$ with $[(\bar{E}) + (\bar{S})]$ at pH 6.5, 15°. The straight line for $1/\tau_6$ was calculated from eq 4 and the curved line for $1/\tau_5$ was calculated from eq 2 using $k_6 = 1.1 \times 10^7\ M^{-1}$ sec^{-1}, $k_{-6} = 2.1 \times 10^4$ sec^{-1}, $k_5 = 0.9 \times 10^4$ sec^{-1}, and $k_{-5} = 2.6 \times 10^4$ sec^{-1}.

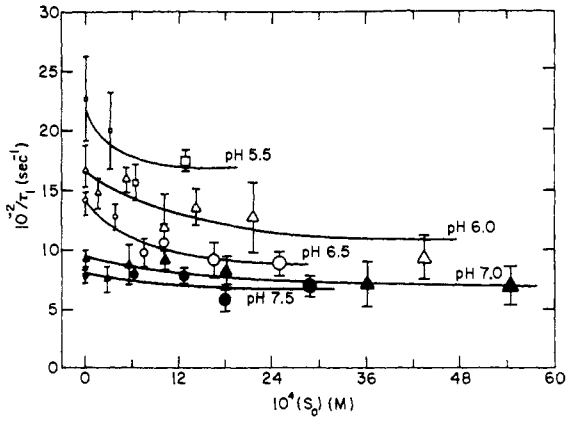
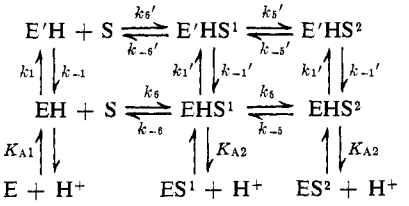


Figure 3. Variation of $1/\tau_1$ with initial concentration of uridine 2′,3′-cyclic phosphate at several pH values, 25°: pH 5.5, □; pH 6.0, △; pH 6.5, ○; pH 7.0, ▲; pH 7.5, ●. The lines have no theoretical significance.

Unfortunately the above analysis of the data in Figures 1 and 2 is not quantitatively correct. Although two distinct relaxation times can be detected at pH 6.5, the experimental precision is insufficient to completely resolve these time constants. Therefore, the relaxation times reported in Figure 2 at low concentrations are almost certainly some sort of average values which are close to, but not identical with the actual relaxation times. However, even if the precise relaxation times could be measured, the analysis of the data which assumes the first step equilibrates much faster than the second is not correct. A more correct analysis would involve solution of the coupled rate equations to give the two relaxation times, each of which would be a function of all four rate constants. However, even this analysis is not sufficient because the isomerization of the free enzyme and an analogous isomerization of the initial enzyme–substrate complex also occurs and is coupled to the binding reactions (see below). Although an exact solution of the entire mechanism is possible in principle, the quantity and precision of the data does not warrant this. Instead the data are best interpreted as being consistent with the mechanism of eq 1. (The more exact solution of the kinetic equations gives qualitatively similar concentration dependences to that predicted by eq 2 and 4.) The calculated rate constants, which are summarized in Table I, should be regarded as semiquantitative and probably are correct to within a factor of 2 or 3.

The relaxation process observed in the presence of free ribonuclease was studied with 10^{-4} *M* enzyme and different initial substrate concentrations at 25° and pH 5.5–7.5. The reciprocal relaxation times are plotted *vs.* the substrate concentrations in Figure 3. The 7.5° temperature jump was applied about 20 msec after mixing when less than 2% of the substrate had reacted. Each data point in Figure 3 was calculated from a minimum of three photograph traces of the relaxation effect. The isomerization process slows down with increasing substrate concentration, the limiting rate at high substrate concentrations increases with decreasing pH, and the relaxation effect can be observed even when the enzyme is 95% saturated with substrate. This latter fact implies the initial enzyme–substrate complex can undergo an isomerization similar to that which occurs with the free enzyme. The simplest mechanism consistent with the data is



(5)

If the horizontal steps and protolytic reactions equilibrate rapidly relative to the isomerization, then the limiting reciprocal relaxation time at low substrate concentrations is

$$1/\tau_1 = \frac{k_1}{1 + K_{A1}/(H^+)} + k_{-1} \qquad (6)$$

and at high substrate concentrations, approaching saturation of the enzyme

$$1/\tau_1 = \frac{k_1'}{1 + K_{A2}/(H^+)} + k_{-1}' \qquad (7)$$

Previous work[8] has shown that $k_1 = 2468$ sec^{-1}, $k_{-1} = 780$ sec^{-1} and $pK_{A1} = 6.1$. Analysis of the data in Figure 3 indicates $k_1' \approx 1300$ sec^{-1}, $k_{-1}' \approx 600$ sec^{-1} and $pK_{A2} = 6.1$. This mechanism can be made more complex by assuming the isomerization rate constants and pK_{Ai}'s are different for the two enzyme–substrate complexes, but the data do not require this. However, as indicated previously, in principle this more complete mechanism should be used to analyze the faster relaxation processes. The difficulty of doing this can be illustrated by noting that the mechanism of eq 5 should have four fast relaxation times (neglecting protolytic reactions) whereas only two are observed experimentally. Most likely some of the relaxation times are degenerate (*e.g.*, $k_6 \approx k_6'$, $k_{-6} \approx k_{-6}'$, $k_5 \approx k_5'$, and $k_{-5} \approx k_{-5}'$) which would reduce the expected number of observed time constants. Clearly an exact analysis of this system is presently not tenable.

