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CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE OF *SERRATIA MARCESCENS*

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SUMMARY

1. A cyclic 3',5'-nucleotide phosphodiesterase from the cells of *Serratia marcescens* was purified over 1000-fold. The purified preparation showed a pH maximum in the region of 7.5–8.5 in Tris–HCl buffer, with a K_m of 0.52 mM for adenosine cyclic 3',5'-phosphate. Gel filtration on a Sephadex G-100 column gave the molecular weight of 51 000.

2. The esterase did not require Mg^{2+} nor Mn^{2+} for activity, but was stimulated by Fe^{2+}, Ca^{2+} or Ba^{2+}. Imidazole did not stimulate the activity.

3. The cyclic 3',5'-nucleotide phosphodiesterase of *S. marcescens* was inhibited competitively by theophylline. The K_i value was $8.3 \cdot 10^{-4}$ M. Proflavine, 8-hydroxyquinoline, Zn^{2+}, Cu^{2+}, Ni^{2+} and 5'-deoxy-5'-(dihydroxyphosphinylmethyl)-adenosine 3'-cyclic ester were also inhibitory. The last compound was the most powerful competitive inhibitor with a K_i of $4.5 \cdot 10^{-6}$ M. ADP, AMP and GTP were also slightly inhibitory.

4. The enzyme was specific for nucleoside cyclic 3',5'-monophosphates. Besides cyclic 3',5'-monophosphates of adenosine, guanosine, inosine, thymidine and uridine the enzyme could hydrolyze cytidine cyclic 3',5'-monophosphate at a considerable rate.

INTRODUCTION

Since the discovery of adenosine cyclic 3',5'-monophosphate (cyclic AMP) in *Brevibacterium liquefaciens*[1] and *Escherichia coli*[2], considerable information has been accumulated on the significance of this nucleotide in the bacterial cell process[3]. The presence of cyclic 3',5'-nucleotide phosphodiesterase (abbreviated as phosphodiesterase) in bacteria has been reported with *E. coli*[4,5] and *B. liquefaciens*[6], but the present knowledge on bacterial phosphodiesterase is fragmentary.

In this paper we report a phosphodiesterase of *S. marcescens*. The enzymic activity was purified over 1000-fold by simple manipulations.

Abbreviations: cyclic AMP, adenosine cyclic 3',5'-monophosphate; cyclic CMP, cytidine cyclic 3',5'-monophosphate; cyclic GMP, guanosine cyclic 3',5'-monophosphate; cyclic IMP, inosine cyclic 3',5'-monophosphate; cyclic UMP, uridine cyclic 3',5'-monophosphate; cyclic TMP, thymidine cyclic 3',5'-monophosphate; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryl adenosine cyclic 3',5'-monophosphate; cyclic phosphonate, 5'-deoxy-5'-(dihydroxyphosphinylmethyl)-adenosine 3'-cyclic ester.

MATERIALS AND METHODS

Cultivation of bacteria

S. marcescens IFO 3736 was kindly supplied by Dr. Takeji Hasegawa of the Institute for Fermentation, Osaka, Japan. The bacterium was cultured for 3 h at 30° in 20 l of a nutrient broth under forced aeration. The cells in the early stationary phase of growth were harvested by centrifugation, and were kept in a freezer at −20°. Usually 90 g of wet cells were obtained.

Assay methods

Phosphodiesterase activity was assayed by two different methods. Method 1 is a modification of the method of BUTCHER AND SUTHERLAND[7]. The standard assay was carried out in a total volume of 0.25 ml containing 12.5 μmoles Tris–HCl (pH 8.0), 1.25 μmoles cyclic AMP and 0.062 μg of the enzyme diluted in 50 mM Tris–HCl (pH 8.0) containing 0.1% bovine serum albumin. The reaction was initiated by the addition of enzyme after temperature equilibration for 10 min at 30°. After 10 min of incubation, the reaction was terminated by adding 50 μl of 4 M HCl, and the reaction tubes were kept in ice for 10 min. The acidified reaction mixture was neutralized with 200 μl of 2 M $KHCO_3$. Immediately after the neutralization, 0.1 mg of *Crotalus atrox* venom in 100 μl of 50 mM Tris–HCl (pH 8.0) was added, and the reaction mixture was incubated again for 10 min at 30°. P_i released was determined by the method of ALLEN[8].

