BBA 65838

CHLORIDE ION AS A MODIFIER OF 2',3'-CYCLIC PHOSPHODIESTERASE PURIFIED FROM HALOPHILIC *VIBRIO ALGINOLYTICUS*

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(Received August 9th, 1968)

SUMMARY

1. 2',3'-Cyclic phosphodiesterase having 3'-nucleotidase activity (which hydrolyzes ribonucleoside 2',3'-cyclic phosphates, ribonucleoside 3'-phosphates and di-*p*-nitrophenyl phosphate) was purified 2000-fold from slightly halophilic *Vibrio alginolyticus*. The activities for these substrates were inseparable by any of the purification steps used.

2. This enzyme had the maximal activity on all of the substrates in the pH ranges from 7.6 to 8.6 and possessed several properties different from those reported (by others) for *Escherichia coli* and *Proteus mirabilis*.

3. Chloride ion acted as an activator for the hydrolysis of 3'-ribonucleotides and di-*p*-nitrophenyl phosphate; however, it acted as an inhibitor for the cyclic phosphodiesterase activity. The kinetic results of the effect of the chloride ion on the hydrolyses of these substrates were well explained in terms of the equation described by FRIEDEN on the single substrate–single modifier case. From these, it was assumed that the chloride ion inversely modifies the two activities in the same protein molecule by affecting the configurational changes in the enzyme structure so as to activate the 3'-nucleotidase and to inhibit the cyclic phosphodiesterase.

INTRODUCTION

ANRAKU[1,2] isolated (from *Escherichia coli*) 2',3'-cyclic phosphodiesterase having 3'-nucleotidase activity, which catalyzes the hydrolyses of ribonucleoside 2',3'-cyclic phosphates, ribonucleoside 3'-phosphates and di-*p*-nitrophenyl phosphate, and it was suggested that the cyclic phosphodiesterase and 3'-nucleotidase activities were associated with the same protein. Recently, CENTER AND BEHAL[3] isolated a similar enzyme with higher specific activity from *Proteus mirabilis* and suggested the existence of the two activities in the same protein molecule.

We purified an enzyme from slightly halophilic *Vibrio alginolyticus* which has substrate specificities similar to those from *E. coli* and *P. mirabilis*. This enzyme, however, possessed several properties distinctly different from those reported for the

above two bacteria[1-3]. The most remarkable example was the effect of salts on the two activities of this enzyme.

This paper deals with the purifications and properties of the cyclic phosphodiesterase of *Vibrio alginolyticus* and the effect of chloride ion on the two activities which are associated with the same protein molecule.

MATERIALS AND METHODS

Materials

3'-Ribonucleotides (Boeringer), other nucleotides and nucleosides (Sigma), DEAE-cellulose (Brown), Sephadex G-100, G-200 and DEAE-Sephadex (Pharmacia), bovine γ-globulin Fr II, egg albumin 2× crystallized, lysozyme 3× crystallized (NBC), and bovine albumin crystallized (Armour) were used. All other reagents used were analytical grade.

Enzyme assays

The reaction was carried out at the optimal pH of the each substrate. The standard reaction mixture (1.0 ml), containing 50 mM Tris–HCl buffer, 200 mM NaCl, 2.0 mM substrate and enzyme, was incubated for 10 min at 37°. For 3'-nucleotidase assay, P_i liberated from the substrate was determined by the method of CHEN, TORIBARA AND WARNER[4] in a final volume of 3.0 ml. When the removal of protein is necessary before P_i determination, the reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid, the mixture was filtered and the 1.0 ml fraction of the filtrate was used for colorimetry. For the assay on the hydrolysis of unstable substances such as ATP and ADP, P_i was determined by the method of ALLEN[5]. For the assay of di-*p*-nitrophenyl phosphate hydrolysis, the reaction was stopped by adding 2.0 ml of 0.1 M NaOH, and the absorbance was read at 400 mμ with a Hitachi Model 101 Spectrophotometer (Hitachi, Ltd.).

Hydrolysis of ribonucleoside 2',3'-cyclic phosphates to the corresponding 3'-ribonucleotides was measured by the increase in absorbance at 286 mμ for Urd-2',3'-*P* and at 290 mμ for Cyd-2',3'-*P*, which is based on the method of RICHARDS[6]. The reaction mixture (2.45 ml), containing 20 mM Tris–H_2SO_4 buffer and substrate, was placed in a 1-cm quarts cuvett equipped with a thermospacer attachment. The reaction was started by adding 0.05 ml of the enzyme solution at 37°. Increments in absorbance at a fixed wavelength were recorded with a Hitachi Recording Spectrophotometer EPS-3. Changes in absorbance caused by the hydrolysis of 1.0 μmole of the substrate per ml was measured at each experimental condition and the results were expressed as μmole of substrate hydrolyzed per ml of the reaction mixture per min.

One unit of activity was defined as that amount which hydrolyzes 1.0 μmole of substrate in 1 min.

