HYDROLYSIS OF URIDINE-5′ *N*-ARYL AND *N*-ALKYL PHOSPHORAMIDATES BY RIBONUCLEOSIDE-5′ PHOSPHORAMIDASE

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1. Introduction

The present paper reports results concerning the mechanism of action of ribonucleoside-5′ phosphoramidase. This enzyme catalyzes hydrolysis of nucleotide amidates displaying a high specificity for the phosphoramidate bond and for the nucleotide fragment of the substrate molecule [1]. The nature of the amidate residue does not determine the specifity of the enzymes action but, as the structure of the substituent at the amide nitrogen becomes more complex, the degree of hydrolysis decreases. A preliminary study of the relative rates of hydrolysis of a number of uridine-5′ phosphoramidates differing in the basicity of the parent amine has resulted in the suggestion that a nucleotide-phosphorylated enzyme may exist as an intermediate in the ribonucleoside-5′ phosphoramidase reaction [2].

In this study the maximum rates of enzymatic hydrolysis of uridine-5′ *N*-aryl and *N*-alkyl phosphoramidates are determined. The results obtained are in agreement with the above suggestion.

2. Materials and methods

Uridine-5′ *N*-aryl and *N*-alkyl phosphoramidates were synthesized by the procedure already used in [2]. Ribonucleoside-5′ phosphoramidase was prepared from rabbit liver as described earlier [1] with some modifications. One enzyme unit is defined as the amount which hydrolyses 1 μmole of uridine-5′ phosphoro-*p*-anisidate per minute in 0.05 M acetate buffer, pH 5.0, at 37°. The mixture (final volume 0.5 ml) containing the substrate ($2 \times 10^{-2} - 1 \times 10^{-3}$ M) and 0.058 units of enzyme in 0.05 M acetate buffer, pH 5.0, was incubated at 37° for 2 min. Under these conditions the degree of hydrolysis of the substrates did not exceed 10%. The reaction was then stopped by adding 0.1 ml of 0.4 N NaOH. After incubation for 10 min 0.1 ml of 0.4 N HCl, 0.1 ml of 1 M glycine buffer pH 10.4, and 0.1 ml solution of intestinal phosphatase were successively added to the sample. After 20 min the reaction was terminated with 2.1 ml of water and the amount of inorganic phosphorus (Pi) liberated was estimated [3]. The maximum velocity and apparent Michaelis constants were estimated from the Lineweaver-Burk plots.

3. Results and discussion

It is known from the literature that the reactions involving amidates of phosphoric acid, including hydrolysis, undergo general acidic catalysis [4]. Hence, it could be thought that the main catalytic function of the enzyme in phosphoramidate hydrolysis is protonation of the phosphoramidate by the proton-donating group of the enzyme, thereby facilitating the nucleophilic attack of the phosphorus atom by a molecule of water. Thus, the mechanism of enzymatic hydrolysis may be that of simple substitution. If this were the case, one would expect

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Table 1
Kinetic parameters for the enzymatic and acidic hydrolysis of uridine-5′ *N*-aryl and *N*-alkyl phosphoramidates.

Parent amine		Respective amide of uridine-5′ phosphate		
		Acidic hydrolysis [2]	Enzymatic hydrolysis	
Compound	pK_a	0.05 N HCl 37° $K \times 10^2$ min^{-1}	K_m (app) (mM)	V_{max} (μg Pi)
p-Nitroaniline	1.0	stable	stable	
Aniline	4.57	0.73	1.80	1.2
p-Anisidine	5.29	1.21	1.00	1.3
Trifluoroethylamine	5.70	1.53	0.09	4.3
Benzylamine	9.34	4.78	0.80	11.4
β-Phenyl isopropyl-amine	10.00	1.50	1.00	11.7
n-Butylamine	10.43	0.83	0.10	11.7
Isopropylamine	10.63	0.55	0.75	11.5
Cyclohexylamine	10.64	0.48	1.85	11.5
Dimethylamine	10.64		0.28	11.4

a parallel change in the rates of the enzymatic and acidic hydrolysis of nucleotide amidates with different basicity of the amide nitrogen.

The data which we have obtained on enzymatic hydrolysis of aryl- and alkylamidates of uridine-5′ phosphate are presented in table 1. The rate constants of acidic hydrolysis of the same compounds are given for comparison.

Comparison of the rate constants of acidic hydrolysis and V_{max} values for weak amine derivatives (aromatic and trifluoroethylamine, pK_a 1.0–5.7) shows that the increase in the basicity of the parent amine is accompanied by an increase in the rate of both acidic and enzymatic hydrolysis. In the series of derivatives of strong aliphatic amines (pK_a 9.34–10.64) the rate of chemical hydrolysis markedly decreases with an increase in the basicity of the parent amine, whereas the rate of enzymatic hydrolysis remains constant though it is higher than that for the most active amidate of the first group.

The results seem to allow the simple substitution mechanism of enzymatic hydrolysis to be ruled out because, if it had been the case, the rates of enzymatic and chemical hydrolysis would have correlated whatever the mechanism of cleavage of the P–N bond. The data can, apparently, be rationalized in terms of double substitution:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_2 \longrightarrow E + P_2$$
$$ES_1 \searrow P_1$$

Thus, the equality of the rates of enzymatic hydrolysis of derivatives of strong amines means that the rate-limiting step is the hydrolysis of the nucleotidyl–enzyme intermediate and $K_{m\,(app)} = K_m \times (k_3/k_2)$ where k_2 and k_3 are rate constants of phosphorylation and dephosphorylation of the enzyme. The differences in V_{max} for derivatives of weak amines seem to be due to the fact that the rate limiting stage in the hydrolysis of these compounds is the phosphorylation of the enzyme by the nucleotide. Its rate, like that of acid hydrolysis, is determined by the pK_a value of the parent amine. For this case $K_m = K_{m\,(app)}$.

Isolation of the covalently bound enzyme–nucleotide complex would be unequivocal proof of the mechanism of action of ribonucleoside-5′ phosphoramidase suggested above.

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