In Method 2 the reaction mixture in a total volume of 0.1 ml contained 5 μmoles Tris–HCl (pH 8.0), an appropriate amount of cyclic [8-^{14}C]AMP (0.34 μC/μmole) and the enzyme. The reaction was performed as described above. After terminating the reaction by adding 10 μl of 55% trichloroacetic acid, 20 μl of carrier solution containing 12.5 mM 5′-AMP and 7.5 mM cyclic AMP were added. Aliquots of slightly turbid reaction mixtures were spotted on Toyō No. 53 (40 cm × 40 cm) filter paper and developed with Solvent 1 (see below). The spots corresponding to 5′-AMP and cyclic AMP were cut out, placed in vials, and the radioactivity was determined in a Beckman DPM-100 liquid scintillation spectrophotometer.

Both assays gave the same results. One unit of enzyme is defined as the amount that caused production of 1 μmole of reaction product per min. Specific activity is expressed in the units of enzyme per mg of protein.

Protein was determined by the measurement of absorbance at 280 nm or by a phenol method[9].

Paper chromatography. Ascending chromatography on Toyō No. 53 paper was performed throughout this work. The solvents used were (1) 95% ethanol–1 M ammonium acetate (pH 7.5, 75:30, by vol.), (2) 95% ethanol–1 M ammonium acetate (pH 3.8, 75:30, by vol.), (3) saturated aqueous $(NH_4)_2SO_4$–1 M sodium acetate–isopropanol (80:18:2, by vol.), (4) isopropanol–concentrated NH_4OH–water (70:10:20, by vol.), (5) 85% saturated aqueous solution of ammonium bicarbonate, and (6) isopropanol–concentrated NH_4OH–0.1 M boric acid (70:10:20, by vol).

Materials

Materials used in this study were obtained from the following sources: cyclic [8-^{14}C]AMP (40.9 mC/mmole), Schwarz; non-radioactive cyclic AMP, Kōjin Co. Ltd. (Tokyo); cytidine cyclic 3′,5′-monophosphate (cyclic CMP), guanosine cyclic 3′,5′-

monophosphate (cyclic GMP), inosine cyclic 3′,5′-monophosphate (cyclic IMP), uridine cyclic 3′,5′-monophosphate (cyclic UMP), thymidine cyclic 3′,5′-monophosphate (cyclic TMP) and N^6,$O^{2'}$-dibutyryl adenosine cyclic 3′,5′-monophosphate (dibutyryl cyclic AMP), Boehringer; 2′,3′-cyclic adenosine monophosphate, various 5′-mononucleotides, *Crotalus atrox* venom, 5′-nucleotidase (venom), chymotrypsinogen, ovalbumin, and bovine serum albumin, Sigma; pancreatic ribonuclease, NBC; Sephadex G-100 and CM-Sephadex C-50, Pharmacia. 5′-Deoxy-5′-(dihydroxyphosphinylmethyl)-adenosine 3′-cyclic ester (cyclic phosphonate)[10] was donated by Dr. John G. Moffatt of Syntex Research Center Palo Alto, Calif., U.S.A. All other reagents were reagent grade.

RESULTS

Enzyme purification

The cells of *S. marcescens* (89 g) were suspended in 178 ml of 50 mM of Tris-HCl buffer (pH 8.0) containing 10 mM of $MgCl_2$, 5 mM of β-mercaptoethanol and 10 μg/ml of pancreatic deoxyribonuclease, and were disrupted in a French pressure cell. The crude cell extract was centrifuged in a Servall RC-2 refrigerated centrifuge for 30 min at 30 000 × *g*. The supernatant, which contained most phosphodiesterase activity, was used as the starting material. All purification procedures were performed at 0–5°.

Step 1: Protamine sulfate treatment. To 240 ml of 30 000 × *g* supernatant of crude cell extract, 3.1 g of protamine sulfate, (1 mg per 3 mg of protein), dissolved in water and adjusted to pH 5.0, were slowly added under continuous stirring. After 20 min the mixture was centrifuged for 30 min at 10 000 × *g*, and the precipitate was discarded.

