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RELATIONSHIP BETWEEN THE ACTIVE SITES OF 2',3'-CYCLIC PHOSPHODIESTERASE WITH 3'-NUCLEOTIDASE ACTIVITY PURIFIED FROM *VIBRIO ALGINOLYTICUS*

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SUMMARY

Using the purified 2',3'-cyclic phosphodiesterase (3'-nucleotidase) of slightly halophilic *Vibrio alginolyticus*, the kinetics of the mutual inhibitions between the substrates and of the inhibitory effects of substrate analogs were studied. The following results were obtained:

1. 3'-Ribonucleotides, 2',3'-cyclic ribonucleotides and *p*-dinitrophenyl phosphate acted as linear competitive inhibitors.

2. The 3'-ribonucleotides all were hydrolyzed at the same active site on the enzyme protein.

3. The activities for 3'-ribonucleotides and for di-*p*-nitrophenyl phosphate were linear competitively inhibited by ribonucleosides and by 5'-ribonucleotides to a lower degree. The decreasing order of their effectiveness (cytidine, uridine, adenosine and guanosine) was similar to the decreasing order of affinities of the 3'-ribonucleotides (3'-CMP, 3'-UMP, 3'-AMP and 3'-GMP) for the 3'-nucleotidase site.

4. The hydrolysis of di-*p*-nitrophenyl phosphate was simple linear noncompetitively inhibited by pyrimidine bases; the increasing order of effectiveness was thymine, uracil, and cytosine. In contrast, the hydrolysis of 3'-AMP is linear competitively inhibited by these bases having a reverse order of effectiveness.

With the results on the chloride modifications for this enzyme, it was considered that di-*p*-nitrophenyl phosphate is not hydrolyzed exactly at either the 3'-nucleotidase or the cyclic phosphodiesterase site and that the active sites for di-*p*-nitrophenyl phosphate and for 3'-nucleotidase possibly overlap at a locus where the ribonucleoside of the 3'-ribonucleotide molecule participates in binding.

The patterns for the product inhibition for the active mechanism of the 3'-nucleotidase are incompatible with the Ordered Uni Bi mechanism established for the phosphatases, and the existence of a phosphorylated enzyme as an intermediate appears unlikely.

INTRODUCTION

We previously described the purification and properties of 2',3'-cyclic phosphodiesterase having 3'-nucleotidase activity from slightly halophilic *Vibrio alginolyticus*. It was discovered that Cl^- activates the 3'-nucleotidase and inhibits the 2',3'-cyclic phosphodiesterase activities of this enzyme[1]. These modifications affected only the Michaelis constant for the substrate di-*p*-nitrophenyl phosphate. Since the two activities residing in the same protein molecule are inversely modified by Cl^-, it was considered that these activities might be located at different sites in the protein molecule. The active site for di-*p*-nitrophenyl phosphate, however, has not been characterized.

Considering the effects of substrate analogs, ANRAKU[2] reported that the enzyme from *Escherichia coli* is inhibited by both soluble and ribosomal RNA's but neither by uridine nor by 5'-UMP. NEU[3] purified the corresponding enzymes of various members of Enterobacteriaceae and briefly noted the inhibitory effect of ribonucleosides and of 5'-ribonucleotides in addition to tRNA and ribopolynucleotides. Types of these inhibitors, however, have not been mentioned by these authors.

Using the purified enzyme of *V. alginolyticus*, we found that the activity for one substrate is inhibited by the presence of another substrate and by several substrate analogs. In an attempt to elucidate the relationships between active sites for various substrates, mutual inhibitions between substrates and the effects of the substrate analogs were examined.

MATERIALS AND METHODS

Materials and methods for enzyme purification and assays were those previously described[1]. The composition of the reaction mixture is given in the legends. Incubation was for 10 min at 37°. 2',3'-Cyclic phosphodiesterase of *V. alginolyticus* possessed the specific activity of about 175 μmoles of P_i released/min per mg protein with 3'-AMP as the substrate. Concentrations of ribonucleosides and ribonucleotides were checked by measuring their absorbances.

