BBA 65943

CYCLIC PHOSPHODIESTERASE HAVING 3'-NUCLEOTIDASE ACTIVITY FROM *BACILLUS SUBTILIS*

PURIFICATION AND SOME PROPERTIES OF THE ENZYME

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(Received March 17th, 1969)

SUMMARY

1 A phosphodiesterase with 3'-nucleotidase activity was partially purified from a culture medium of *Bacillus subtilis* Phosphodiesterase activity against *p*-dinitrophenyl phosphate was also detected in the same fraction

2 The three enzyme activities were not separated by physical and chemical procedures used so far

3 The purified enzyme preparation cleaved the phosphodiester bond of ribonucleoside 2',3'-cyclic phosphates rapidly and that of *p*-dinitrophenyl phosphate slowly It released P_i from ribonucleoside, and deoxyribonucleoside 3'-monophosphates but not from corresponding 5'- and 2'-isomers and from *p*-nitrophenyl phosphate

4 The final preparation was free of nonspecific acid or alkaline phosphatase, 5'-nucleotidase, phosphodiesterase against adenosine 3',5'-cyclic monophosphate, deoxyribonuclease and ribonuclease

5 The purified enzyme was nearly homogeneous by hydroxylapatite chromatography, sedimentation, Sephadex G-200 gel filtration and polyacrylamide gel electrophoresis

6 The $s_{20,w}$ was 4 93 S with an estimated molecular weight of about 50 000

7 Three enzyme activities were greatly activated by heat treatment (70°, 5 min) in the presence of Co^{2+} or Mn^{2+} (0 1 mM) This temperature-dependent activation was specific for Co^{2+} or Mn^{2+}

INTRODUCTION

As a part of the studies aimed at developing methods for the enzymatic analysis of nucleotides, we have been investigating the properties and mode of action of 3'-nucleotidase from *Bacillus subtilis*

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IGARASI AND KAKINUMA[1] and IGARASI[2] reported the presence of 3′-nucleotidase activity in the culture filtrate of *B subtilis* and purified it about 14-fold In this investigation, we have purified this enzyme as a nearly homogeneous protein and have characterized it as a 3′-nucleotidase having phosphodiesterase activities for ribonucleoside 2′,3′-cyclic phosphates and for *p*-dinitrophenyl phosphate

A similar enzyme, first reported by ANRAKU[3,4] and ANRAKU AND MIZUNO[5], catalyzed the hydrolysis of ribonucleoside 3′-phosphates, ribonucleoside 2′,3′-cyclic phosphates and *p*-dinitrophenyl phosphate Similar enzymes have been subsequently reported in various microorganisms[6–9]

A distinctive feature of *B subtilis* 3′-nucleotidase is its activity against deoxyribonucleoside 3′-monophosphates, which can be used to detect slight contaminations of these isomers in the preparation of deoxyribonucleoside 5′-monophosphates This activity of other cyclic phosphodiesterases has not been clarified, except for the partially purified *Escherichia coli* B preparation by ANRAKU AND MIZUNO[5] which has slight nonspecific monophosphatase activity against various nucleoside monophosphates

MATERIALS AND METHODS

Enzymes

Phosphomonoesterase was donated by Dr H Shimono of our laboratory It was purified from human prostate gland as described by SCHMIDT[10] Micrococcal nuclease and crystalline pancreatic ribonuclease were purchased from Sigma Part of the micrococcal nuclease, prepared as described by CUNNINGHAM *et al* [11], was supplied by Mr Y Kozai and Mr K Hamada of our laboratory Spleen phosphodiesterase was purified from calf spleen by the procedure of HILMOE[12]

Nucleic acids and nucleotides

Commercial yeast RNA (Nakarai Chemicals, Japan) was used after purification according to the procedure of SEVAG *et al* [13] A mixture of ribonucleoside 2′- and 3′-monophosphates was prepared by alkaline hydrolysis of purified RNA Calf thymus DNA, isolated by the method of KAY *et al* [14], was further purified by alkaline treatment followed by acid precipitation to remove contaminating RNA A mixture of deoxyribonucleoside 3′-monophosphates was prepared from the purified DNA preparation by sequential treatment with micrococcal nuclease and spleen phosphodiesterase[15]

Each nucleoside monophosphate was separated and purified by chromatography on a column of Dowex 1-X8 (formate form) (200–400 mesh)[16] 3′-UMP was prepared by enzymatic hydrolysis of 2′,3′-cyclic UMP with crystalline pancreatic ribonuclease as described by SMRT AND ŠORM[17] Contaminating salts were removed by charcoal treatment or by lyophilization

^{32}P-labeled ribonucleoside 2′- and 3′-monophosphates

Cells of *E coli* B were grown in the presence of $^{32}P_i$ and were harvested as described by LEHMAN *et al* [18] A mixture of ^{32}P-labeled DNA and ^{32}P-labeled RNA was extracted from the cells according to MARMUR[19] and was subjected to alkaline hydrolysis in 0 5 M KOH for 18 h at room temperature $HClO_4$ was added to the

hydrolysate and the resulting DNA and $KClO_4$ precipitates were removed by centrifugation The supernatant fluid, which contained ^{32}P-labeled nucleoside 2'- and 3'-monophosphates ($5\,7 \cdot 10^6$ counts/μmole) was adjusted to pH 7 0

