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Phenyl Ester of Adenosine 3'-Phosphate as a Novel Probe for the Rate-Limiting Step in RNA Hydrolysis

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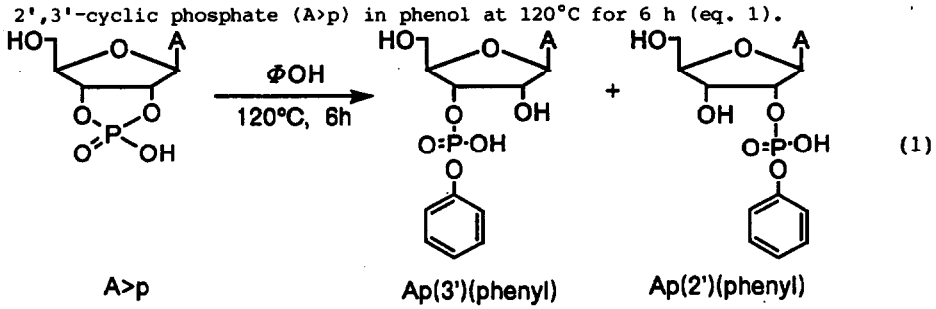
Abstract: Phenyl ester of adenosine 3'-phosphate is synthesized as a probe for the rate-limiting steps in enzymatic and non-enzymatic RNA hydrolyses.

Significant interests have been focusing onto catalytic hydrolyses of RNAs, mainly because of potential applications to artificial ribonucleases.[1-3] The hydrolysis proceeds via pentacoordinate intermediate, formed by the intramolecular attack of the 2'-hydroxyl group of ribose toward the phosphorus atom.[4] However, rather little has been known on whether the formation of the intermediate is rate-limiting or its decomposition is.[3,5,6] These informations are definitely required for understanding of the catalytic mechanisms and for the molecular design of still more advanced catalysts.

We present here the first successful preparation of phenyl ester of adenosine 3'-phosphate (Ap(3')(phenyl)) as quite a useful probe for the purpose. This probe has a similar structure as an RNA dimer adenylyl(3'-5')adenosine (ApA), except for the better leaving group (phenolate of pK_a 9.8) than that of ApA (5'-OH of the ribonucleoside: pK_a>13).[7] If the formation of the pentacoordinate intermediate is rate-limiting, the probe should be hydrolyzed at a comparable rate with RNA. On the contrary, the probe should be hydrolyzed much faster than ApA if the decomposition of the intermediate to the products is rate-limiting.[8]

Ap(3')(phenyl) was synthesized, together with the corresponding 2'-phenyl ester (Ap(2')(phenyl)), in a ratio of around 1:1, by the treatment of adenosine 2',3'-cyclic phosphate (A>p) in phenol at 120°C for 6 h (eq. 1).



A>p Ap(3')(phenyl) Ap(2')(phenyl)

Separation of Ap(3')(phenyl) from the reaction mixture and its purification were carried out by HPLC: an ODS column (Merck LiChrosphere RP-18(e)) with a linear gradient from 95/5 A/B mixture at 0 min to 50/50 at 15 min, and then to 0/100 at 20 min (A, pH 6.5 triethylamine-acetate buffer; B, acetonitrile). The retention times for Ap(2')(phenyl) and Ap(3')(phenyl) were 7.5 and 9.9 min, respectively. The structures of these products were confirmed by ^{1}H-NMR spectroscopy.[9] The hydrolyses of Ap(3')(phenyl) and ApA were followed by periodical HPLC analysis (ODS column). Of the two phenyl phosphate esters, only Ap(3')(phenyl) was susceptible to the catalysis by ribonuclease T_2, consistently with the strict selection of 3'-5' phosphodiester linkage by the enzyme.

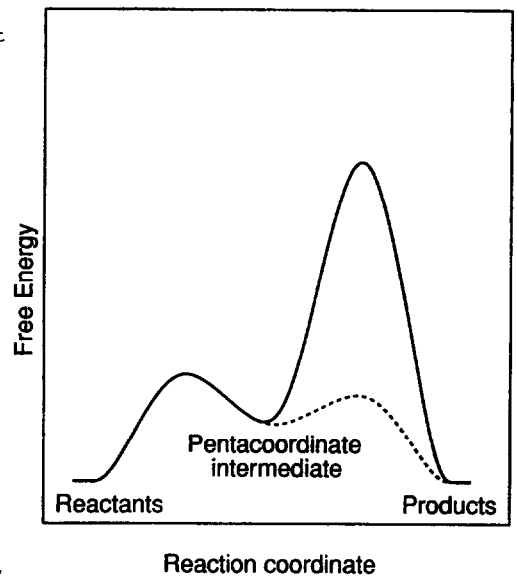


Fig. 1 Schematic energy profiles for the alkaline hydrolyses of ApA (the solid line) and of Ap(3')(phenyl) (the dotted line).