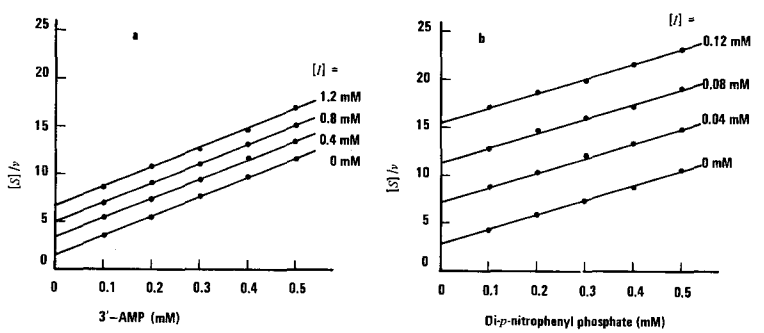

Fig. 1. Competitive correlation between 3'-AMP and di-*p*-nitrophenyl phosphate hydrolyses. The reaction mixture (1.0 ml) contained 20 mM Tris-H_2SO_4 (pH 8.3), 200 mM NaCl, 0.025 μg of enzyme and various concentrations of 3'-AMP and di-*p*-nitrophenyl phosphate. The concentrations of the inhibitor([*I*]) added were indicated in the figures (a). Effect of di-*p*-nitrophenyl phosphate (*I*) on the hydrolysis of 3'-AMP. Velocities (*v*) were expressed in μmoles of P_i released per 10 min per 0.025 μg of enzyme. (b) Effect of 3'-AMP (*I*) on the hydrolysis of di-*p*-nitrophenyl phosphate. Velocities were expressed in μmoles of *p*-nitrophenol released per 10 min per 0.025 μg of enzyme.

RESULTS

Mutual inhibition between the substrates

As shown in Fig. 1, the hydrolysis of 3'-AMP is competitively inhibited by the presence of di-*p*-nitrophenyl phosphate and *vice versa*. Experiments similar to those shown in Fig. 1 were carried out using different concentrations of the enzyme and of NaCl. The values of K_m/v_{max} with and without various inhibitor concentrations were obtained from the vertical intercepts of $[S]/v$ *versus* $[S]$ plots. As shown in Fig. 2, these values are replotted against the concentrations of the inhibitor. The plots are linear in all cases indicating that the inhibition is linear competitive, according to the nomen-

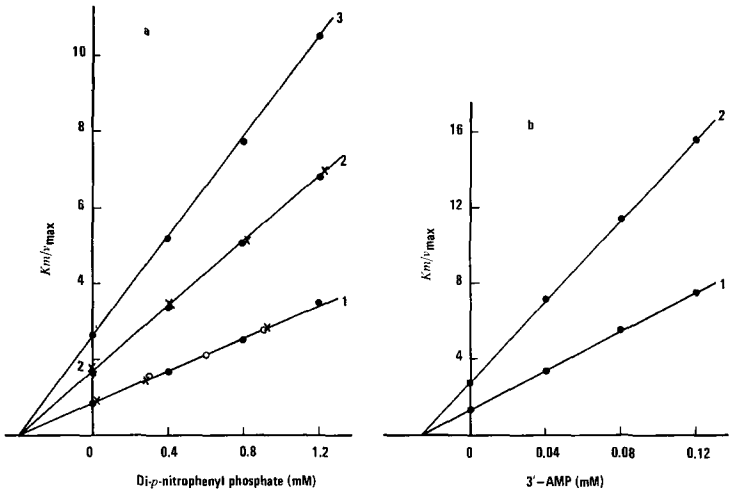

Fig. 2. (a) Effect of di-*p*-nitrophenyl phosphate (I) on the 3'-AMP hydrolysis. (b) Effect of 3'-AMP (I) on the di-*p*-nitrophenyl phosphate hydrolysis. The values of K_m/v_{max} in the presence and absence of the inhibitor were obtained from the vertical intercepts of $[S]/v$ *versus* $[S]$ plots, and they were plotted against inhibitor concentrations. The amounts of enzyme and NaCl used are: (1) of a and b, 0.05 μg of enzyme and 200 mM NaCl; (2) of a and b, 0.025 μg of enzyme and 200 mM NaCl; (3) of a, 0.025 μg of enzyme and 40 mM NaCl. Other conditions are the same as shown in Fig. 1. Different symbols used for the same experimental conditions denote values obtained from the independent experiment.