Unlabeled and ^{32}P-labeled ribonucleoside 2', 3'-cyclic phosphates

These were prepared by the method of SMITH *et al* [20] Barium salts were converted to sodium salts before use by treatment with Dowex 50W-X8 (sodium form) Part of the unlabeled cyclic phosphates samples were donated by Dr C Ukita

Other chemicals

Calcium *p*-dinitrophenyl phosphate was purchased from Tokyo Kasei Chemical Co (Japan) Sodium *p*-nitrophenyl phosphate was obtained from Nakarai Chemicals (Japan) Sephadex G-200 was obtained from Pharmacia Hydroxylapatite was prepared as described by LEVIN[21] DEAE-cellulose (0 9 mequiv /g, Brown Co) was obtained from the Biochemical Corp (Japan) Other chemicals of reagent grade were obtained from commercial sources

Assay of 3'-nucleotidase activity (Assays Ia and Ib)

This assay measured the release of P_i from nucleoside 3'-monophosphates or $^{32}P_i$ from ^{32}P-labeled nucleoside 3'-monophosphates The reaction mixture (0 5 ml) contained 10 μmoles of Tris–HCl (pH 7 5), 0 1 μmole of substrate, and enzyme The mixture was incubated for 20 min at 37° When unlabeled substrates were used, the reaction was stopped by the addition of 0 04 ml of 5 M H_2SO_4 The color was then developed, according to FISKE AND SUBBAROW[22], in a final volume of 1 ml and was read at 650 mμ with a Beckman DU spectrophotometer (Assay Ia)

When ^{32}P-labeled substrates (1 10^6–3 10^6 counts/μmole), were used, charcoal treatment determined the amount of P_i released Reaction was halted by the addition of 0 2 ml of a mixture containing 0 01 M HCl and 0 6 M formic acid, 0 1 ml of 0 01 M KH_2PO_4, 0 1 ml of 1 mM of unlabeled nucleoside monophosphate and 0 3 ml of a 10% (w/v) Norit A suspension After 5 min at 0°, charcoal was removed by centrifugation, and 0 8 ml of the supernatant fluid was transferred to a tube containing 0 3 ml of charcoal suspension After standing 5 min in the cold, the tube was centrifuged, and 0 5 ml of the supernatant fluid was directly plated on an aluminum planchet which was dried, and its radioactivity was measured (Assay Ib)

Assay of cyclic phosphodiesterase activity

This assay measured the release of P_i from ribonucleoside 2',3'-cyclic phosphates (Assay IIa) or $^{32}P_i$ from ^{32}P-labeled ribonucleoside 2',3'-cyclic phosphates (Assay IIb) The incubation mixture (0 5 ml) contained 10 μmoles of Tris–HCl (pH 7 5), 0 1 μmole of substrate and enzyme After 20 min at 37°, the reaction was stopped by heating at 100° for 10 min and was quickly cooled* To this solution were added 0 1 ml of 0 66 M acetate buffer (pH 5 0) containing 66 mM $MgCl_2$ and 0 1 ml of prostate phosphomonoesterase at an appropriate concentration After 20 min at 37°, the reaction was stopped by the addition of 0 045 ml of 5 M H_2SO_4 (Assay IIa)

* In the presence of Co^{2+} or Mn^{2+} ,three activities become equally heat stable In such cases, the reaction was terminated by adding 1 μmole of EDTA, followed by heat treatment (100°, 10 min)

or by the addition of 0 2 ml of a mixture containing 0 01 M HCl and 0 6 M formic acid, 0 1 ml of 0 01 M KH_2SO_4 and 0 1 ml of 1 mM of unlabeled cyclic phosphate (Assay IIb) P_i (Assay IIa) or $^{32}P_i$ (Assay IIb) was determined as described in the assay of 3'-nucleotidase

Assay of phosphodiesterase activity against p-dinitrophenyl phosphate

The incubation mixture (0 5 ml) contained 20 μmoles of Tris–HCl (pH 7 5), 0 23 μmoles of *p*-dinitrophenyl phosphate and enzyme Incubation was conducted for 20 min at 37° The reaction was stopped by the addition of 2 5 ml of 4% K_2HPO_4 solution The amount of *p*-nitrophenol released was determined in a Beckman DU spectrophotometer at 410 mμ (Assay III)

Unit of enzyme activity

An unit of enzyme activity is defined as the amount of enzyme which causes the release 1 μmole of P_i or *p*-nitrophenol from each substrate per h

Specific activity was expressed in terms of units/mg of protein

Other enzyme activities

Ribonuclease and deoxyribonuclease activities were assayed by the procedure described by McDONALD[23] with several modifications The reaction mixture (0 5 ml) contained 60 μmoles of Tris–HCl (pH 7 5), substrate (3 mg of RNA or 0 6 mg of DNA) and enzyme After incubation for 20 min at 37°, the reaction was stopped by the addition of 2 5 ml of 0 8% $HClO_4$ containing 0 08% uranium acetate After standing in the cold for 30 min, the precipitate was removed by centrifugation, and 0 5 ml of the supernatant was diluted with 2 5 ml of water to determine its absorbance at 260 mμ An increase in absorbance of 1 0 at 260 mμ in a 1-cm length cuvette was defined as 1 unit of enzyme activity under the described conditions