Bacterial growth

Vibrio alginolyticus 138-2 (isolated and characterized by Prof. K. AISO of this Institute) was grown at 37° in a high phosphate medium containing 0.5% yeast extracts, 0.5% polypeptone, 0.4% K_2HPO_4, 0.2% glucose, and 3% NaCl (pH 7.2). The cells were grown under aeration and harvested during the exponential phase of

growth in a Sharpless centrifuge. About 200 g wet weight of the cells were obtained from two 50-l cultures.

Procedures for osmotic shock of cells

Since the cells of *V. alginolyticus* are very fragile in a hypotonic environment, procedures for the osmotic shock[7,8] were modified to prevent a significant lysis of the cells. For analytical purposes the cells were harvested at the exponential phase of growth by centrifugation and washed twice with 1 M NaCl in 50 mM Tris–HCl (pH 8.0).

The washed cells were suspended in 10 ml of the washing medium at the concentration of 4.2 mg protein per ml. The cell suspensions were centrifuged, and the sedimented cells were rapidly dispersed in 10 ml of 0.25 M NaCl and 10 mM $MgCl_2$ in 50 mM Tris–HCl (pH 8.0) (shock medium) and centrifuged. The supernatant was removed and used for assays of enzymic activities. Samples were taken immediately before centrifugation for measurements of absorbances at 500 mμ and viabilities of the cell suspensions.

Preparation of crude extracts

A rather dilute salt solution was used as a medium for the osmotic shock to facilitate the release of the cyclic phosphodiesterase from high concentrations of the cells of *V. alginolyticus*. After washing the cells (200 g wet weight) 3 times with cold 1 M NaCl in 50 mM Tris–HCl (pH 8.0), they were subjected to a sudden osmotic shock by being rapidly dispersed in 1000 ml of cold 0.2 M NaCl and 2 mM $MgCl_2$ in

TABLE I

PURIFICATION OF 2′,3′-CYCLIC PHOSPHODIESTERASE FROM *V. alginolyticus*

Fraction	*Vol. (ml)*	*Protein*		*3′-AMP*		*Di-p-nitrophenyl phosphate (units/ml)*	*Activity ratio (di-p-nitrophenyl phosphate/ 3′-AMP)*
		(mg/ml)	*(mg)*	*(units/ml)*	*(units/mg protein)*		
Sonic extracts*	1000	30.8	30 800	2.80	0.09	2.24	0.80
I Shock fluids, pH 4.5 supernatant	3400	1.4	4 760	0.55	0.39	0.61	1.11
II $(NH_4)_2SO_4$ fraction, dialyzed	161	8.2	1 320	11.0	1.34	13.9	1.26
III Second pH 4.5 supernatant	166	5.6	930	9.0	1.61	11.4	1.27
IV DEAE-cellulose fraction, conc. and dialyzed	49	2.0	98	28.9	14.5	30.7	1.06
V DEAE-Sephadex fraction, conc. and dialyzed	11.7	2.5	29	100	40.0	114	1.14
VI Sephadex G-100 fraction, conc. and dialyzed	10.0	0.87	8.7	112	129	114	1.02
VII Sephadex G-200 fraction, conc. and dialyzed	4.0	1.3	5.2	228	175	264	1.16

* Assays on a sonic extracts are shown for comparison.

50 mM Tris–HCl (pH 8.0). The mixture was centrifuged and the supernatant was collected. The pellets were further extracted 5 times with 500 ml each of the same medium, and the extracts were combined with the above supernatant.

Purification of the cyclic phosphodiesterase

All procedures were performed at 0–4°. Results on the purifications are summarized in Table I and Fig. 1.

The pH of the combined extracts was adjusted to 4.5 by dropwise addition of 1 M acetic acid, and the newly formed precipitates were removed by centrifugation. The supernatant, which contained all of the cyclic phosphodiesterase activity, was adjusted to pH 8.0 with 1 M Tris (Fraction I in Table I). Fraction I was further fractionated with ammonium sulfate and the fraction precipitated between 50 and 80% saturation was collected and dialyzed against 20 mM Tris–HCl (pH 8.0) (Fraction II). The pH of Fraction II was again adjusted to 4.5 and the precipitates were removed. The supernatant, after correction of the pH to 8.0 (Fraction III), was then applied to DEAE-cellulose column as indicated in Fig. 1a. Tubes No. 55–66 were combined, concentrated and dialyzed by negative pressure dialysis in a collodion bag (Carl Schleicher and Schüll) (Fraction IV). Fraction IV was applied to a DEAE-Sephadex column (Fig. 1b), and tubes No. 37–42 were combined, concentrated and dialyzed (Fraction V). Fraction V was further treated with Sephadex G-100 (Fig. 1c), and tubes No. 13–17 were combined, concentrated and dialyzed (Fraction VI). Fraction VI thus obtained was again treated with Sephadex G-200 (Fig. 1d), and tubes No. 44–52 were combined, concentrated and dialyzed (Fraction VII).