clature proposed by CLELAND[4]. Fig. 2a shows that the inhibitor constant of di-*p*-nitrophenyl phosphate for 3'-AMP hydrolysis is unaffected by NaCl concentrations.

The activity for di-*p*-nitrophenyl phosphate was also linear competitively inhibited by other 3'-ribonucleotides and by 2',3'-cyclic ribonucleotides. The inhibitory effects of 3'-ribonucleotides were about the same as the corresponding cyclic ribonucleotides, and the order of effectiveness was found to be as follows: 3'-CMP > 3'-UMP > 3'-AMP > 3'-GMP.

The hydrolysis of 2',3'-cyclic ribonucleotides to the corresponding 3'-ribonucleotides was inhibited by the presence of 3'-ribonucleotides. Fig. 3 is the effect of 3'-CMP on the hydrolysis of Urd-2',3'-*P*. The hydrolysis of Cyd-2',3'-*P* was also linear competitively inhibited by 3'-CMP. It was found that the 3'-ribonucleotides became less inhibitory for the cyclic phosphodiesterase activity in the order: 3'-CMP, 3'-UMP, 3'-AMP and 3'-GMP.

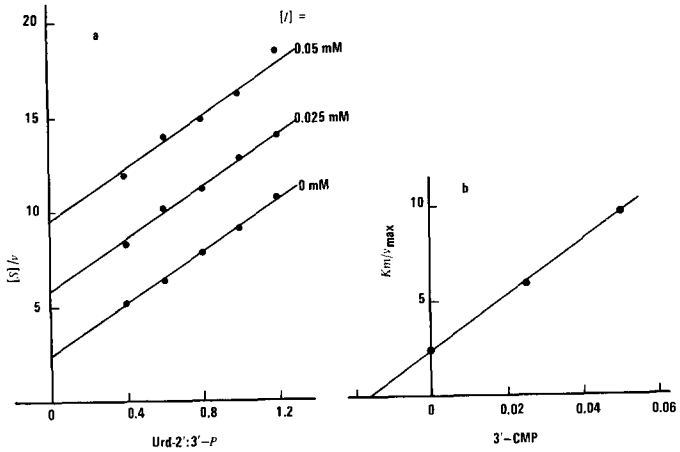

Fig. 3. Effect of 3′-CMP on the hydrolysis of Urd-2′,3′-*P*. The hydrolysis of Urd-2′3′-*P* was measured by the increase in absorbance at 286 mμ. The reaction mixture (2.5 ml) contained 20 mM Tris–H_2SO_4 (pH 8.6), 0.1 μg of enzyme per ml and 3′-CMP(*I*) as indicated above. Velocities (*v*) were expressed in μmoles of Urd-2′,3′-*P* hydrolyzed per min per 0.1 μg of enzyme. (a) $[S]/v$ *versus* $[S]$ plots in the presence and absence of 3′-CMP(*I*). (b) K_m/v_{max} *versus* $[I]$ plots.

Table I indicates the Michaelis and inhibitor constants for several substrates. With respect to the mutual effects between 3′-AMP and di-*p*-nitrophenyl phosphate, the inhibitor constant of di-*p*-nitrophenyl phosphate for 3′-AMP hydrolysis does not coincide with the Michaelis constant for di-*p*-nitrophenyl phosphate and *vice versa*. This might indicate that these two substrates are not hydrolyzed at exactly the same active site on the enzyme protein.