Other methods

Protein was determined by the method of LOWRY *et al* [24] with bovine serum albumin as standard In the last three fractions, concentration was also determined spectrophotometrically from absorbance at 280 mμ The absorbance of a solution of 1 mg/ml, determined by the method of LOWRY *et al* [24], was about 1 1 at 280 mμ in a 1-cm length cuvette with 0 05 M Tris–HCl (pH 7 5)*

^{32}P was measured in a Nuclear Chicago thin-window gas-flow counter

Sedimentation velocity

Sedimentation velocity was determined in a Spinco, Model E analytical ultracentrifuge at 59 780 rev /min with schlieren optics The temperature was controlled near 22° The sample was placed in 20 mM potassium phosphate (pH 7 2), containing 0 1 M NaCl and the sedimentation coefficient was corrected for water at 20° (see ref 25)

Gel filtration on a Sephadex G-200 column

A column (2 1 cm × 61 cm) of Sephadex G-200 was prepared and equilibrated

* The pH value was determined at 25° and a concentration of 0 5 M ,then diluted with distilled water

with 0 05 M Tris–HCl (pH 7 5) Samples (1 5 ml) dissolved in the Tris–HCl buffer were applied to the Sephadex G-200 column and eluted with the same buffer at a flow rate of 13 ml/h Fractions of 1 68 ml were collected Beef pancreatic chymotrypsinogen (10 mg), ovalbumin (10 mg), bovine serum albumin (10 mg), human γ-globulin (10 mg), and beef liver catalase (5 mg) were used as reference standard for molecular weight determinations Void volume denotes the elution volume of Blue dextran 2000 (0 5 mg) The following substances were determined by their specific absorptions at stated wavelengths. marker proteins, 280 mμ and Blue dextran, 630 mμ Catalase activity was determined by the procedure of BEERS *et al* [26] A plot of log molecular weight against the ratio of elution volume (V_e) to void volume (V_0) was figured

Polyacrylamide gel electrophoresis

Electrophoresis was performed with a modified method of ORNSTEIN AND DAVIS[27], in 7 5% polyacrylamide gel at 4° in Tris–glycine buffer (pH 8 3)

Instead of preparing space gel, samples dissolved in the reservoir buffer containing 10% sucrose were layered on coarse gel After performing electrophoresis, cylindrical polyacrylamide gel was cut into two equal portions longitudinally One-half of the gel was stained with Amido black 10B, and the other half was sliced to 2-mm thickness, each piece being eluted with 2 ml of water for 12 h at 0° and assayed according to standard assay procedures

Stoichiometry

The reaction mixture contained 0 5 μmole of 3'-AMP or 0 243 μmole of 2',3'-cyclic AMP, 5 μmoles of Tris–HCl buffer (pH 7 5) and 0 014 μg of Hydroxylapatite IIB fraction, incubated in a total volume of 0 5 ml at 37° The reaction was stopped by heating at 100° for 10 min and was spotted on Toyo Roshi No 52 filter paper Compounds were separated by paper electrophoresis in 0 05 M Tris–HCl (pH 7 5), at 15 V/cm for 1 h, and each spot was eluted with 5 ml of 0 01 M HCl The amounts of 3'-AMP, 2',3'-cyclic AMP and adenosine were determined by the absorbance at 260 mμ P_i was located with $^{32}P_1$, eluted with water, and estimated using the method of FISKE AND SUBBAROW[22]

RESULTS

Purification of enzyme

A summary of enzyme purification from the culture filtrate of *B subtilis* (IFO No 3032) is presented in Table I The first and second steps were essentially the same as those described by IGARASI[2]

Step 1 Culture filtrate The culture filtrate was obtained according to the procedure of IGARASI[1] A culture medium containing soluble starch (3%), polypeptone (0 5%), corn steep liquor (3%) and calcium carbonate (1 3%) was neutralized and sterilized at 120° for 10 min After inoculation the bottle was placed on a reciprocal shaker and was incubated at 28° for 45 h All subsequent steps were carried out at 0–4°, unless otherwise specified Cells and supernatant fluid were separated by centrifugation The supernatant fluid was clarified by filtering through a Seitz filter

Step 2 Precipitation with acetone To 20 l of the culture filtrate (adjusted to

pH 7 5 with NaOH) were added 20 l of acetone (−20°) with constant rapid stirring over a 30-min period After standing for 12 h, the precipitate was collected by centrifugation and was dissolved in 1 l of 0 05 M Tris–HCl (pH 7 5) (Buffer A) Insoluble materials were removed by centrifugation The supernatant fluid is designated as the Acetone fraction

Step 3 $(NH_4)_2SO_4$ fractionation To 1 l of the acetone fraction were added 176 g of $(NH_4)_2SO_4$ with stirring over a 15-min period After 30 min, the precipitate was removed by centrifugation The supernatant fluid (845 ml) was adjusted to pH 7 5 with about 2 0 ml of 1 M NH_4OH, and an additional 265 g of $(NH_4)_2SO_4$ were added with stirring over a 15-min period After 90 min, the resulting precipitate was collected by centrifugation This precipitate was dissolved in 100 ml of Buffer A, and 90 ml of the solution were dialyzed overnight against two changes of 1800 ml of Buffer A, the precipitate which formed during dialysis was removed by centrifugation The supernatant fluid (106 ml) is referred to as the $(NH_4)_2SO_4$ fraction