Disc gel electrophoresis

Gel and buffer solutions were prepared according to the method of ORNSTEIN AND DAVIS[9]. Electrophoresis was carried out at 5° for 4 h at a constant current of 2 mA per tube (4.5 mm × 43 mm). The gel was extruded from the tube and divided into two parts along the longitudinal axis. One half was stained with Amido Black 10B, and the other was incubated with the several substrates under standard conditions. The position of the activity for di-*p*-nitrophenyl phosphate was located with a yellow band. P_i released from the substrates was detected by the reagent used for P_i determination.

Gel filtration for molecular weight determination

Sephadex G-200 gel column (1.5 cm × 85 cm) was prepared and calibrated with bovine γ-globulin, bovine albumin, egg albumin and lysozyme according to the procedure of LEACH AND OSHEA[10]. The column was developed with 0.2 M KCl in 50 mM Tris–HCl (pH 8.3) at a flow rate of 4.5 ml/cm² per h. The column void volume was checked with Blue Dextran 2000 (Pharmacia, mol. wt., $2 \cdot 10^6$). A linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume to the column void volume was obtained.

Other methods

Protein was determined by the method of LOWRY *et al.*[11] using bovine albumin as a standard. Since the color development of protein was affected by the presence of Tris, protein was determined after dialysis against water.

Since the pH of Tris buffer is affected by temperature, all of the Tris buffers were adjusted to the requisite final pH at the concentrations and temperature used for experiments with a Hitachi–Horiba M-5 pH meter, unless otherwise indicated.

RESULTS AND DISCUSSION

2',3'-Cyclic phosphodiesterase of Vibrio alginolyticus

Localization of the enzyme. By the osmotic shock procedure described in METHODS, more than 70% of the total amount of cyclic phosphodiesterase was released from the exponentially growing cells of *V. alginolyticus*. After the osmotic shock, 37% of the cells were viable, and the absorbances of the cell suspensions in 1 M NaCl were unchanged. Since the amount of protein released in the shock medium

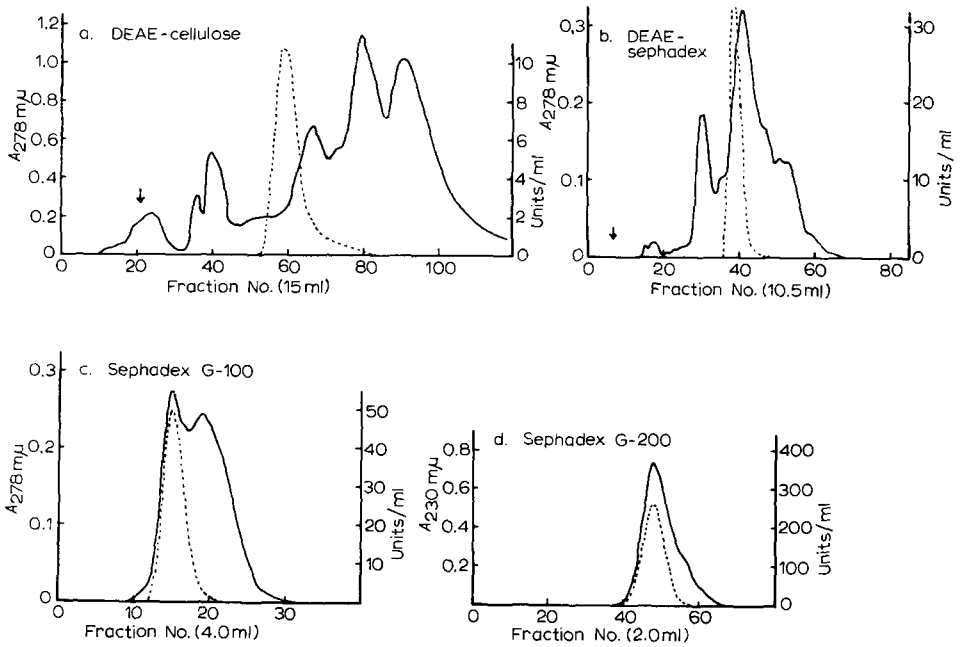


Fig. 1. Chromatography of the cyclic phosphodiesterase of *V. alginolyticus*. a. DEAE-cellulose column (3.2 cm × 23 cm) was equilibrated with 10 mM Tris–HCl (pH 8.1), and 140 ml of the enzyme of Fraction III indicated in Table I was applied. The column was washed with 175 ml of the buffer, and a linear gradient was begun (indicated by arrow). The mixing vessel contained 800 ml of the buffer, and the reservoir 800 ml of the buffer containing 0.4 M NaCl. b. DEAE-Sephadex column (1.5 cm × 16 cm) was equilibrated with 50 mM Tris–HCl (pH 8.3), and 35 ml of the enzyme of the Fraction IV was applied. The column was washed with 38 ml of the buffer, and a linear gradient was begun (indicated by arrow). The mixing vessel contained 500 ml of the buffer, and the reservoir an equal volume of the buffer containing 0.4 M NaCl. c, Sephadex G-100 column (2.0 cm × 41 cm) was washed with 20 mM Tris–H_2SO_4 (pH 8.0) prior to use, and 6 ml of the enzyme of Fraction V was applied. The column was developed with the same buffer at a flow rate of 10 ml/cm² per h. d. Sephadex G-200 column (1.5 cm × 85 cm) was washed with 200 mM KCl in 50 mM Tris–HCl (pH 8.3), and 1.4 ml of the concentrated enzyme corresponding to 3.5 ml of Fraction VI was applied. The column was developed with the washing solution at a flow rate of 4.5 ml/cm² per h. ———, absorbance at the indicated wavelength; — — — —, enzyme activity for 3'-AMP. The activity for di-*p*-nitrophenyl phosphate was eluted as coincident peaks with those for 3'-AMP.

