THE HYDROLYSIS OF URIDINE CYCLIC PHOSPHONATE CATALYZED BY RIBONUCLEASE-A: IMPLICATIONS FOR THE MECHANISM OF ACTION OF THE ENZYME

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Abstract.—Uridine 2',3'-cyclic phosphonate (I) is slowly hydrolyzed by ribonuclease-A with $k_2$ and $K_m$ values at pH6 that are respectively 1900 and 15 times smaller than the same parameters at the same pH for the related phosphate (II). Since the ratio of rate constants for hydroxide ion catalyzed hydrolysis is about 4, this result is consistent with, but does not prove a mechanism for the enzymic reaction that requires a pseudorotation.

The action of ribonuclease on a dinucleoside phosphate or cyclic phosphate substrate can be considered to proceed by either an “in-line” or an “adjacent” mechanism, according to the relative geometry of the entering and leaving groups in the displacement reaction. An adjacent mechanism implies the existence of a pentacoordinate intermediate and requires that this intermediate undergo pseudorotation before product can form. There is no such restriction for the in-line case. In principle, therefore, it should be possible to distinguish between these alternative pathways by employing a substrate for which the formation or pseudorotation of the intermediate is inhibited. It is obviously necessary that in all other respects the test compound should resemble a normal substrate.

We here report the initial results of our investigation of the ribonuclease-catalyzed hydrolysis of uridine 2',3'-cyclic phosphonate (I), a substance closely related to a normal substrate of the enzyme, but for which the rate of formation of a pentacoordinate intermediate by the adjacent path should be significantly reduced.

Experimental.—Bovine pancreatic ribonuclease-A was purchased from Worthington Biochemical Corporation as a phosphate-free lyophilized powder. Uridine 2',3'-cyclic phosphonate was prepared from the 3'-phosphonate by treatment with dicyclohexyl-carbodiimide, and was isolated as the calcium salt. Analysis by means of specific ion
electrodes, by ultraviolet and atomic absorption spectroscopy, and by thin-layer chromatography showed that this compound was contaminated with sodium and calcium chlorides, but contained a negligible amount of any other impurity, including ring-opened material. Before use, the calcium salt was converted to the sodium form by passage through a column of ion exchange resin (Dowex 50-Na⁺); in buffer solution at pH 6 and 4°C the sodium salt was stable for at least ten weeks. The apparent extinction coefficient of I based on the phosphorus content was 10,100 at λmax 261.5 mμ(9,550 at 259 mμ for the phosphate). All enzyme kinetic runs were made in Tris acetate/sodium acetate/acetic acid buffer at pH 6.00 ± 0.01, with the addition of KCl to give an ionic strength of 0.2. Delta ε for the cyclic and ring-opened forms was determined for both I and II at 10⁻⁴M between 270 and 290 mμ at every 1 mμ at a constant slit width of 0.2 mm, in buffer at pH 6 and ionic strength 0.20. The Amax/Amin ratio was 0.32 for I and 0.24 for II. A Cary 15 spectrophotometer equipped with a thermostated cell block (25.00°C ± 0.01) was used for all absorption measurements and kinetic runs. The acid and the base hydrolyses were followed at 280 mμ. Other details and the rate constants are given in Table 1.

The enzyme catalyzed hydrolyses were followed at 280, 281, or 286 mμ using the 0.1 slidewire. For the phosphate, an enzyme concentration of 3.5 × 10⁻⁷ M was used; six runs were made with substrate concentrations varying from 2.2 × 10⁻⁴ to 4.7 × 10⁻⁴ M. For the phosphonate the enzyme concentration was 3.4 × 10⁻⁴ M; seven runs were made with substrate concentrations varying from 1.5 × 10⁻⁴ to 1.9 × 10⁻⁴ M. For the phosphate, the integrated form of the Michaelis-Menten equation (including product inhibition) was adjusted directly to the experimental plots of substrate concentration versus time using the nonlinear weighted least-squares method described by Wentworth. The values of k₂, Kₗ, and Kₛ, obtained in this way were poorly defined but were used only to calculate a value of the initial velocity (v₀). The same value of v₀ was obtained whether a two-parameter fit (kₗ and Kₗ varying, with Kₛ constant) at 8.2 × 10⁻⁵ or three-parameter fit was used. The covariance term for kₗ and Kₗ was included in the calculation of the standard deviation of v₀. For the slower reaction of the phosphonate, values of v₀ were obtained from plots of ln (S₀/S) versus time. For both esters, k₂ and Kₗ were calculated from the values of v₀ and S₀ using the nonlinear weighted least-squares method, and weights were taken as inversely proportional to the variances of the individual values of v₀. The results are given in Table 1.