Mutual effects of the 3′-ribonucleotides were examined by measuring the initial velocities of the mixed substrates. If two substrates are assumed to be hydrolyzed at the same active site on the enzyme protein, the initial velocities of the mixed substrates should follow this rate equation[5]:

$$v_t = v_a + v_b = \frac{a \cdot V_a/K_a + b \cdot V_b/K_b}{1 + a/K_a + b/K_b}$$

TABLE I

LINEAR COMPETITIVE INHIBITION BETWEEN TWO SUBSTRATES

Substrate	K_m *(mM)*	*Inhibitor*	*Velocity determined*	K_i *(mM)*
3′-AMP	0.08	Di-*p*-nitrophenyl phosphate	P_i	0.40
Di-*p*-nitrophenyl phosphate	0.20	3′-AMP	*p*-Nitrophenol	0.026
Cyd-2′,3′-*P*	0.10	3′-CMP	Increase in absorbance at 290 mμ	0.06
Urd-2′,3′-*P*	0.36	3′-CMP	Increase in absorbance at 286 mμ	0.016

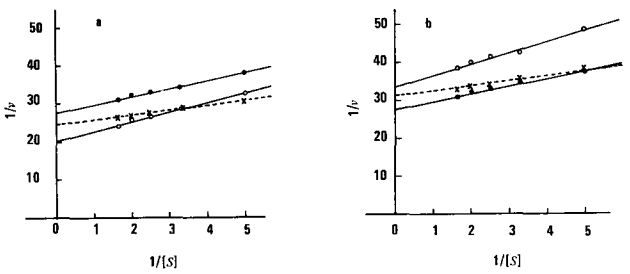

Fig. 4. $1/v$ *versus* $1/[S]$ plots for single and an equimolar mixture of 3′-ribonucleotides. The reaction mixture (1.0 ml) contained 50 mM Tris–HCl (pH 8.3), 200 mM NaCl, 0.02 μg of enzyme and the various concentrations of the substrate. Velocities(v) were expressed in μmoles of P_i released per 10 min per 0.02 μg of enzyme. In an equimolar mixture of the substrates, $[S]$ represents the concentrations of the each substrate mixed. (a) ●—●, 3′-AMP; ○—○, 3′-GMP; ×, plots for the equimolar mixture of 3′-AMP and 3′-GMP; — — —, theoretical lines calculated from v_{max} and K_m values for 3′-AMP and 3′-GMP. (b) ●—●, 3′-AMP; ○—○, 3′-UMP; ×, plots for the equimolar mixture of 3′-AMP and 3′-UMP; — — —, theoretical lines calculated.

v_t = velocity of total activity; v_a, v_b = velocities for the substrates A and B; a, b = substrate concentrations for the substrates A and B; K_a, K_b = Michaelis constants; V_a, V_b = maximal velocities. In an equimolar mixture of A and B, *i.e.*, $a = b = [S]$, v_t becomes $(V_a/K_a + V_b/K_b)/(1/[S] + 1/K_a + 1/K_b)$. In this case, the maximal velocity attainable is $(V_a \cdot K_b + V_b \cdot K_a)/(K_a + K_b)$, and the Michaelis constant is $K_a \cdot K_b/(K_a + K_b)$. Fig. 4 shows $1/v$ *versus* $1/[S]$ plots for respective and an equimolar mixture of 3′-ribonucleotides. Theoretical lines for the mixed substrates were calculated from the former equation using the Michaelis constants and the maximal velocities for the respective substrates and are in good agreement with the experimental results obtained for the mixed substrates.

Effects of substrate analogs

The hydrolyses of 3′-AMP and *p*-dinitrophenyl phosphate, respectively, were inhibited by ribonucleosides and 5′-ribonucleotides. The effects of these substances

TABLE II

SUMMARY OF INHIBITOR CONSTANTS OF LINEAR COMPETITIVE INHIBITORS

Linear competitive inhibitor	*K_i (mM) for the hydrolysis of*	
	3′-AMP	*Di-p-nitrophenyl phosphate*
Cytidine	0.020	0.0056
Uridine	0.023	0.009
Adenosine	0.045	0.018
Guanosine	0.32	0.095
5′-CMP	0.04	0.02
5′-UMP	0.22	0.095
5′-AMP	0.30	0.165
5′-GMP	0.32	0.165
2′-AMP	0.16	0.075
p-Nitrophenyl phosphate	—	6.5