was only 2% of the total, there might be no significant lysis of the cells by this treatment. Addition of 1 mM EDTA to the first medium (1 M NaCl in 50 mM Tris–HCl) showed no effect on the release of this enzyme. However, when 1 M sucrose was used as the first medium for osmotic shock, addition of EDTA resulted in the release of 60% of the enzyme in the sucrose medium. These results might indicate a surface localization of this enzyme in this organism, as in the case of *Escherichia coli*[7,8] and other Enterobacteriaceae[12].

In contrast to the case of *E. coli*, this organism released no 5′-nucleotidase and ribonucleoside triphosphatase into the shock medium. We previously reported that these enzymes are localized in the ghost fraction obtained from the spheroplast lysate of *V. parahaemolyticus*[13]. This was confirmed in the case of *V. alginolyticus*. Details on the nature of these enzymes will be reported elsewhere.

Substrate specificities and coexistence of the activities in the same protein molecule. The preparation of crude extracts and purifications of the cyclic phosphodiesterase were carried out as described in METHODS and the results are summerized in Table I and Fig. 1. The purified enzyme catalyzed the hydrolyses of 2′,3′-cyclic ribonucleotides to the corresponding 3′-ribonucleotides, and then catalyzed the hydrolyses of the latter to the ribonucleosides and P_i. It also hydrolyzed di-*p*-nitrophenyl phosphate at a rate comparable to the hydrolyses of 3′-ribonucleotides. For the hydrolyses of 3′-ribonucleotides and di-*p*-nitrophenyl phosphate, about 200 mM NaCl were required for maximal activities, but the cyclic phosphodiesterase activity was inhibited by the presence of NaCl. Details on the effect of salts will be described later. Table II indicates the summary of optimal pH, K_m and v_{max} in the presence or absence of NaCl, and $M_{1/2}$ for the several substrates.

This enzyme was unable to hydrolyze adenosine 3′,5′-cyclic phosphate, 2′- and 5′-ribonucleotides, ADP, ribonucleoside triphosphates, glucose 1-phosphate, glucose 6-phosphate, *p*-nitrophenyl phosphate, yeast RNA and native and heat-denatured

TABLE II

SUMMARY OF OPTIMAL pH, K_m, v_{max} AND $M_{1/2}$ FOR SEVERAL SUBSTRATES

Reaction velocities at the various concentrations of each substrate were measured at the optimal pH of the substrate using 20 mM Tris–H_2SO_4 buffer with or without 200 mM NaCl, and K_m and v_{max} values were estimated from $1/v$ *versus* $1/[S]$ and $[S]/v$ *versus* $[S]$ plots. v_{max} was expressed as μmoles of substrate hydrolyzed/min per mg of protein. $M_{1/2}$ represents the concentration of NaCl equivalent to the velocity lying halfway between the velocity in the absence of NaCl and the velocity in the presence of saturating amounts of NaCl, and it was estimated from $v/[M]$ *versus* $[M]$ plots in which $[M]$ represents the concentration of NaCl added, and v is the velocity corrected for the velocity without added NaCl.

Substrate	*Optimal pH*	*Without NaCl*		*With 200 mM NaCl*		$M_{1/2}$ *(mM)*
		K_m *(mM)*	v_{max}	K_m *(mM)*	v_{max}	
3′-CMP	7.8	0.01	27	0.01	180	10
3′-UMP	7.6	0.05	6	0.05	170	18
3′-AMP	8.3	0.08	11	0.08	170	20
3′-GMP	8.1	0.10	9	0.10	260	50
Di-*p*-nitrophenyl phosphate	8.3	0.45	170	0.20	286	20
Cyd-2′,3′-*P*	8.4	0.10	380	0.10	200	30
Urd-2′,3′-*P*	8.6	0.36	1560	0.36	600	40

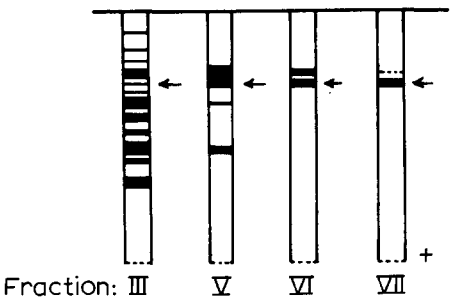


Fig. 2. Tracings of the electrophoretic patterns of proteins on acrylamide gel. The amounts of protein applied to the acrylamide gel column are: Fraction III, 200 μg; Fraction V, 75 μg; Fraction VI, 13 μg; Fraction VII, 6 μg. The protein band indicated by the arrow coincided with the position of enzyme activities for di-*p*-nitrophenyl phosphate, 3′-ribonucleotides, and 2′,3′-cyclic ribonucleotides.

calf thymus DNA. The activities for these substances, if present, were less than 0.2% of that for 3′-AMP.

