The Kinetics of Ribonuclease Action on Cytidine-2',3'-cyclic Phosphate*

MICHAEL LITT

From the Department of Chemistry, Reed College, Portland 2, Oregon

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The enzyme ribonuclease has been studied extensively from the structural point of view. The amino acid sequence has been completely worked out (1, 2) and information about the active center has recently become available (3, 4). However, very few studies of the kinetics of this enzyme have been made, and most of the previous kinetic work is difficult to interpret because ribonucleic acid was used as the substrate. The use of such a complicated substrate is not conducive to the interpretation of kinetic studies because many different reactions may be occurring simultaneously. In view of the current interest in ribonuclease, it seemed desirable to investigate the kinetics with a small, well-defined substrate molecule such as cytidine-2',3'-cyclic phosphate (5).

Several workers have used pyrimidine cyclic phosphates as substrates for kinetic studies. Davis and Allen, using both cytidine and uridine-2',3'-cyclic phosphates, found that ribonuclease was inhibited by mononucleotides, by Cu++, and by Zn++ (6). Hummel, Flores, and Nelson, with the use of cytidine-2',3'-cyclic phosphate, measured the Michaelis constant and maximal velocity at a single pH, 6.7 (7). Vithayathil and Richards used cytidine-2',3'-cyclic phosphate to make measurements of Michaelis constants and maximal velocities of ribonuclease and subtilisin-modified ribonuclease (8).

The present paper describes a study of the kinetics of ribonuclease hydrolysis of cytidine-2',3'-cyclic phosphate at four different pH values. The response of the Michaelis constant and maximal velocity to changes in pH is observed and interpreted in terms of ionization of the histidine residue in the active center.

EXPERIMENTAL PROCEDURE

Pancreatic RNase (Worthington Biochemical Corporation) was fractionated on a carboxymethyl cellulose column, according to the method of Taborsky (9). The major component, presumably identical to Taborsky's Fraction D, was used. Fractions containing this component were pooled, deionized by passage through a mixed bed ion exchange column, and lyophilized. Stock solutions of RNase were prepared as necessary.

KCl, 0.5 M, was used for preparation of stock solutions to prevent undue absorption of the enzyme on glass surfaces (10). The concentrations of the stock solutions of RNase were determined spectrophotometrically. The molar extinction coefficient at 278 µM, pH 6.5, was taken as 9800 (11).

Cytidine-2',3'-cyclic phosphate was prepared by the method of Shugar and Wierzchowski (12). It was chromatographically pure in the solvent system, isopropanol-water-concentrated aqueous ammonia, 7:2:1 (volume for volume for volume) (13). Solutions of the cyclic phosphate thus prepared were passed through a column of Dowex 50 in the K+ form to remove unwanted cations. The resulting solutions of the potassium salt were lyophilized and refrigerated. Stock solutions were made up in doubly distilled water as required. The concentration of the stock solutions were determined spectrophotometrically.

The molar extinction coefficient at the absorption maximum of 266 mµ was determined as 8170 by allowing a solution of the cyclic phosphate with known absorbancy to undergo complete enzymatic hydrolysis to 3'-CMP. The molar extinction coefficient of 3'-CMP at pH 7.0 and 272 mµ was taken as 8970 (14).

Reagents used in the preparation of buffers were analytical grade. Water was redistilled from alkaline permanganate. All buffers were tested for traces of Zn++, Cu++, and other heavy metal ions by extraction with a 0.001% solution of dithizone in CHCl₃ (15). Buffers which gave positive tests for trace metals were extracted with dithizone in CHCl₃ until they no longer gave positive tests.

Rate measurements were made by observing the change in absorbancy at 256 mµ, as suggested by Crook, Mathias, and Rabin (16). Beer's law plots at 286 mµ were made for both cytidine-2',3'-cyclic phosphate and for 3'-CMP in 0.10 M, pH 7.0 Tris buffer. In agreement with Crook et al., it was found that the concentration range over which Beer's law was valid depended on the slit width. A slit width of 0.9 mm was found to give linear plots up to absorbancies of about 1.0. This was the maximal slit width used in the kinetic runs. When cells of a 10-mm path length are used, an absorbancy of 1.0 at 286 mµ corresponds to a substrate concentration of 3.5 × 10⁻⁴ M or to a product concentration of 2.3 × 10⁻⁴ M. The latter figure is the upper limit for the substrate concentration that can be used if the rate is to be followed over the entire course of the reaction. By use of cells with 5-mm and 1-mm path lengths, this upper limit of concentration was increased by factors of 5 and 10, respectively.