Step 4 Chromatography on DEAE-cellulose A column of DEAE-cellulose (3 0 cm × 26 cm) was equilibrated with 3 l of Buffer A 90 ml of the $(NH_4)_2SO_4$ fraction (1 83 g of protein) were applied to the column After washing with 1 l of 0 02 M NaCl in Buffer A, the column was developed with 200 ml of 0 2 M NaCl in Buffer A The flow rate was 0 9 ml/min, and 17 2-ml fractions were collected Approx 65% of the three enzyme activities applied to the column was recovered in the eluate Fractions 7–16 were combined to yield the DEAE-I fraction (172 ml) This DEAE-I fraction was dialyzed for 3 h against two changes of 2 l of Buffer A Dialysis at this step resulted in about a 30% loss of activity However, the remaining activity was quite stable in the subsequent steps

Step 5 Rechromatography on DEAE-cellulose A column (1 7 cm × 11 cm) was prepared as described above The dialyzed sample (136 ml) of the DEAE-I fraction (306 mg of protein) was applied to the column, which was then washed with 500 ml of 0 02 M NaCl in Buffer A A linear gradient of the elution from 0 02 to 0 2 M NaCl in Buffer A was then applied to the column The total gradient volume was 300 ml The flow rate was 0 6 ml/min, and 17 2-ml fractions were collected The elution profile showed that the three enzyme activities were eluted coincidently Fractions 5–15 were pooled to give the DEAE-II fraction (185 ml), and 175 ml of this were concentrated to less than 0 5 ml by ultrafiltration, using a collodion bag (Membranfilter, Goettingen, Germany) The final volume was adjusted to 5 1 ml with Buffer A

Step 6 Chromatography on Sephadex G-200 To a Sephadex G-200 column (2 0 cm × 61 cm) previously equilibrated with 1 l of Buffer A was applied a 5-ml concentrated sample of the DEAE-II fraction The column was then developed with the same buffer at a flow rate of about 12 ml/h, and 3 7-ml fractions were collected Fractions 32–39 were pooled to yield the Sephadex-I fraction (29 6 ml) 26 ml of the pooled fraction were concentrated to 5 ml by ultrafiltration with a collodion bag

Step 7 Rechromatography on Sephadex G-200 Exactly the same procedure as that used in Step 6 was repeated Fractions 32–39 were pooled and concentrated to 3 0 ml (Sephadex-II fraction)

Step 8 Chromatography on hydroxylapatite A column of hydroxylapatite (3 3 cm × 1 1 cm) was equilibrated with 500 ml of 1 mM potassium phosphate (pH 7 5) A portion of the pooled Sephadex-II fraction (28 9 mg as protein) was

applied to the column and was washed with 100 ml of 1 mM potassium phosphate (pH 7 5) The enzyme was then eluted with 150 ml of 0 2 M potassium phosphate (pH 7 5) Fractions were collected in 9 5-ml portions at a flow rate of about 9 5 ml/h Fractions 2–6 were pooled and dialyzed against Buffer A to yield the Hydroxylapatite-I fraction (47 ml) An aliquot (46 5 ml) was concentrated to 4 7 ml by ultrafiltration and was stored

Step 9 Rechromatography on hydroxylapatite After equilibrating the same column (Step 8) as described above, 4 ml of the concentrated Hydroxylapatite-I fraction was adsorbed and washed with 400 ml of 5 mM of potassium phosphate (pH 7 5) The column was then eluted successively with the following potassium phosphate solutions (pH 7 5) 0 01 M, 200 ml, 0 02 M, 200 ml, 0,05 M, 100 ml, and finally 0 5 M, 100 ml Fractions were collected in 9 5-ml portions at a flow rate of about 9 5 ml/h The enzyme was recovered in the last three eluates The eluate with 0 02 M buffer was concentrated and dialyzed against Buffer A to yield the Hydroxylapatite-IIA fraction (1 8 ml) The last two eluates were combined, concentrated and dialyzed in the same way to yield the Hydroxylapatite-IIB fraction (3 0 ml). These preparations were stored at $-20°$

These preparations showed almost the same properties so far as tested, except for a slight difference in their specific activities, as shown in Table I

Properties of purified enzyme

Estimation of molecular weight An estimation of molecular weight was obtained by gel filtration with Sephadex G-200 (Fig 1) Assuming that the shape of this enzyme is the same as that of the calibrating proteins, the position occupied by this enzyme corresponds to a molecular weight of about 50 000 The $s_{20,w}$ of this enzyme was found to be 4 93 S

TABLE I

PURIFICATION OF *B subtilis* 3'-NUCLEOTIDASE

3'-Nucleotidase activity was measured according to Assay Ia, with 3'-AMP as substrate

Fraction	*Vol (ml)*	*Total activity* $\times 10^{-4}$ *(units)*	*Yield* (%)*	*Specific activity (units/mg protein)*	*Protein content (mg/ml)*
Culture filtrate	$2\ 10^4$	110 0	100	13 6	4 05
Acetone (0–50%)	$1\ 10^3$	57 2	52 1	83 1	6 86
$(NH_4)_2SO_4$ (30–75%)	118	42 6	38 8	177	20 30
DEAE-I	222	18 7	17 0	366	2 25
DEAE-II	302	11 7	10 7	384	1 01
Sephadex-I	51 2	2 5	2 32	474	1 05
Sephadex-II	5 9	2 0	1 83	653	5 15
Hydroxylapatite-I**	49 5	1 4	1 30	934	0 31
Hydroxylapatite-IIA	2 3	0 45	0 41	1060	1 87
Hydroxylapatite-IIB	3 7	0 62	0 56	1140	1 44