As shown in Table I, this enzyme was purified 2000-fold, and the activity ratio of di-*p*-nitrophenyl phosphate to 3′-AMP remained constant during purification. Fig. 2 shows the results of acrylamide disc gel electrophoresis. Although the final preparation contained two proteins of different mobilities, all of the positions of the activities for di-*p*-nitrophenyl phosphate, 3′-ribonucleotides and 2′,3′-cyclic ribonucleotides coincided with only one main protein band. An enzyme with similar substrate specificities has been isolated from *E. coli* by ANRAKU[1,2] and from *Proteus mirabilis* by CENTER AND BEHAL[3]. These authors pointed out that the cyclic phosphodiesterase and 3′-nucleotidase activities are associated with the same protein molecule. The present results also strongly suggest the coexistence of the two activities in the same protein molecule. In another experiment, we purified an enzyme having similar substrate specificities from *Serratia marcescens*[14], and in this case the two activities were also inseparable during purification. The existence of the cyclic phosphodiesterase and 3′-nucleotidase activities in a coupled state seems to be common to this enzyme isolated from gram-negative bacteria.

Molecular weight determination. The molecular weight of this enzyme protein was determined by gel filtration. The enzyme protein and the bovine albumin were

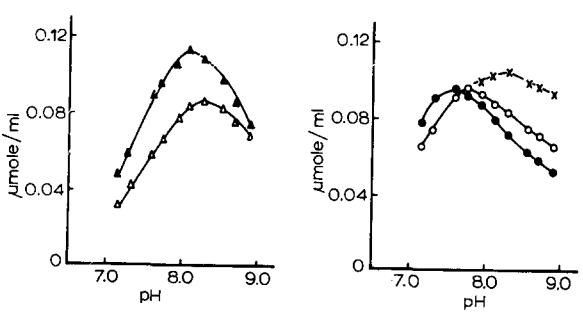


Fig. 3. Effect of pH on the enzyme activities. Activities for 3′-AMP (△—△); 3′-GMP (▲—▲); 3′-CMP (○—○); 3′-UMP (●—●); and di-*p*-nitrophenyl phosphate (×—×) were measured under standard conditions using 0.05 μg of enzyme and 50 mM Tris–HCl buffer.

eluted as coincident peaks. From the calibration curve, the molecular weight was estimated to be about 65 000, a value which was close to that reported on the enzyme from *E. coli* (68 000) by ANRAKU[15]. When the column was developed with 20 mM Tris–H_2SO_4 (pH 8.3), the elution volume of the enzyme protein was unaffected, indicating that the size of this enzyme is independent of the concentrations of salts, even in the presence or absence of Cl^-.

Optimal pH. As shown in Fig. 3, the optimal pH for 3′-ribonucleotides varied, depending on the substrates, and ranged from 7.6 to 8.3. The optimal pH for the 2′,3′-cyclic ribonucleotides was slightly higher than the corresponding 3′-ribonucleotides (Table II).

Enzyme stability. This enzyme was stable for at least 6 months when stored at −20° in concentrations above 50 μg/ml in 20 mM Tris buffer (pH 8.0). As shown in Fig. 4a, it was unstable in acid media and lost its activity below pH 5.0 at 37°. Above pH 6.0, it was stable for at least 2 h at 37°. Fig. 4b indicates the effect of temperature

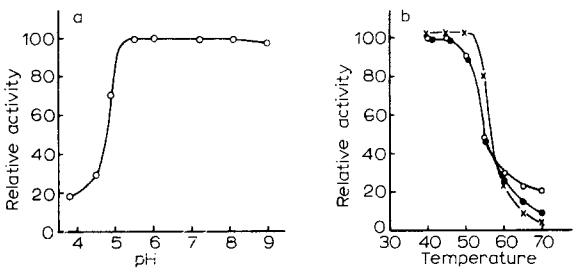


Fig. 4. Effect of pH and temperature on the stability of the enzyme. a. Effect of pH. The reaction mixture containing 0.5 μg of enzyme/ml in 20 mM acetate buffer (pH 3.8–6.0) or 20 mM Tris–H_2SO_4 (pH 7.2–9.0) was incubated for 10 min at 37°, and the activity for 3′-AMP was measured under standard conditions using 0.05 μg of enzyme. b. Effect of temperature. The reaction mixture (0.8 ml), containing 0.025 μg of enzyme in 50 mM Tris–HCl (pH 8.3 at 37°), was incubated for 5 min at various temperatures in the presence or absence of metal ions, and rapidly cooled in ice. After that the activity for 3′-AMP was measured under standard conditions. ○—○, no addition; ●—●, 0.2 mM $CoCl_2$; ×—×, 0.2 mM $MnCl_2$.

on the enzyme activity. The remaining activity for 3′-AMP after treatment for 5 min at 55° in the absence of salts was 45% of the original activity, whereas, in the presence of 0.2 mM $MnCl_2$, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 200 mM NaCl or 0.2 mM $CoCl_2$, the remaining activity was 87, 75, 56, 47 or 45%, respectively. Among these, $MnCl_2$ provided most protection against heat inactivation. The heat activation reported by ANRAKU[1,2] was not observed with this enzyme.