TABLE III

COMPARISON OF THE EFFECTS OF RIBONUCLEOSIDES FOR THE HYDROLYSIS OF 3′-RIBONUCLEOTIDES AND OF DI-*p*-NITROPHENYL PHOSPHATE

The reaction mixture (1.0 ml) contained 50 mM Tris–HCl (pH 8.3), 200 mM NaCl, 0.5 mM substrate, 0.2 mM inhibitor and 0.025 μg of enzyme. Activities were expressed in relative activities. Values in parentheses indicate the Michelis constant in mM for the respective substrates.

Inhibitor added	*Relative activity*				
	3′-CMP (0.01)	*3′-UMP (0.05)*	*3′-AMP (0.08)*	*3′-GMP (0.10)*	*p-Dinitrophenyl phosphate (0.20)*
None	100	100	100	100	100
Cytidine	80	59	40	25	8.5
Uridine	84	64	44	29	13
Adenosine	88	77	60	40	22
Guanosine	99	95	92	82	62

were examined by a method similar to those in Figs. 1 and 2 and they all proved to be linear competitive inhibitors. The inhibitor constants obtained for both substrates are summarized in Table II which shows that the ribonucleosides are more inhibitory than the corresponding 5′-ribonucleotides and that the order of effectiveness is Cyd > Urd > Ado > Guo. Deoxyribonucleosides were found to act as linear competitive inhibitors, and their effectiveness was similar to the corresponding ribonucleosides.

In Table III the effects of the ribonucleosides on the hydrolyses of 3′-ribonucleotides and of di-*p*-nitrophenyl phosphate are compared. The order of their effectiveness is the same as above for all substrates examined. In addition, the respective ribonucleosides become more inhibitory for the substrates in the order 3′-CMP, 3′-UMP, 3′-AMP, 3′-GMP and di-*p*-nitrophenyl phosphate. This order coincides with the increasing magnitudes in the Michaelis constants for the respective substrates. These results suggest a close correlation between the active sites for 3′-ribonucleotides and di-*p*-nitrophenyl phosphate.

In contrast to the results reported for the enzymes of Enterobacteriaceae[3], this enzyme is unable to hydrolyze *p*-nitrophenyl phosphate at an appreciable rate, and it was found that *p*-nitrophenyl phosphate is a linear competitive inhibitor of the di-*p*-nitrophenyl phosphate hydrolysis. The inhibitor constants obtained are listed in Table II.

This enzyme was not inhibited by the presence of P_i. With 0.5 mM of di-*p*-nitrophenyl phosphate as the substrate, 5.0 mM of P_i inhibited only 3% of the activity. The activity for 0.5 mM of 3′-AMP was unaffected by 1.0 mM of P_i. NEU[3] has reported no inhibitory effect of P_i with the enzymes of Enterobacteriaceae.

Inhibitory effects of pyrimidine and purine bases

The activity for di-*p*-nitrophenyl phosphate was inhibited by pyrimidine bases. Fig. 5 demonstrates the effect of uracil; the values of $1/v_{max}$ and K_m/v_{max} in the presence and absence of uracil were obtained from the vertical intercepts of Figs. 5a and 5b, respectively. As shown in Fig. 6, these values are replotted against the concen-