The results shown in Figures 1 and 2 were obtained with initial enzyme concentrations of about 5×10^{-4} *M*. Other experiments using 1×10^{-4} *M* enzyme were unsuccessful in detecting distinct concentration dependences of the relaxation times due to coupling of the

enzyme–substrate interaction effects with proton transfer reactions. These reactions are slowest at about pH 6.5. With an enzyme concentration of 1×10^{-4} M at pH 6.5, the proton transfer reactions have a relaxation time of 21 μsec; with 5×10^{-4} M enzyme the relaxation time is less than or equal to the heating time constant ($\sim$10 μsec). A relaxation effect was observed at pH 5–7.5, using 1×10^{-4} M initial enzyme concentration, whose rate did not vary significantly with a tenfold change in concentration of uridine 2′,3′-cyclic phosphate. This is probably a coupled effect dependent both upon proton-transfer reactions and enzyme–substrate interactions. The calculated relaxation times from about four different substrate concentrations per pH are: 140 $\pm$ 30 μsec at pH 5, 46 $\pm$ 5 μsec at pH 5.5, 31 $\pm$ 4 μsec at pH 6, 52 $\pm$ 9 μsec at pH 6.5, 46 $\pm$ 6 μsec at pH 7, and 71 $\pm$ 10 μsec at pH 7.5. These data are presented only for the sake of completeness and will not be considered further.

Discussion

The relaxation phenomena reported here are certainly associated with the interaction of ribonuclease and uridine 2′,3′-cyclic phosphate. Less than 2% of the cyclic phosphate has been converted to 3′-phosphate when the measurements were made. Under these conditions, the concentration of the 3′-phosphate is too low to produce discernible competing relaxation effects with ribonuclease. The only possible exceptions to this statement are the measurements of τ_1 at the highest concentrations of cyclic phosphate where the possibility exists that coupling with the ribonuclease–uridine 3′-phosphate conformational change (corresponding to τ_3) might occur. However, even in this instance τ_3 is generally observed only at relatively high concentrations of uridine 3′-phosphate ($\sim 10^{-3}$ M). Furthermore, the two relaxation processes have opposite amplitudes, *i.e.*, the ribonuclease–3′-nucleotide isomerization is accompanied by a release of protons, while the free enzyme isomerization shows a net uptake of protons, so that they are easily distinguishable.

The relaxation spectra of the uridine cyclic phosphate–ribonuclease interaction are virtually identical with those associated with the cytidine cyclic phosphate–ribonuclease interaction.[5] Two relaxation times related to the binding process are observed, and an isomerization process analogous to that of the free enzyme can be observed when the enzyme is essentially saturated with substrate. Thus, the general mechanism must be the same in both cases: namely formation of an initial enzyme–substrate complex followed by a conformational change, with the enzyme–substrate complexes able to undergo a conformational change similar to that which the free enzyme undergoes. The rate constants associated with the reactions of the two substrates are quite similar. Because of the difficulty of obtaining precise rate constants for these systems a meaningful quantitative comparison cannot be made. For the mechanisms of interaction of the enzyme with uridine and cytidine 3′-phosphate the rate constants are quantitatively the same except for the rate constant for the dissociation of the initial enzyme–substrate complex.

The fact that the enzyme–substrate complexes can isomerize in a manner analogous to that of the free enzyme appears to be a feature found with the cyclic phosphate substrates. This is not observed with the 3′-phosphate or cytidylyl-3′,5′-cytidine; although the enzyme–substrate complexes formed with these substances undergo conformational changes, the dynamics of these changes are not associated with τ_1. Consideration of detailed balance for the mechanism of eq 5 indicates that within experimental error both of the isomers bind the substrate equivalently. (Strictly speaking the reported parameters demand that k_6/k_{-6} be slightly greater than k_6'/k_{-6}' but the experimental precision is not sufficient to be certain that this is correct.) In contrast, the data obtained with the 3′-phosphate suggest the "acid-stable" isomer may bind the substrate somewhat more strongly.[7] However, in this case also a definite conclusion cannot be reached.

The general mechanism for the binding of all substrates to ribonuclease involves formation of an initial complex with a second-order rate constant of 10^7–10^8 M^{-1} sec^{-1} followed by a conformational change of the enzyme–substrate complex. A molecular interpretation of this mechanism has already been proposed,[7] namely formation of an initial complex, primarily with histidines 12 and 119 in the groove of ribonuclease, followed by a slight closing of the groove with lysine 41 taking over part of the binding function so that histidines 12 and 119 can take part in the catalysis. The exact nature and stereochemistry of the acid–base catalysis is still obscure, although this question is being actively pursued and several possible mechanisms have been proposed.[14–17] Nevertheless, the dynamics of the ribonuclease reactions appear to be at least partially understood in molecular terms. The occurrence of conformational changes appears to be a general feature of enzymatic reactions; possible implications of this observation for the mechanism of action of enzymes have been discussed elsewhere.[15]

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