Effect of metal ions and EDTA. This enzyme was inhibited by several metal ions. The inhibition of the activity for di-*p*-nitrophenyl phosphate by 0.2 mM each of Zn^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+} and Cu^{2+} was 90, 35, 30, 17 and 17%, respectively, and that for 3′-AMP was 78, 36, 20, 14 and 2%, respectively. The 3′-nucleotidase activity was not stimulated significantly by Mg^{2+}, Ca^{2+} and Co^{2+} under standard conditions (in the presence of 200 mM NaCl), and only a slight stimulation ranging from 6 to 10% was noted by 0.2 mM Mn^{2+}.

The activities for 3′-ribonucleotides and di-*p*-nitrophenyl phosphate were inhibited from 7 to 29% by 0.2 mM EDTA, and the inhibition was independent of

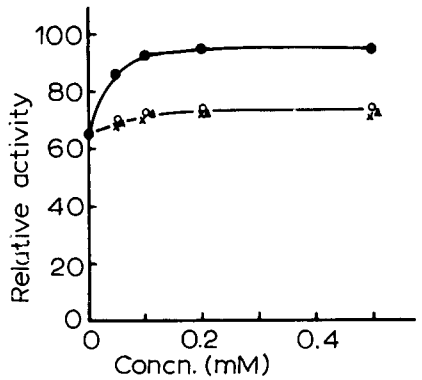


Fig. 5. Effect of metal ions on the EDTA-treated enzyme. The reaction mixture containing 1 mM EDTA and enzyme (2.5 μg/ml) in 20 mM Tris–H_2SO_4 (pH 8.1) was incubated for 10 min at 37° and cooled in ice. This was diluted 5-fold with the same buffer, and a 0.1-ml fraction was used to measure the activity for 3'-CMP under the standard conditions in the presence or absence of various concentrations of metal ions indicated. A value of 100 was assigned to the activity of enzyme for 3'-CMP before EDTA treatment. ●—●, $MnCl_2$; ○—○, $CoCl_2$; ▲—▲, $MgCl_2$; ×—×, $CaCl_2$.

the concentration of EDTA. When they were preincubated for 15 min with 1 mM EDTA at 37°, the activities for these substrates were inhibited 40 to 47%. The activity for Cyd-2',3'-*P* was also inhibited to the same extent. As shown in Fig. 5, the enzyme partially inactivated with EDTA was reactivated by Mn^{2+} (up to 95% of the original activity), and only slight activation was noted with Mg^{2+}, Ca^{2+} and Co^{2+}. These results might indicate the participation of metals, especially Mn^{2+}, in the active sites of this enzyme. Prolonged incubation with EDTA resulted in an irreversible denaturation, but sufficient activation by Mn^{2+} could not be attained with such preparations.

Effect of salts on the enzyme activities. The hydrolyses of 3'-ribonucleotides and di-*p*-nitrophenyl phosphate were activated by a number of salts. Fig. 6 indicates the activation curves for several salts. The form of the activation curves conformed with Michaelis–Menten kinetics. Among monovalent anions, Cl^- was the most effective,

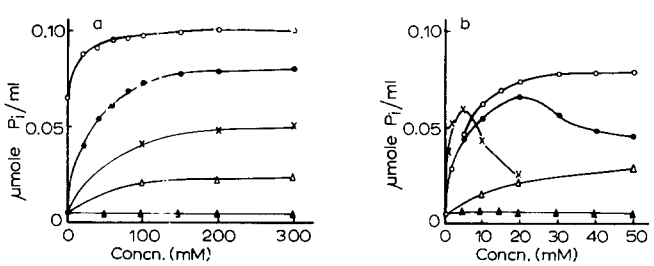


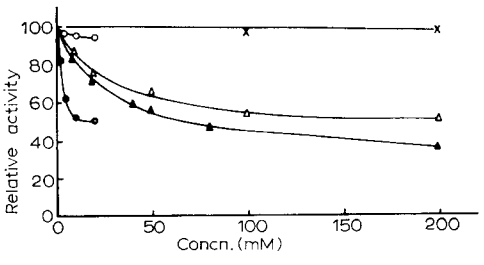
Fig. 6. Effect of salts on the enzyme activity. The reaction mixture (1.0 ml) contained 20 mM Tris–H_2SO_4 (pH 8.3), 0.05 μg of enzyme, 2 mM substrate and the various amounts of salt as indicated. a, Di-*p*-nitrophenyl phosphate as substrate (activity was expressed as μmole *p*-nitrophenol/ml): ○—○, NaCl or KCl. 3'-AMP as substrate: ●—●, NaCl or KCl; ×—×, HCOONa; △—△, CH_3COONa; ▲—▲, Na_2SO_4. b, 3'-AMP as substrate: ○—○, $MgCl_2$; ●—●, $CaCl_2$; ×—×, $MnCl_2$; △—△, $Mg(CH_3COO)_2$; ▲—▲, $MgSO_4$.