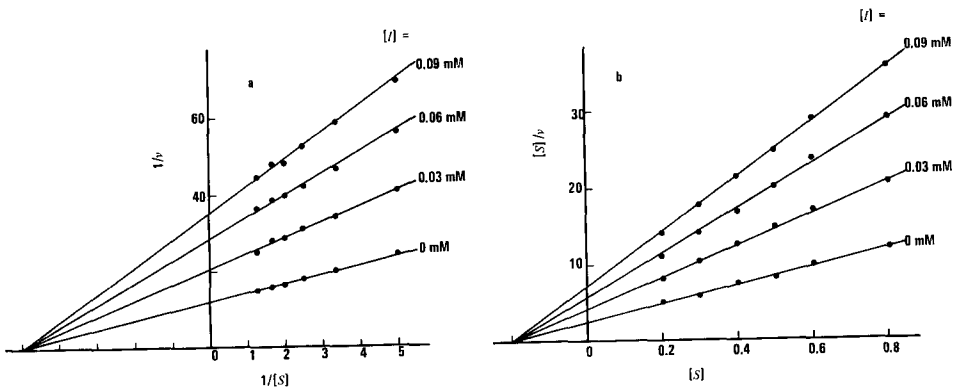

Fig. 5. Effect of uracil on the hydrolysis of di-*p*-nitrophenyl phosphate. The reaction mixture (1.0 ml) contained 50 mM Tris–HCl (pH 8.3), 200 mM NaCl, 0.03 μg of enzyme and the various concentrations of di-*p*-nitrophenyl phosphate ([*S*]) and uracil (*I*) as indicated above. Velocities (v) were expressed in μmoles of *p*-nitrophenol released/10 min per 0.03 μg of enzyme. (a) $1/v$ *versus* $1/[S]$ plots. (b) $[S]/v$ *versus* $[S]$ plots.

trations of uracil. The $K_{\text{intercept}}$ and K_{slope} which are obtained from the horizontal intercepts of Figs. 6a and 6b are in excellent agreement indicating that uracil is a simple linear noncompetitive inhibitor. This was also confirmed for thymine and cytosine. In contrast to di-*p*-nitrophenyl phosphate, the pyrimidine bases were less inhibitory for 3'-AMP, and it was found that they act as linear competitive inhibitors.

The inhibitory effects of purine bases were slight for both substrates. Adenine was found to act as a linear competitive inhibitor for 3'-AMP. For the hydrolysis of di-*p*-nitrophenyl phosphate, plots of $1/v_{\max}$ *versus* $[I]$ and $K_m/v_{\max}$ *versus* $[I]$ were linear, but the values of $K_{\text{i(intercept)}}$ and $K_{\text{i(slope)}}$ were not in agreement, indicating that adenine is a linear noncompetitive inhibitor. Guanine showed no appreciable effect on the activities of both substrates. Similar patterns of inhibition have been observed with the enzyme of *Serratia marcescens*[6].

Table IV summarizes the types of inhibition and the inhibitor constants of

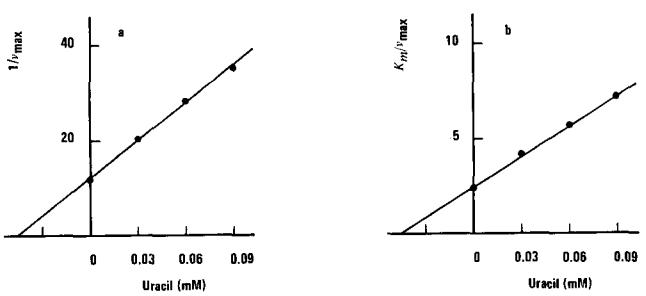

Fig. 6. Effect of uracil on the hydrolysis of di-*p*-nitrophenyl phosphate. The values of $1/v_{\max}$ and $K_m/v_{\max}$ in the absence and presence of the various concentrations of uracil were obtained from the vertical intercepts of Figs. 5a and 5b, respectively, and they were plotted against the concentrations of uracil .(a) $1/v_{\max}$ *versus* $[I]$ plots. (b) $K_m/v_{\max}$ *versus* $[I]$ plots.

TABLE IV

EFFECT OF PYRIMIDINE AND PURINE BASES ON THE HYDROLYSIS OF 3′-AMP AND DI-*p*-NITROPHENYL PHOSPHATE

Inhibitor	*K_i (mM) for*				
	3′-AMP		*Di-p-nitrophenyl phosphate*		
	Type of inhibition	*K_i*	*Type of inhibition*	*$K_{i(slope)}$*	*$K_{i(intercept)}$*
Thymine	linear competitive	2.0	simple linear noncompetitive	0.036	0.036
Uracil	linear competitive	1.5	simple linear noncompetitive	0.045	0.045
Cytosine	linear competitive	0.4	simple linear noncompetitive	0.15	0.15
Adenine	linear competitive	1.27	linear noncompetitive	0.50	4.0

pyrimidine and purine bases. It shows that the inhibitory effects of pyrimidine bases are in the order of cytosine > uracil > thymine for 3′-AMP hydrolysis. In contrast, the reverse order is true for di-*p*-nitrophenyl phosphate.