* At each purification step, aliquots were removed The yield values in this column were adjusted to indicate the yield that would have been obtained if the entire fraction had been used in the subsequent step

** In this step, several batches of the Sephadex-II fractions were pooled

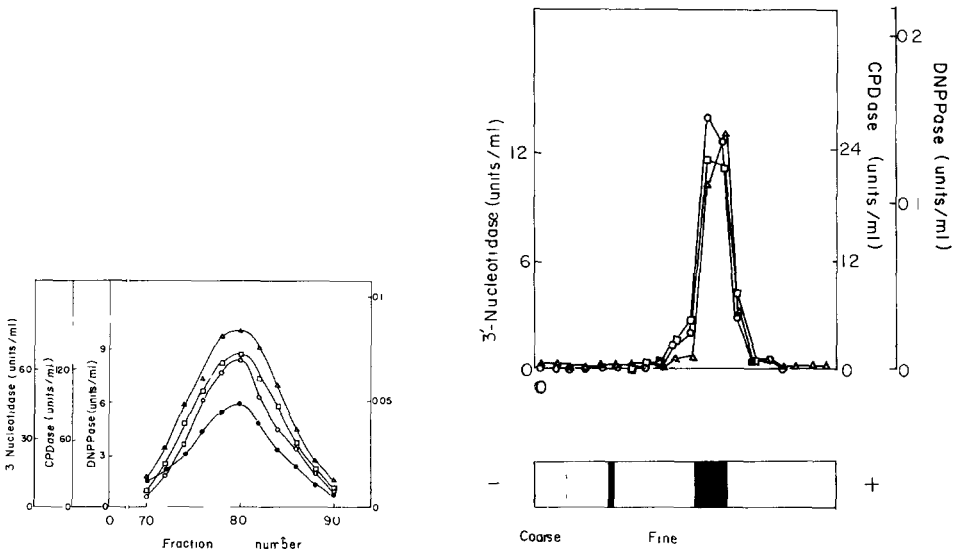


Fig 1 Elution diagram of the Hydroxylapatite-IIA fraction (1 03 mg as protein) from a Sephadex G-200 column For details see text □—□, cyclic phosphodiesterase (2',3'-cyclic AMP) (CPDase), ○—○, 3'-nucleotidase (3'-AMP), △—△, phosphodiesterase (*p*-dinitrophenyl phosphate) (DNPPase), ●—●, protein

Fig 2 Disc electrophoresis of the Hydroxylapatite-IIA fraction (0 102 mg as protein) For details see text The current was 5 mA for 150 min □—□, cyclic phosphodiesterase (2',3'-cyclic AMP) (CPDase), ○—○, 3'-nucleotidase(3'-AMP), △—△, phosphodiesterase (*p*-dinitrophenyl phosphate) (DNPPase)

Homogeneity as protein The ratios of the three specific activities remained constant throughout the elution pattern from the Sephadex G-200 column (Fig 1) There was one major band having three enzyme activities and a minor band No activity was recognized in the minor band using polyacrylamide gel electrophoresis (Fig 2) The sedimentation boundary observed with schlieren optics was found to be very nearly symmetrical No significant amounts of protein having sedimentation coefficients greater or less than that of the main component could be detected

Substrate specificity Results are summarized in Table II The enzyme was quite specific for nucleoside 3'-monophosphates Activities on nucleoside 5'- and 2'-monophosphates were less than 0 01% of that on 3'-AMP, under the standard assay conditions In addition, it should be noted that deoxyribonucleoside 3'-monophosphates of the same order were dephosphorylated by this enzyme, showing that the 2'-hydroxyl sugar group was not essential for its action *p*-Nitrophenyl phosphate was resistant to this enzyme under the standard assay conditions as shown in Table II

The phosphodiester bonds of ribonucleoside 2',3'-cyclic monophosphates were cleaved with velocities apparently 2 times as fast as in the hydrolysis of ribonucleoside 3'-monophosphates Relative phosphodiesterase activity against *p*-dinitrophenyl phosphate was about 8% of that of 3'-AMP, but ribonuclease and deoxyribonuclease was not detected by the method described Cyclic phosphodiesterase for 3',5'-cyclic AMP and cyclic phosphodiesterase for ribonucleoside 2',3'-cyclic mono-

TABLE II

RELATIVE ENZYME REACTION VELOCITIES ON VARIOUS PHOSPHATE COMPOUNDS

Relative reaction velocities were measured under standard conditions of Assays Ia, IIa, or III The reaction mixture contained 0 0375 μg of Hydroxylapatite-IIA for Assays Ia and IIa, and 0 375 μg for Assay III The compounds tested are as indicated

Assay method	*Substrate*	*Relative activity*
Ia	3′-AMP	100
	3′-GMP	100
	3′-CMP	86
	3′-UMP	100
	3′-dAMP	46
	3′-dGMP	63
	3′-dCMP	28
	3′-dTMP	11
	2′-AMP, 2′-GMP, 2′-CMP, 2′-UMP	0 01
	5′-AMP, 5′-GMP, 5′-CMP, 5′-UMP	0 01
IIa	2′,3′-cyclic AMP	200
	2′,3′-cyclic GMP	161
	3′,5′-cyclic AMP	0 01
III	*p*-Dinitrophenyl phosphate	0 86
	p-Nitrophenyl phosphate	0 0001

phosphates (2′,3′-cyclic NMP) were not recognized as yielding nucleoside 2′-monophosphates