Step 2: $(NH_4)_2SO_4$ treatment. To the supernatant fraction from the preceding step, solid $(NH_4)_2SO_4$ was added slowly with continuous stirring until the solution was 45% saturated. After 30 min the preparation was centrifuged for 20 min at 15 000 × *g* and the precipitate was discarded. Additional $(NH_4)_2SO_4$ was added to the supernatant fraction to increase the concentration to 60% saturation and, after being stirred for 30 min, the mixture was centrifuged for 30 min. The supernatant portion was discarded, and the precipitate was dissolved in 30.0 ml of 50 mM sodium acetate buffer (pH 5.5) containing 5 mM of β-mercaptoethanol, and was dialyzed overnight against 1 l of the same buffer.

Step 3: Chromatography on CM-Sephadex. A tube, 1.8 cm in diameter and 50 cm long, was filled with CM-Sephadex C-50 which had been preequilibrated in the same buffer as used in Step 2. The dialyzate obtained in Step 2 was centrifuged to remove the precipitate, and the clear supernatant was applied to the column. The column was washed with 220 ml of the same buffer and then the enzyme was eluted with a linear gradient produced by 750 ml each of the same buffer containing 0.1 and 0.4 M NaCl, respectively. The flow rate was 10 ml/h, and 9.75-ml fractions were collected. The enzyme activity was found only in one peak eluted by 0.25 M NaCl. Active fractions were pooled and concentrated under vacuum with a collodion bag (Sartrius Membranfilter) dipped in 50 mM Tris–HCl (pH 8.0), and stored at a protein concentration of 0.67 mg/ml. The enzyme, when kept at −20°, lost 20% of its original activity during 1 month. A summary of the purification achieved at each step in the procedure is shown in Table I. Over-all purification exceeded 1100-fold in 38.4% yield.

TABLE I

SUMMARY OF PURIFICATION OF PHOSPHODIESTERASE

89 g of cells were disrupted and processed as described in the text. Standard assay Method 1 was employed for the determination of activity.

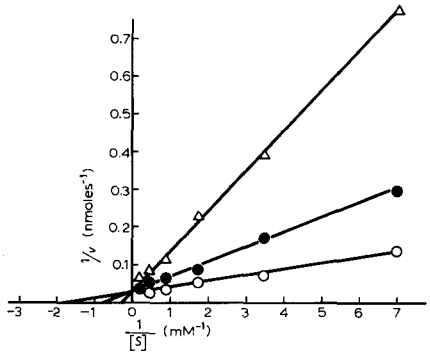
Fraction	*Total vol. (ml)*	*Total protein (mg)*	*Total units*	*Specific activity*	*Yield (%)*
30 000 × *g* supernatant	240	10430	2500	0.239	100
Protamine supernatant	308	3540	1940	0.55	77.5
$(NH_4)_2SO_4$ (45–60%)	30	1035	1464	1.42	58.6
CM-Sephadex concentrate	5.6	3.58	962	268	38.4

General properties

Under the standard conditions the reaction proceeded linearly for at least 15 min. There was a linear relationship between initial reaction velocity and protein concentration up to 0.077 μg per tube under the standard assay condition of Method 1. The enzyme had a K_m of $5.25 \cdot 10^{-4}$ M. V was calculated as 295 μmoles/mg protein per min (Fig. 1). The optimal pH was between 7.5 and 8.5 (Fig. 2). In contrast to the phosphodiesterase of animal origin[7,11,12], the enzyme of *S. marcescens* was not stimulated by imidazole buffer.

Molecular weight

The molecular weight of the enzyme was determined by the molecular sieve principle according to WHITAKER[13]. By using Sephadex G-100 as a molecular sieve and ribonuclease, α-chymotrypsinogen, ovalbumin and bovine serum albumin as the standards, we assessed the molecular weight of our phosphodiesterase as 51 000.



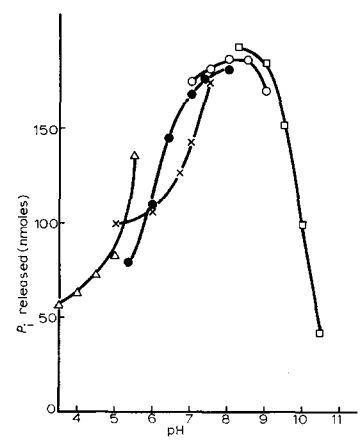


Fig. 1. Double reciprocal plots of enzyme activity as a function of cyclic AMP concentration in the presence or absence of cyclic phosphonate. Assay Method 2 was used. Enzyme used was 0.0145 μg. ○—○, no inhibitor; ●—●, 0.01 mM of inhibitor; and △—△, 0.03 mM of inhibitor.