A typical run was made as follows. Equal volumes (3.0 ml, in the case of 10-mm cells) of identical substrate solutions were placed in each of two clean, dry absorption cells. After the cells had reached thermal equilibrium with the thermostatic cell compartment, a small volume (usually 10 µl) of a concentrated stock solution of RNase was added to one of the cells. After rapid mixing, the difference in absorbancy between the two cells was measured as a function of time. The final value of the absorbancy was taken as the value reached after the solutions had stood overnight.

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The temperature of the Beckman model DU spectrophotometer cell compartment was controlled at 25 ± 0.1° by circulating water from a constant temperature bath through thermospacers. Measurements were made at pH 5.0, 6.0, 7.0, and 8.0. At the two lower pH values, sodium acetate-acetic acid buffers were used. At the two higher pH values, Tris-HCl buffers were used. In all cases, the ionic strength was 0.1 M.

In many of the runs, the total concentration of RNAse in the reaction mixture was 1.26 × 10⁻⁵ M. This was arbitrarily taken as the standard enzyme concentration. In cases in which the RNAse concentration differed from this value, the measured initial rates were corrected to take account of this. When the correction was made, it was assumed that the initial rate was directly proportional to the enzyme concentration.

RESULTS

For the runs at pH 7.0 and 8.0, the reactions followed a first order law, usually up to 30% to 50% completion. At higher degrees of reaction, the rates fell off more rapidly than predicted by a first order law. This was presumably due to product inhibition. A typical rate curve is shown in Fig. 1. The initial slopes of these first order plots were used to evaluate the initial rates according to the method of Crook et al. (16). Michaelis constants and maximal velocities were evaluated from plots of (S)₀/s versus (S)₀ (17). (S)₀ is the initial molar concentration of substrate and v₀ is the initial reaction velocity. The plots for pH 7.0 and 8.0 are shown in Fig. 2.

At pH 5.0 and 6.0, product inhibition was so extreme that plots such as those of Fig. 1 showed a large curvature in the neighborhood of zero time; this made accurate evaluation of the initial rates impossible. In order to interpret the data at pH 5.0 and 6.0, an integrated rate equation was required. It was found that the data could be fitted to the integrated rate equation derived by Alberty (18) for a reversible, one-intermediate Michaelis-Menten reaction scheme:

\[ E + S \rightleftharpoons \frac{k_1}{k_2} ES \rightleftharpoons \frac{k_3}{k_4} E + P \]

This reaction scheme is symmetrical; it may be assigned Michaelis constants Kᵣ and Kₛ referring to the forward and reverse reactions, respectively. Maximal velocities Vᵣ and Vₛ may also be assigned to the forward and reverse reactions. In terms of the velocity constants of the reaction scheme, the K and V values are expressed as follows:

\[ Kᵣ = \frac{k_2 + k_3}{k_1} ; \quad Kₛ = \frac{k_2 + k_3}{k_4} ; \quad Vᵣ = k_3(E)₀ ; \quad Vₛ = k_2(E)₀ \]

(E)₀ is the total concentration of enzyme.

In the hydrolysis of cytidine-2',3'-cyclic phosphate, the equilibrium constant is undoubtedly quite large; no unreacted cyclic phosphate can be detected chromatographically after equilibrium has been established. For this case, (Kᵣₛ ≫ 1), the integrated rate equation may be written (18):

\[ \frac{1}{t} \ln \left[ 1 - \frac{(P)}{(S)₀} \right] = \left[ \frac{(Kᵣ/Kₛ - 1)}{Kᵣ + (S)₀} \right] (P)/t - \frac{Vᵣ Kᵣ}{Kᵣ + (S)₀} ; \quad (1) \]

According to this equation, a plot of

\[ \frac{1}{t} \ln \left[ 1 - \frac{(P)}{(S)₀} \right] \text{ versus } (P)/t \]

should be linear with slope m and intercept b, in which

\[ m = \frac{(Kᵣ/Kₛ - 1)}{Kᵣ + (S)₀} \quad \text{ and } \quad b = -\frac{Vᵣ Kᵣ}{Kᵣ + (S)₀} \]

Furthermore, the ratio b:m should be independent of substrate concentration. We have called a plot according to Equation 1 an Alberty plot. Typical Alberty plots for runs at pH 5.0 and 6.0 are shown in Fig. 3. It is seen that Equation 1 fits the data quite well over nearly the entire course of the reaction. The b:m ratio for runs at a given pH turns out to be independent of concentration, as required.