the order of effectiveness being $Cl^- > Br^- = I^- > NO_3^- > HCOO^- > CH_3COO^-$. The relative activities for 3'-AMP in the presence of optimal concentrations of the sodium salt of the above anions were 100, 93, 92, 65, 58, and 28, respectively. The activation by NaCl was saturated at about 200 mM. Although the activity for di-*p*-nitrophenyl phosphate obtained in the absence of salt was 65% of that in the presence of 200 mM NaCl, the activity for 3'-AMP in the absence of salt was only 7% of that in 200 mM NaCl. Thus 3'-nucleotidase activity was much more dependent on the concentration of Cl^-. Monovalent cations such as Li^+, Na^+, K^+, Rb^+, Cs^+, and $Tris^+$ could be substituted for one another, and NH_4^+ was less efficient.

Fig. 6b indicates the effect of divalent cations. On a molar basis, $MgCl_2$ was much more effective than NaCl. But the maximal velocities obtained at the optimal concentrations of NaCl and $MgCl_2$ were identical and, in the presence of saturating amounts of NaCl, no further activation was observed with $MgCl_2$ or *vice versa*. Since Na_2SO_4, $MgSO_4$ and sucrose showed no activating effect on the activity, the presence of Cl^- appears to be essential for the activation. $MnCl_2$ and $CaCl_2$ showed inhibition above 5 mM and 20 mM, respectively, and no activating effect was observed by any concentrations of $CoCl_2$.

On the other hand, the cyclic phosphodiesterase activity was inhibited by the presence of NaCl as indicated in Fig. 7. The inhibition curves were more or less rectangular hyperbolas. In the presence of 200 mM NaCl, the activities for Cyd-2',3'-*P* and Urd-2',3'-*P* were inhibited 50 and 65%, respectively, of those in the absence of NaCl. $MgCl_2$ also showed inhibition at low concentrations, and the inhibition reached a maximum at 20 mM. Since Na_2SO_4 and $MgSO_4$ showed only slight inhibition on the cyclic phosphodiesterase activity, the presence of Cl^- appears to be essential for the inhibitory action.

Effect of chloride ion on the enzyme kinetics. Fig. 8 shows the activity–pH curves for 3'-AMP in the presence and absence of NaCl. There was a small shift in optimal



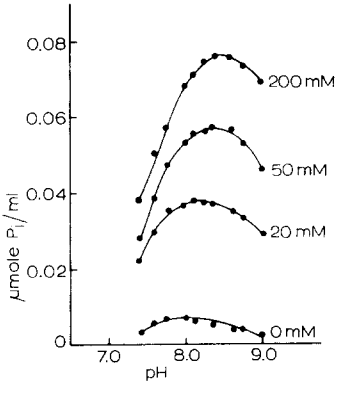


Fig. 7. Effect of salts on the cyclic phosphodiesterase activities. The reaction mixture contained 20 mM Tris–H_2SO_4 (pH 8.4 for Cyd-2',3'-*P* and pH 8.6 for Urd-2',3'-*P*), 1.0 mM substrate, and the various amounts of salt as indicated. Cyd-2',3'-*P* as substrate: △—△, NaCl or KCl; ×--×, Na_2SO_4; ●—●, $MgCl_2$; ○—○, $MgSO_4$. Urd-2',3'-*P* as substrate: ▲—▲, NaCl or KCl.

Fig. 8. Effect of the concentrations of NaCl on the optimal pH. The reaction mixture (1.0 ml) contained 20 mM Tris–H_2SO_4, 2.0 mM 3'-AMP, 0.05 μg of enzyme, and NaCl as indicated above each curve.

pH. However, even if the activities are compared at their optimal pH, the activation by Cl^- is very marked. Thus the Cl^- activation cannot be explained in terms of a shift in the dissociation constants controlling the pH curve.

Fig. 9 shows the effect of Cl^- on the hydrolysis of di-*p*-nitrophenyl phosphate. All the reciprocal plots ($1/v$ *versus* $1/[S]$) were linear in the presence and absence of NaCl and had a common point. By increasing the concentration of NaCl, v_{max} increased and apparent K_m decreased. These effects were saturated at about 200 mM.