Fig. 2. Phosphodiesterase activity as a function of pH. Standard conditions of Method 1 were employed for the assay except that the pH of the reaction mixture was adjusted with 50 mM (final) of the following buffers: acetate (△—△), imidazole–HCl (×—×), Tris–maleate (●—●), Tris–HCl (○—○) and glycine–NaOH (□—□).

Effects of metal ions

Mg^{2+} or Mn^{2+} is required for full activity of animal phosphodiesterase, and metal chelating agents such as EDTA inhibit the activity[7,14]. In contrast, our enzyme was not stimulated by these metal ions up to 50 mM, nor inhibited by EDTA. Instead, the enzyme was stimulated by Fe^{2+}, Ca^{2+} and Ba^{2+}. Cu^{2+}, Zn^{2+} and Ni^{2+} were inhibitory (Table II).

TABLE II

EFFECTS OF METALS

The effect of metals was studied at 1 mM concentration. The assay was performed under the standard conditions of Method 1. In reaction tubes containing Cu^{2+} or Zn^{2+}, venom treatment was performed in the presence of 2 mM of EDTA.

Metal	*Relative activity*
$BaCl_2$	168
$CaCl_2$	169
$CoCl_2$	41
$CuSO_4$	22
$FeCl_2$	210
$FeCl_3$	84
$MgCl_2$	101
$MnCl_2$	97
$NiCl_2$	33
$ZnSO_4$	20
Control	100

Substrate specificity

Most reports concerning cyclic 3′,5′-nucleotide phosphodiesterase have stated that the enzyme has a preference for nucleotides having purine bases[11,14,15]. As is evident from Table III, our preparation hydrolyzed cyclic UMP and cyclic TMP at rates comparable with those for cyclic GMP and cyclic IMP. One outstanding observation is that our preparation could attack cyclic CMP at 0.174 times the rate for

TABLE III

SUBSTRATE SPECIFICITY OF PHOSPHODIESTERASE FROM VARIOUS SOURCES

The percentages given for the enzymic hydrolysis of different substrates are relative to those for cyclic AMP. The present results were obtained by Method 1, using 5 mM of various cyclic 3′,5′-nucleoside monophosphates as substrates. In a separate experiment it was confirmed that the amount of venom was enough to hydrolyze 5′-nucleotides formed by phosphodiesterase reaction.

Substrate	*S. marcescens (present results)*	*Dog heart (ref. 11)*	*Rat liver (ref. 14)*	*Rabbit brain (ref. 15)*
Cyclic AMP	100	100	100	100
Dibutyryl cyclic AMP	0		0	
Cyclic GMP	52.2	33	49	33
Cyclic IMP	69.8	55–65	86	
Cyclic CMP	17.4	0	0–5	0
Cyclic UMP	51.7	12–15	8–9	11
Cyclic TMP	53.6			

cyclic AMP under the standard assay conditions (5 mM of substrate). K_m for cyclic CMP was about 20 mM. The enzyme did not attack dibutyryl cyclic AMP.

In separate experiments we tested the hydrolysis of cyclic 2′,3′-AMP, 5′-AMP and 3′-AMP in similar conditions to those for Table III except that 2.5 μg of enzyme (40 times the amount used in standard assay Method 1) was used. No hydrolysis product (2′-AMP, 3′-AMP, adenosine, *etc.*) was detected on the paper chromatogram with Solvent 3. The enzyme did not attack *p*-nitrophenyl phosphate (10 mM) at pH 8.0, but hydrolyzed it at the rate of 2.5 nmoles/mg protein per min at pH 5.0. The specific activity of this acid phosphatase was about 0.001% of its phosphodiesterase activity. These activities could be separated by gelfiltration on a Sephadex G-100 column.

Inhibitors

It has been reported that methylxanthines interfere with animal phosphodiesterase but not with the enzymes from microbial sources[4,7,11,12,14–16]. Our phosphodiesterase was inhibited by theophylline (Table IV). The inhibition was competitive, and K_i was $8.3 \cdot 10^{-4}$ M. Table IV also shows that proflavine, 8-hydroxyquinoline, and cyclic phosphonate[10], a phosphonate analog of cyclic AMP, were inhibitory. The inhibition by the last compound was very pronounced, and the K_i was 4.5 μM (Fig. 1).