Discussion.—To the extent that the Michaelis constant (Kₗ) measures binding—and this is probably a reasonable assumption here—the phosphonate is bound to the enzyme somewhat more strongly than is the phosphate. However, the striking feature of the results shown in Table 1 is the large difference (×1900) between the value of k₂ for the phosphate and for the phosphonate. By contrast, the rates of nonenzymic hydrolysis of the two compounds in base (or in acid) are quite similar, so that the ratio of ratios: (k₂ phosphate/k₂ phosphonate) (kOH phosphate/kOH phosphonate) is about 500. Since the phosphate has available two ester bonds, whereas the phosphonate has but one, it could be argued that the “chemical” hydrolysis unfairly favors the phosphate (both 2' and 3' esters are indeed formed on acid or base catalyzed hydrolysis of the cyclic phosphate; the enzymic reaction gives only the 3' ester, so that this ratio perhaps underestimates the actual difference. This large rate difference could be due to a combination of several factors:

1. The phosphonate could bind to the enzyme with an orientation that precludes efficient catalysis. This explanation is suggested by previous findings that small changes in the structure of the pyrimidine base can result in a large decrease in k₂ (often with little change in Kₗ). In the present case, however, the base is unchanged, and although the specificity of the enzyme with respect to
substitution on the ribose ring is not known in detail, examination of the probable
mode of binding of the phosphonate using the three-dimensional model of the
structure of ribonuclease-S did not indicate any unfavorable steric interactions
between the enzyme and the new CH₃ group of the substrate. In addition, the
conformation of the phostone ring is under the same severe restraint that applies
to the phosphate.

(2) Although the pKₐ values of the phosphate and phosphonate are probably
similar, the pH/rate optimum of the phosphonate may differ from that of the
phosphate, and since measurements were made at but one pH, the phosphonate
may appear a worse substrate than it really is. However, at about pH 6, the
value of k₂ for the phosphate is not changing rapidly with pH, and if this is also
true for the phosphonate it seems unlikely that a major part of the rate difference
would be due to this cause. This possibility will be further investigated as more
material becomes available.

(3) Attack of water on the phosphorus atom to give a pentacoordinate interme-
diate or transition state can occur from either of two directions (see Fig. 1).
If the approach is from the left side (in-line), both the incoming water molecule
and the 2' oxygen take up apical positions, and the resulting trigonal bipyramid
can decompose directly to products. There is no requirement for an interme-
diate, but it is not excluded. If the approach is from the right (adjacent mech-
nism), the incoming water molecule and the 3' CH₃ would take up apical posi-
tions, while the 2' oxygen would therefore initially assume a basal position. In
order for product to form, this pentacoordinate intermediate must first undergo a
pseudorotation to place the 2' oxygen in an apical position. However, the formation of this initial intermediate with a CH₃ group in an apical position is

![Diagram]

FIG. 1 The directions of attack for the in-line and adjacent mechanisms. Attack from
above or below the phostone ring is considered unlikely as it would result in a five-membered
ring spanning two basal positions.
would probably with a mechanism, in-line whether the first reverse nonenzymic is the expected not an ROH be strongly considered not to be slow. It is possible that the presence of the CH₂ group could hinder the approach of water from the left side, and thus reduce the rate of an in-line mechanism, but this seems unlikely in view of the similarity of the rates of the nonenzymic hydrolyses for I and II which, in the former case, almost certainly involves an in-line mechanism.

The two steps of RNase action: It has usually been the practice to equate water with a free nucleoside in the two steps that are catalyzed by RNase, and so discussions of mechanism have shown the ring opening step as the microscopic reverse of the ring closure, with ROH replaced by HOH. Although likely, this is not an absolute requirement and arguments favoring one mechanism for, e.g., the reaction of a dinucleoside phosphate, should not be applied uncritically to the ring-opening reaction (unless, of course, the ring opening is carried out by the same ROH to reform the diester). In principle, there are four different over-all mechanisms, involving all possible combinations of in-line and adjacent mechanism for the two steps. Each of these could be further subdivided according to whether the first step requires the catalytic groups in the same, or in a different, protonated form from the second step. Many of these possibilities may be discarded as improbable.

In-line or adjacent? A central point of this investigation was to try to establish whether pseudorotation is obligatory in the action of ribonuclease; we have not proved that it is not, and therefore the adjacent mechanism cannot be excluded on the basis of these experiments. However, two strong claims have recently been made that pseudorotation is either not involved or is not required in the action of the enzyme. Roberts et al. considered the nmr and X-ray evidence of the binding of cytidine 3' phosphate and other inhibitors, assumed that a similar mode of binding would be shown by a phosphate diester substrate, and concluded that for the first step only an in-line mechanism is possible. This may be substantiated when the measurements are repeated on models that are more closely related to a dinucleoside phosphate substrate. Eckstein has prepared the cyclic thiophosphate III and separated the two diastereoisomers a and b. On acid hydrolysis this compound exchanged sulfur into the solvent, in agreement with the work of Haake and Westheimer on ethylene phosphate.
When the hydrolysis of III was catalyzed by ribonuclease, no sulfur could be detected in the solvent, and the conclusion was drawn that pseudorotation could not have taken place. However, in this case pseudorotation is perhaps a necessary, but certainly not sufficient, condition for the observation of sulfur exchange. Whereas in the acid hydrolysis the three unesterified atoms (oxygen and sulfur) of the trigonal bipyramid become equivalent through pseudorotation, the enzyme would be expected to place some restrictions on this process, since these atoms will probably be closely associated with functional groups at the active site. In addition, the partitioning ratio of the intermediate (to give starting material or product) is not known, and could clearly be different for the acid and enzymic hydrolyses. Irrespective of these arguments, the availability of the isomers IIIa and b fortunately should allow a completely unambiguous assignment of mechanism for the first step.