DISCUSSION

In the previous paper[1], we have reported that the 2′,3′-cyclic phosphodiesterase of *V. alginolyticus* catalyzes the hydrolyses of 2′,3′-cyclic ribonucleotides, 3′-ribonucleotides and di-*p*-nitrophenyl phosphate and that these substrate activities are associated with the same protein molecule. The present results on the mutual effects of the substrates showed that they interact as linear competitive inhibitors (Table I). These competitive correlations indicate that the active sites for these substrates are not independent of one another. With respect to the 3′-ribonucleotide activities, they are hydrolyzed at the same active site of the enzyme protein (Fig. 4). Similarly, all 2′,3′-cyclic ribonucleotides may be suppossed to hydrolyzed at the cyclic phosphodiesterase site.

However, the kinetic results on the mutual inhibition between 3′-AMP and di-*p*-nitrophenyl phosphate showed that they are not hydrolyzed exactly at the same active site (see text). This was supported by the facts that the inhibitory effects of pyrimidine and purine bases differ not only in the types of inhibition but also in the order of their effectiveness (Table IV). Moreover, it has been pointed out[1] that the activity for the latter substrate is modified by Cl^- in a manner quite different from 3′-nucleotidase and the cyclic phosphodiesterase.

On the other hand, the hydrolysis of di-*p*-nitrophenyl phosphate is linear competitively inhibited by ribonucleosides and by ribonucleotides, the order of effectiveness being the same as that for 3′-nucleotidase (Tables II and III). These results may be explained by considering that a part of its active site is common and overlapped with the 3′-nucleotidase site possibly at a locus where the base and sugar parts of the 3′-ribonucleotide molecule participate in binding. Since pyrimidine and purine bases inhibit 3′-AMP hydrolysis linear competitively, they will probably combine at a part of this common locus in a manner showing no effect on the Michaelis constant for di-*p*-nitrophenyl phosphate.

Since 3′-nucleotidase and cyclic phosphodiesterase activities are inversely

modified by Cl^- (ref. 1), the two active sites are not exactly identical. ANRAKU[2] has suggested different sites for the two activities of the *E. coli* enzyme. The linear competitive inhibition of the latter activity by the 3'-ribonucleotides may result from product inhibition. In this case, the increasing order of inhibitory effects of the 3'-ribonucleotides coincides with the increasing order of their affinities for the 3'-nucleotidase site. Therefore, the binding locus of the two active sites corresponding to the ribonucleoside part of the substrate may have a similar structure. Whether this binding locus is included as a common structure in each active site could not be clarified at present.

On the active mechanism of nonspecific phosphatase, HSU *et al.*[7] performed a full kinetic study on potato phosphatase with *p*-nitrophenyl phosphate and 3-glycerophosphate as substrates; a mechanism has been established for the ordered release of products, the alcoholic product first and P_i second. This mechanism was thought to be general for other phosphatases[8]. Similar patterns in competitive inhibition by P_i and in noncompetitive inhibition by the alcoholic product, however, were not observed with the 3'-nucleotidase of this enzyme. For the hydrolysis of 3'-AMP, adenosine is a linear competitive inhibitor, and P_i does not affect the activity. In addition, the 3'-nucleotidase activity is not inhibited by 1.0 mM of diisopropyl phosphofluoridate. Therefore, the action mechanism for 3'-nucleotidase may be different from that for the above phosphatases. Although a precise mechanism for the 3'-nucleotidase could not be established at present, the existence of a phosphorylated enzyme as an intermediate seems unlikely.

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