Effects of pH on activity The pH optima for the three enzyme activities coincide and are close to 6 5 Cyclic phosphodiesterase activity against 2′,3′-cyclic NMP, 3′-nucleotidase activity and phosphodiesterase activity against *p*-dinitrophenyl phosphate were at pH 5 0, 34%, 16% and 56%, at pH 9.5, 54%, 20% and 28% of the corresponding value at pH 7 5, respectively 2′,3′-Cyclic AMP, 3′-AMP or *p*-dinitrophenyl phosphate was used as substrate The following buffers were used Tris–HCl (pH 7 0–9 0) sodium acetate (pH 3 5–6 5) and glycine–NaOH (pH 9 5–10 5), respectively

Stability The three enzyme activities were relatively stable when stored at 0° or −20° in 0 05 M Tris–HCl buffer (pH 7 5) and were not affected by repeated freezing and thawing When enzyme preparation in 0 05 M Tris–HCl buffer (pH 7 5) was heated at 70° for 5 min, 3′-nucleotidase activity, cyclic phosphodiesterase activity against 2′,3′-cyclic NMP and phosphodiesterase activity against *p*-dinitrophenyl phosphate retained 88%, 110% and 37%, respectively, of the corresponding activities of the unheated sample The three activities were, however, completely inactivated when heated at 100° for 10 min under the same conditions

Stoichiometry When 3′-AMP was incubated with the enzyme for 40 min, 0 094 μmole of 3′-AMP decreased, and 0 107 μmole of adenosine and 0 099 μmole of P_i were produced When 2′,3′-cyclic AMP was incubated with the enzyme for 20 min, 0 120 μmole of 2′,3′-cyclic AMP was reduced, and 0 095 μmole of 3′-AMP and 0 040 μmole of adenosine were formed In this case, P_i was not estimated

Effects of various metal ions and other substances As shown in Table III, phospho-

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON THE THREE ENZYME ACTIVITIES

Assays were performed according to Assays Ia, IIa and III The reaction mixture contained 0 0375 μg of Hydroxylapatite-IIA for Assay Ia and IIa, and 0 375 μg for Assay III The compounds tested are as indicated

Compound	*Concn (mM)*	*Relative activity*		
		Phosphodiesterase (p-Dinitrophenyl phosphate)	*Cyclic phosphodiesterase (2',3'-cyclic AMP)*	*3'-Nucleotidase (3'-AMP)*
None		100	100	100
$MnCl_2$	20	179	30	0
	10	211	55	0
	1	233	69	99
	0 1	199	69	101
	0 01	140	74	115
$MgCl_2$	20	85	100	80
	10	88	95	85
	1	108	98	82
$CoCl_2$	20	177	30	0
	10	197	36	0
	1	208	80	41
	0 1	192	106	120
	0 01	113	101	—*
$CaCl_2$	10	68	93	0
	1	77	93	116
$NiCl_2$	10	60	28	16
	1	56	35	32
	0 1	75	87	91
$ZnCl_2$	1	30	3	11
	0 1	37	76	18
EDTA	10	22	99	44
	1	53	96	28
	0 1	95	99	70
	0 01	94	100	108
NaF	10	102	—	98
	1	100	—	92

* No test was made

diesterase activity against *p*-dinitrophenyl phosphate was greatly activated by Co^{2+} or Mn^{2+} at relatively low concentrations 3'-Nucleotidase activity and cyclic phosphodiesterase activity against 2',3'-cyclic NMP were not enhanced at any of these ion concentrations and were greatly inhibited by both ions at high concentrations which were still stimulatory to phosphodiesterase activity against *p*-dinitrophenyl phosphate Mg^{2+} had no effect on the three activities up to 20 mM, Ca^{2+} inhibited only 3'-nucleotidase activity Cu^{2+}, Ni^{2+} and Zn^{2+} inhibited the three enzyme activities at relatively low concentrations

3'-Nucleotidase activity and phosphodiesterase activity against *p*-dinitrophenyl phosphate were inhibited by EDTA at relatively low concentrations, and this inhibitory effect of EDTA was reversed by Mg^{2+}, Co^{2+} or Mn^{2+}, specifically Cyclic phosphodiesterase activity against 2',3'-cyclic NMP was not inhibited by EDTA even at high concentrations

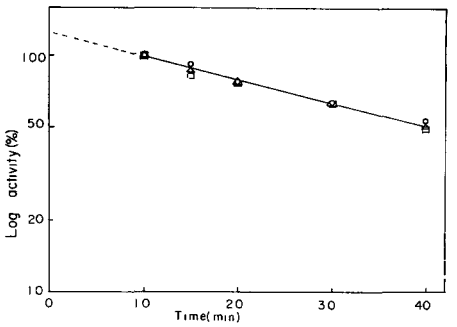


Fig 3 Heat treatment of enzyme preparation Heat treatment the enzyme preparation (1 44 μg of the Hydroxylapatite-IIB fraction per ml) in 0 05 M Tris–HCl (pH 7 5) containing 0 1 mM $CoCl_2$, was kept at 70° for the indicated period of time and was quickly cooled to 0° Three enzyme activities were measured according to Assays Ia, IIa and III, with 0 144 μg of the enzyme treated in the presence of 0 1 mM $CoCl_2$ Activities were expressed as the percentages of those activities obtained after 10 min at 70° ○—○, 3′-nucleotidase (3′-AMP), □—□, cyclic phosphodiesterase (2′,3′-cyclic CMP), △—△, phosphodiesterase (*p*-dinitrophenyl phosphate)