Ap(3')(phenyl) is promptly hydrolyzed at pH 7-10 and 50°C to phenol and A>p (in 1:1 ratio as expected). The A>p is then slowly converted to adenosine 2'- and 3'-phosphates. The pathway of the hydrolysis is exactly identical with that of ApA hydrolysis. Logarithm of the rate constant for the conversion of Ap(3')(phenyl) to A>p and phenolate increases linearly with pH (slope 1.0).

Quite importantly, the alkaline hydrolysis of Ap(3')(phenyl) is 10^5 fold faster than that of ApA at the same pH. Thus the hydrolysis rate is definitely governed by the basicity of the leaving group. Apparently, decomposition of the pentacoordinate intermediate to products is rate-limiting in the alkaline hydrolysis of ApA (see Fig. 1).

The rate-limiting decomposition in the ApA hydrolysis is furthermore confirmed by the results in Table 1. All the non-enzymatic catalysts (ethylenediamine,[1d] $[Co(triethylenetetramine)(H_2O)_2]^{3+}$,[1c] and Tm^{3+} ion [1f]) accelerate the hydrolysis of ApA in much larger magnitudes than the corresponding values for the hydrolysis of Ap(3')(phenyl). The k(Ap(3')(phenyl))/k(ApA) ratio monotonously decreases, in the range of 10^5 fold, with increase in the catalytic activity. Thus, the rate-limiting step in ApA hydrolysis is different from that (the formation of the pentacoordinate intermediate) in the hydrolysis of Ap(3')(phenyl). The assignment for the latter is based on the rate-limiting formation of the intermediate in the hydrolysis of diaryl phosphates.[10]

Table 1. Pseudo-First Order Rate Constants k's for the Enzymatic and Non-Enzymatic Hydrolysis of ApA and Ap(3')(phenyl) [a]

Catalyst	Rate constant k (min^{-1})		k(Ap(3')(phenyl)) / k(ApA)
	Ap(3')phenyl	ApA	
OH^- (alkaline hydrolysis)	7×10^{-3}	7×10^{-8} [b]	1×10^5
ethylenediamine (1.0 M)	8×10^{-2}	8×10^{-5}	1×10^3
[Co(triethylenetetramine)-$(H_2O)_2]^{+3}$ (0.1 M) [c]	3×10^{-2}	3×10^{-4}	1×10^2
Tm^{3+} (0.01 M)	2×10^{-1}	8×10^{-2}	2.5
ribonuclease T_2 [d]	4×10^{-2}	3×10^{-2}	1.3

a. At pH 8, 50°C unless otherwise noted.
b. Estimated from the pH-rate constant profile of slope 1.0.
c. At pH 7.
d. At pH 4.5, 37°C; $[enzyme]_0$ = 0.05 units/ml.

With rather less efficient catalysts ethylenediamine and the Co(III) complex, the k(Ap(3')(phenyl))/k(ApA) ratio greatly exceeds 1 (Table 1): decomposition of the intermediate is still rate-limiting here, although accelerated significantly. With the more active catalyst Tm^{3+} ion, however, the decomposition of the intermediate is sufficiently accelerated so that its formation is now partially rate-limiting (k(Ap(3')(phenyl))/k(ApA) = 2.5). For the enzyme ribonuclease T_2, the ratio is equal to 1.3.

Previously the mechanisms of non-enzymatic RNA hydrolyses as well as of the hydrolyses by ribozymes have been, in most of the cases, interpreted in terms of the assumption that formation of the pentacoordinate intermediate is rate-limiting. Apparently this is not always the case, as evidenced above by use of the present probe.

In conclusion, phenyl esters of ribonucleoside phosphates, prepared by one-step reaction from the cyclic phosphates, provide valuable clues for the rate-limiting step in RNA hydrolysis. It has been shown that <u>any catalyst for RNA hydrolysis must promote decomposition of the pentacoordinate intermediate.</u> Application of the result to molecular design of highly active catalysts and artificial ribonucleases is currently under way.

Acknowledgments

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4) RNA hydrolysis is a two-step reaction with 2',3'-cyclic phosphate of ribonucleotide as a stable reaction intermediate. The present paper refers to the first step (formation of the cyclic phosphate intermediate) alone, which is rate-determining in the total hydrolysis. The term "intermediate" is used only for the pentacoordinate intermediate in the first step, in order to avoid unnecessary confusion.

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9) Typical ^{1}H-NMR data for Ap(3')(phenyl) (in DMSO-d_6, 400 MHz): δ8.35 (s, 1H), 8.13 (s, 1H), 6.99-7.25 (m, 5H), 5.88 (d, 1H), 4.66 (overlapping two q, 2H; 2'H and 3'H of the ribose), 4.03 (m, 1H). For Ap(2')(phenyl): 8.28 (s, 1H), 8.10 (s, 1H), 6.86-7.05 (m, 5H), 5.98 (d, 1H), 5.02 (q, 1H; 2'H of the ribose), 4.38 (q, 1H; 3'H of the ribose), 4.00 (m, 1H). The signal assignments were confirmed by 2D NMR.

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