CHEUNG[12] reported inhibition of animal phosphodiesterase by ribonucleoside triphosphates, pyrophosphate and citrate. Although ATP, ADP and AMP slightly inhibited the *S. marcescens* enzyme at 10 mM concentration, the inhibitory activities of GTP, UTP, CTP, pyrophosphate and citrate were, if any, negligibly small.

TABLE IV

INHIBITORS OF PHOSPHODIESTERASE

The assay was performed by Method 2 using 0.585 mM of cyclic [8-^{14}C]AMP as the substrate.

Compound	*Concentration (mM)*	*Inhibition (%)*
None		0
Theophylline	1	21.2
	10	79.2
Proflavine	1	72.5
	10	88.9
8-Hydroxyquinoline	1	40.5
	10	80.9
Cyclic phosphonate	0.05	86.6
	0.1	93.3
EDTA	1	0
	10	0
AMP	1	11.0
	10	42.3
ADP	1	16.5
	10	42.3
ATP	1	0
	10	31.7
GTP	10	24.3
UTP	10	20.5
CTP	10	0
Sodium pyrophosphate	10	0
Sodium citrate	10	0

As is evident from Table IV, EDTA up to 10 mM did not show any inhibitory effect.

Identification of reaction product and stoichiometry

For the identification of reaction product, cyclic [8-^{14}C]AMP (1.25 μmoles) was incubated with 1.3 μg of enzyme, and the reaction product was separated by paper chromatography with Solvent 4. Radioactivity, which was initially associated with cyclic AMP, was quantitatively recovered (1.21 μmoles as 5'-AMP) from the new ultraviolet-absorbing substance. This was eluted with a small volume of water, and small aliquots were cochromatographed with 5'-AMP using Solvents 1–5. All radioactivity was associated with 5'-AMP.

Another aliquot (0.54 μmole on the basis of radioactivity) was hydrolyzed with excess (1.3 mg) 5'-nucleotidase. The reaction product was identified as adenosine by paper chromatography with Solvents 1–5. Thus the only reaction product was 5'-AMP, and the stoichiometric conversion of cyclic AMP to 5'-AMP was confirmed. Reaction products of cyclic nucleotide 3',5'-phosphates other than cyclic AMP (see Table III) were also identified as nucleoside 5'-monophosphates by paper chromatography with Solvents 1, 3 and 6.

DISCUSSION

Purification of cyclic 3',5'-nucleotide phosphodiesterase activity from various sources has been hampered by the low enzyme levels and large losses in activity during the purification procedure[12,15]. *S. marcescens* is an excellent source of phosphodiesterase, purification exceeding 1000-fold being achieved by simple procedures.

There are several outstanding characteristics of the enzyme which differ from those of enzymes obtained from other sources. (1) The enzyme can hydrolyze cyclic CMP, which has been said to be resistant to hitherto reported cyclic 3',5'-nucleotide phosphodiesterases. (2) The molecular weight of our enzyme is 51 000 whereas those of others are over 200 000 (refs. 5, 14, 16), although it is possible that our preparation is a monomeric unit of enzyme and the other preparations are oligomers of the monomeric unit. (3) Our enzyme does not require Mg^{2+} or Mn^{2+} for maximal activity, and the addition of EDTA to a metal-free reaction mixture did not reduce the enzymic activity. This observation may cast a doubt, at least for the *S. marcescens* enzyme, on the suggestion of CHEUNG[12] that a metalloenzyme complex might be the active form of phosphodiesterase, although the possibility remains that metal(s) other than Mg^{2+} and Mn^{2+} are implicated, because external addition of Fe^{2+}, Ca^{2+} and Ba^{2+} stimulated the enzyme action and 8-hydroxyquinoline inhibited the activity. (4) Phosphodiesterase of *S. marcescens* was inhibited competitively by theophylline. In this respect the enzyme resembles animal phosphodiesterase but is distinct from those of *E. coli*[4] and a slime mold, *Dictyostelium discoideum*[16].

Besides theophylline, some other compounds inhibited the activity; of these, cyclic phosphonate was the most potent. We also confirmed that this compound inhibits phosphodiesterase from a wide variety of bacteria and from a yeast. Since there has been no description of inhibitors of microbial phosphodiesterase, some compounds listed in Table IV may be useful in future studies on microbial adenyl cyclase and phosphodiesterase.

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