NaF had no effect on 3′-nucleotidase activity and phosphodiesterase activity against *p*-dinitrophenyl phosphate The effect of NaF on cyclic phosphodiesterase activity against 2′,3′-cyclic NMP was not tested because this was a potent inhibitor for the prostate phosphomonoesterase used in the enzyme assay

Heat activation and metal ions Heat treatment at 70° for 5 min activated cyclic phosphodiesterase activity against 2′,3′-cyclic NMP and 3′-nucleotidase about 2-fold and phosphodiesterase activity against *p*-dinitrophenyl phosphate about 40-fold in the presence of Co^{2+} or Mn^{2+} (0 1 mM) This temperature-dependent activation was specific to Co^{2+} or Mn^{2+}, and attempts to replace Co^{2+} with other bivalent ions (Mg^{2+} or Ca^{2+}) of comparable ionic strength failed to show similar activation effects Activated enzyme activities were inactivated at the same rate at 70° in the presence of 0 1 mM Co^{2+} After 40 min of heating at 70°, about 50% of each activity remained, percentages based on activities observed under these conditions after 10 min are shown in Fig 3

TABLE IV

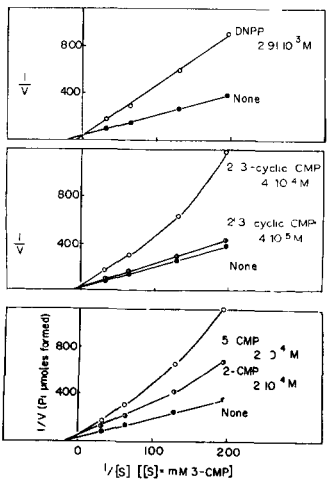
MICHAELIS CONSTANT AND MAXIMUM VELOCITY FOR VARIOUS SUBSTRATES BY LINEWEAVER–BURK METHOD

Experimental details were given in the legends of Figs 4–5

Substrate	$K_m \times 10^5$ *(M)*	v_{max} *(μmoles/h per mg protein)*	$K_i \times 10^4$ *(M)*				
			2′-CMP	*5′-CMP*	*3′-CMP*	*2′,3′-cyclic CMP*	*p-Dinitrophenyl phosphate*
3′-CMP	5 0	5 85 10^5	3 70	1 33		2 70	29.10
2′,3′-cyclic AMP	4 0	2 70 10^5	0 83	1 15	2 10		12 50
p-Dinitrophenyl phosphate	72 0	77	—*	2 10	1 36	1 23	

* No test was made

Kinetics The K_m and v_{max} values with several substrates are listed in Table IV When 3'-CMP was used as substrate, 2'-CMP, 5'-CMP, 2',3'-cyclic CMP and *p*-dinitrophenyl phosphate inhibited 3'-nucleotidase competitively (Fig 4) The cause(s) for a slight upward deviation tendency of the plot when inhibitors were added was not clear As shown in Fig 5, nucleoside monophosphates including 2'-CMP and 5'-CMP, inert substrates, and 3'-CMP, susceptible to 3'-nucleotidase,



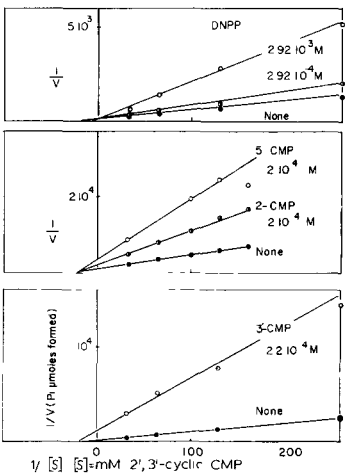


Fig 4 Competitive inhibition of 3'-nucleotidase activity (3'-CMP as substrate) by *p*-dinitrophenyl phosphate (DNPP), 2',3'-cyclic CMP, 5'-CMP or 2'-CMP The reaction mixture contained 10 μmoles of Tris–HCl buffer (pH 7 5) and 1 44 10^{-3} μg of the Hydroxylapatite-IIB fraction, incubated with indicated amounts of ^{32}P-labeled 3'-CMP(1 07 10^6 counts/μmole) and various substances The assay was performed according to the procedure for Assay Ib

Fig 5 Competitive inhibition of cyclic phosphodiesterase (2',3'-cyclic CMP as substrate) by p-dinitrophenyl phosphate (DNPP) and uncompetitive inhibition by 5'-CMP, 2'-CMP or 3'-CMP The reaction mixture contained 10 μmoles of Tris–HCl buffer (pH 7 5) and 2 88 10^{-3} μg of the Hydroxylapatite-IIB fraction, incubated with indicated amounts of ^{32}P-labeled 2',3'-cyclic CMP (2 16 10^6 counts/μmole) and various substances The assay was performed according to the procedure for Assay IIb

seem to be noncompetitive inhibitors to the substrate 2',3'-cyclic CMP *p*-Dinitrophenyl phosphate, on the other hand, inhibits competitively