According to Equation 1, the reciprocal slopes (1/m) and reciprocals of intercepts (1/b) should both be linear functions of the initial substrate concentration:

\[ \frac{1}{m} = \frac{Kᵣ}{(Kᵣ/Kₛ - 1)} + \frac{(S)₀}{(Kᵣ/Kₛ - 1)} ; \quad \frac{1}{b} = \frac{Kᵣ}{Vᵣ} + \frac{Kₛ}{Vₛ Kᵣ} \]

Thus, plots of 1/m and 1/b versus (S)₀ should be straight lines. From the slopes and intercepts of these secondary plots, all the kinetic constants may, in theory, be evaluated.

In Figs. 4 and 5, secondary plots for two series of runs, one at pH 5.0, the other at pH 6.0, are shown. At both pH values the slopes of the secondary plots may be evaluated quite accurately; however, the intercepts of the plots are so close to the
TABLE I

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_a$ (M)</th>
<th>$V_{max}/(E)_0$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>3.2 X 10$^{-3}$</td>
<td>6.0 X 10$^{-2}$</td>
</tr>
<tr>
<td>6.0</td>
<td>2.0 X 10$^{-3}$</td>
<td>8.0 X 10$^{-2}$</td>
</tr>
</tbody>
</table>

**DISCUSSION**

It is interesting to note that the values of $K_a$ and $V_{max}/(E)_0$ obtained at pH 7.0 agree quite well with those measured by Hummel et al. at pH 6.7 (7). Hummel reported $K_a = 9.9 \times 10^{-4}$ M. Although no value for $V_{max}/(E)_0$ is stated by Hummel et al. (7), it was evaluated from data given in the article. Hummel's value of $V_{max}/(E)_0$ is 1.6 sec$^{-1}$.

Two notable features of Table I are the pH independence of $V_{max}/(E)_0$ over the pH range from 5.0 to 8.0 and the decrease of $K_a$ as the pH decreases within this same range.

These results can be interpreted in terms of the simple reversible, one-intermediate scheme above. The pH independence of $V_{max}/(E)_0$ within the pH range of imidazole ionization suggests that the ionizable group of the histidine residue in the active center of the enzyme (3, 4) is bound by the substrate in the enzyme-substrate complex. Although the data are rather meager, the variation of $K_a$ within this same pH range is consistent with the idea that the active histidine residue in the free enzyme participates in the initial binding of the substrate and must be protonated in order to do this.

It is well known that many enzyme-substrate reaction mechanisms are not adequately represented by the one-intermediate scheme, but require two or more intermediate complexes (18). It is important to consider what modifications would be required in the above interpretation of the pH dependence of the kinetic constants if this were true for the RNAse-cytidine-2',3'-cyclic phosphate system. Krupka and Laidler (19) have shown that measurements of initial rates in the limit of low substrate concentrations can yield unambiguous information about the pK$_a$ values of groups in the active site of the free enzyme, regardless of the number of intermediate complexes that may be formed. In the limit of low substrate concentration, the initial rate is given by:

$$v_0 = V_{max}/(E)_0/K_a$$

As this study shows $V_{max}$ to be independent of pH in the range investigated, the pH dependence shown by $v_0$ at low substrate...
concentrations will be identical to that shown by $1/K_e$. Therefore, the conclusion that the free enzyme has an ionizable group in the active site with a $pK_e$ in the neutral region is unaffected by the possibility of multiple intermediates.

The situation with regard to $V_0$ is more complicated. In terms of the one-intermediate mechanism, $V_0 = k_d(E)_o$. If a series of intermediates exists, $V_0 = k_{app}(E)_o$ in which $k_{app}$ is a rather complex constant which may incorporate rate constants for all of the steps in the reaction except the initial binding of enzyme and substrate (17). However, if one of the steps is slow enough to control the entire reaction sequence, $k_{app}$ may be identified with the rate constant for the rate-controlling step.

The above analysis severely limits the interpretation that can be given to the lack of pH dependence of $V_0$. The most that can be said is that if the histidine residue in the active center of one of the intermediates is free, then breakdown of that particular intermediate does not constitute the rate-determining step.

**SUMMARY**

The kinetics of the ribonuclease catalyzed hydrolysis of cytidine-2',3'-cyclic phosphate has been studied at four different pH values in the pH range 5.0 to 8.0. At pH values of 7.0 and above, the initial rates can be evaluated from the data, and Michaelis constants and maximal velocities determined from initial rates. At pH 6.0 and below, product inhibition becomes so extensive that initial rates cannot be evaluated. An integrated rate equation is used to treat the low pH data.

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Michael Litt


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