Reversal of acid/base roles? The in-line mechanism is usually shown as involving a reversal of the acid/base roles of the two imidazole groups in the second step. Thus one imidazole (B₁) is pictured as a general base toward the 2'-OH, while the other (B₂) acts as a general acid, protonating the 5'-0 leaving group. This leaves the imidazole groups in the opposite state of protonation, and there has been much discussion as to whether the original state is first regained before the ring opening step. It has been assumed in a number of places that this question may be subject to an experimental test, since a reversal of the roles of the imidazoles (which differ in pKₐ) should give a different dependence of k₂/Kₘ on pH for the two steps. This assumption is in error, as is easily seen from a consideration of the relevant equilibria (Figure 2). Reaction via EH or HE gives precisely the same pH dependence, since the ratio of EH to HE is pH in-

\[
\frac{[EH]}{[HE]} = \frac{K_4}{K_3}; \quad \frac{[EH]}{[E]} \text{ (total free)} = \frac{1}{1 + \frac{K_2}{[H] + \frac{K_4}{[H] + \frac{H}{K_2}}}}
\]

Fig. 2. The microscopic acid dissociation equilibria for a hypothetical enzyme with two groups at the active site.
dependent. This error presumably arose from neglecting the difference between the macro- and the microscopic \( pK_a \) values of the groups at the active site:\(^{24} \)

\[
K_{a_1} \text{(macro)} = K_1 K_2 / (K_1 + K_2); \quad K_{a_2} \text{(macro)} = K_3 + K_4.
\]

Thus the similarity of the shapes of the pH/rate profiles for the two steps of ribonuclease action cannot be used as an argument to favor either mechanism over the other.

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Abbreviations: RNase, Bovine pancreatic ribonuclease A (EC 2.7.7.16); \( K_m \), Michaelis constant; \( K_p \), inhibition constant for product; \( k_o \), apparent first-order rate constant for decomposition of the enzyme-substrate complex.

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2 In a recent paper\(^4\) the terms “Linear” and “Pseudorotation” have been used to describe the two mechanisms (in-line and adjacent, respectively).
6 We thank Dr. R. Skogerboe of the Material Science Center at Cornell for these determinations.
7 The equation used was:

\[
k_o E_d = K_m (1 + S_o / K_p) \ln \left( \frac{S_o}{S} + (1 - K_m / K_p)(S_o - S) \right),
\]

where \( E_d \), \( S_o \), and \( S \) are the concentrations of total enzyme, added substrate, and substrate at time \( t \), respectively.
11 Abrash, H. I., C. S. Cheung, and J. C. Davis, Biochemistry, 6, 1298 (1967).
14 Our results, together with those of Abrash et al.\(^{11}\) and Eckstein,\(^{29}\) indicate that the hydrolysis rate of II is of second order in hydrogen ion between 0.01 and 0.1 N acid, \( (I = 0.1) \), but of less than second order above this range (\( I = 0.4 \)). The rate difference between the phosphate and phosphonate is greater in 0.4 N than in 0.1 N acid: ignoring specific salt effects this corresponds to a \( pK_a \) for I very roughly 0.5 units higher than for II. The observed rate difference thus represents a combination of rate and equilibria effects. However, to the extent that the enzymic reaction involves general acid catalysis, the same types of factors would be expected to determine the relative rates of this reaction, and thus the acid catalyzed hydrolysis may also serve as a reasonable model for the intrinsic reactivity difference of I and II on the enzyme.
16 The calculated standard deviations of \( k_o \) and \( K_m \) do not take account of the possible presence of systematic error. However, the more accurately defined ratio \( k_o / K_m \) is still over 100 times greater for the phosphate.
Wyckoff, H. W., K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, J. Biol. Chem., 242, 3984 (1967). We thank Dr. F. M. Richards for an opportunity to study in detail the model of ribonuclease-S.

We have not yet determined whether I is a competitive inhibitor of the reaction of II.


Eckstein claims that the sulfur atom is not closely associated with the enzyme in one isomer, but the pH of his determination was not stated, and the pH optima of $K_m$ and $k_2$ may be different for the phosphate and the thiophosphates. The large difference ($\times 200$) in the rates of acid catalyzed hydrolysis of the two compounds may reflect a large difference in $pK_a$ (see reference 14).


Note added in proof: The possibility of different mechanisms for the two steps of RNase action has recently been stressed, although on other premises, by D. C. Ward, W. Fuller, and E. Reich, these Proceedings, 62, 581 (1969).