In the case of phosphodiesterase activity against *p*-dinitrophenyl phosphate, preliminary experiments indicate that all inhibitors containing 2'-CMP, 5'-CMP, 3'-CMP and 2',3'-cyclic CMP act competitively

DISCUSSION

Each of these three enzyme activities was purified about 80-fold Still present in the purified 3'-nucleotidase, however, are cyclic phosphodiesterase activity against 2',3'-cyclic NMP and phosphodiesterase activity against *p*-dinitrophenyl phosphate All attempts to separate these activities have been unsuccessful This raises the question as to whether a single protein is responsible for these activities The present work was directed toward the elucidation of this problem

Since a similar enzyme from *E coli* B was first reported by ANRAKU[3] and was classified on the basis of its substrate specificity as a 3'-nucleotidase having cyclic phosphodiesterase activity for 2',3'-cyclic NMP and phosphodiesterase activity for *p*-dinitrophenyl phosphate, similar enzymes from various kinds of microorganisms have been reported[6–9] Several differences are found among them *B subtilis* enzyme is unique in that it (a) hydrolyzes deoxyribonucleoside 3'-monophosphates in the same order as compared with ribonucleoside 3'-monophosphates and (b) has a slight, but definite, phosphodiesterase activity for *p*-dinitrophenyl phosphate as compared with 2',3'-cyclic NMP

Although the assay conditions are somewhat different for each, the three enzyme activities of *B subtilis* are relatively stable at 70° for 5 min in 0 05 M Tris–HCl buffer (pH 7 5), as are those of *P. mirabilis*[6] On the other hand, *E. coli* enzyme[4] activities are completely inactivated under these conditions

As for pH optima, 6 5 is observed for *B subtilis* activities, similar to the *E coli*[3] value but not to those (7 0–8 0) for *Proteus mirabilis*[6] and other Enterobacteriacae[7]

The addition of Co^{2+} activates only the phosphodiesterase activity against *p*-dinitrophenyl phosphate of the *B subtilis* enzyme, all three activities of *E. coli*[3] (and other Enterobacteriacae[7]) but none of the activities of *P. mirabilis*[6]

The fact that phosphodiesterase activity against *p*-dinitrophenyl phosphate reached the same level as compared with the two other activities by heat treatment in the presence of Co^{2+} or Mn^{2+} and that the three enzyme activities were inactivated in parallel by heat, independent of differences in heat activation, metal ion effects and active sites, provides further evidence that the same protein is responsible for all these activities

The above observations indicate that Co^{2+} or Mn^{2+} probably function to induce a specific conformational change in the enzyme molecule which is necessary for its catalytic activities Co^{2+} or Mn^{2+} do more simply protect the enzyme against heat denaturation, it appears that at 65–75° the enzyme molecule undergoes a change in its structure and that in the presence of Co^{2+} or Mn^{2+} a new conformation is stabilized

The 3'-nucleotidase from *B subtilis* has its optimum pH in a nearly neutral region, as described above The alkaline phosphatase optimum from this bacteria[28,29] is at pH 10 5 Co^{2+}, Ni^{2+} and Mn^{2+} stimulate the alkaline phosphatase at 0.2 mM. 3'-Nucleotidase at this concentration is not activated by these ions but is strongly inhibited at higher concentrations (Table III) Furthermore, *p*-nitrophenyl phosphate is an excellent substrate for alkaline and acid phosphatases but is completely resistant to 3'-nucleotidase (Table II) These facts clearly distinguish 3'-nucleotidase from other phosphomonoesterases and indicate that the final preparation is free of non-specific acid and alkaline phosphatases and of 5'-nucleotidase activities In hydrolysis of *p*-nitrophenyl phosphate, this substrate was rapidly hydrolyzed with 3'-nucleotidase preparations from Enterobacteriacae[7] including *E coli* B, which gave single protein bands in polyacrylamide gel electrophoresis Preparations from *P mirabilis*[6] and *E coli* B[4] showed negligible activity In this respect, the results obtained with *E coli* B enzymes were not consistent with each other.

Phosphodiesterase activities for *p*-dinitrophenyl phosphate were recognized in *B subtilis* and were partially purified by several authors[30] One of these phosphodiesterases, called "Pdase I" (see ref 30), may be the same as the enzyme described here

This paper determines for the first time that this activity must be due to a single protein having two other activities

From kinetic data, 3'-nucleotidase seems to be competitively inhibited by *p*-dinitrophenyl phosphate, 2'-CMP, 5'-CMP and 2',3'-cyclic CMP (Fig 4) The inhibition of cyclic phosphodiesterase activity against 2',3'-cyclic NMP by nucleoside monophosphates appears to be noncompetitive but that by *p*-dinitrophenyl phosphate appears to be competitive (Fig 5) Phosphodiesterase activity against *p*-dinitrophenyl phosphate is inhibited competitively by nucleoside monophosphates and 2',3'-cyclic CMP

Recently CENTER *et al* [31], using the enzyme preparation from *P mirabilis*, suggested that the same active site is involved in the hydrolysis of all substrates possessing a diester bond, while a second interacting site is involved in the hydrolysis of substrates possessing a phosphomonoester bond

ACKNOWLEDGMENTS

The authors wish to express their thanks to Drs E Ohmura and Y Nakao at the Takeda Chemical Industries, Research Laboratory, Japan, for generous supplies of the culture filtrate This investigation was supported in part by Scientific Research Grants from the Ministry of Education of Japan

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