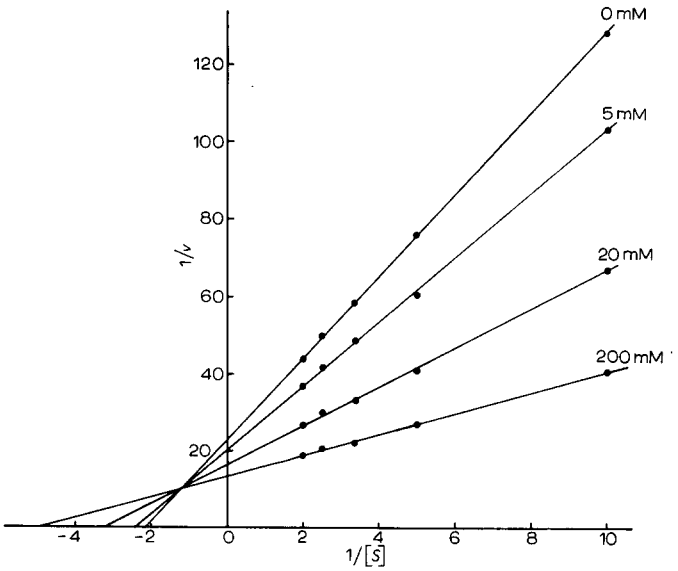


Fig. 9. Lineweaver–Burk plots ($1/v$ *versus* $1/[S]$) at different concentrations of NaCl with di-*p*-nitrophenyl phosphate as substrate. Reaction velocities at the various concentrations of di-*p*-nitrophenyl phosphate were measured using 0.025 μg of enzyme and 20 mM Tris–H_2SO_4 (pH 8.3) in the presence or absence of NaCl as indicated above. v: μmole *p*-nitrophenol 10 min per 0.025 μg of enzyme. $[S]$: the concentration of di-*p*-nitrophenyl phosphate in mM.

Fig. 10 indicates the effect of NaCl on the hydrolysis of 3′-AMP. This showed that Cl^- has no effect on K_m. The increase in v_{max} was saturated at about 200 mM. The activities for the other 3′-ribonucleotides were also modified in a manner quite similar to the case of 3′-AMP. $MgCl_2$ also modified the 3′-nucleotidase as NaCl except that the activation was saturated at low concentrations, *i.e.*, at about 20 mM. $M_{1/2}$ of $MgCl_2$ for 3′-AMP hydrolysis was 4.0 mM.

Fig. 11 shows the effect of NaCl on the hydrolysis of Urd-2′,3′-*P*. As in the case of 3′-nucleotidase, Cl^- has no effect on K_m and the decrease in v_{max} was also saturated at about 200 mM. Similar results were obtained in the hydrolysis of Cyd-2′,3′-*P*. The maximal velocities in the presence or absence of 200 mM NaCl and $M_{1/2}$ values for several substrates are presented in Table II.

These results clearly indicate that the Cl^- activation of the 3′-nucleotidase and the Cl^- inhibition of the cyclic phosphodiesterase are caused by affecting only v_{max}. But the case of di-*p*-nitrophenyl phosphate hydrolysis is rather complicated. Suppose Cl^- modifies the activity by binding to a site other than the active sites of the enzyme,

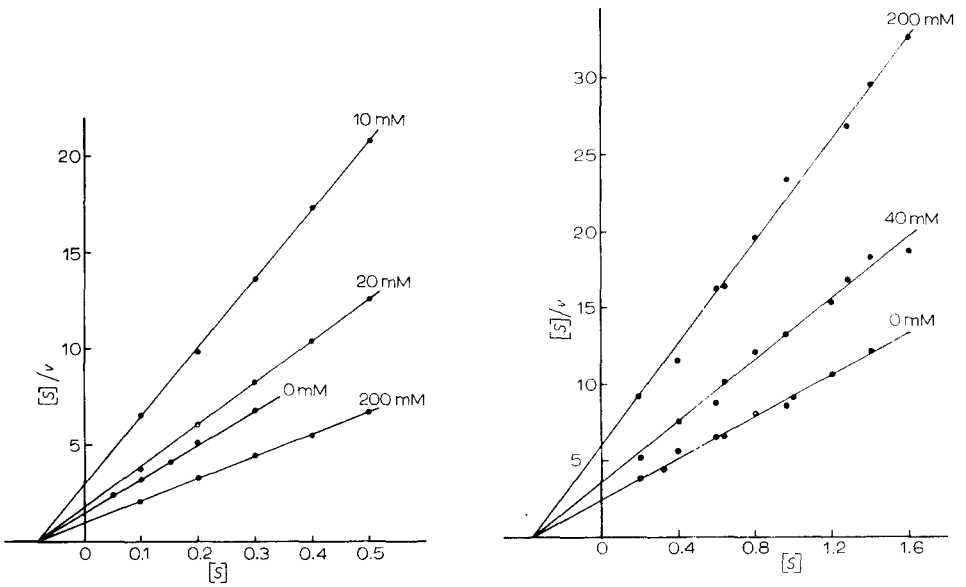


Fig. 10. $[S]/v$ *versus* $[S]$ plots at different concentrations of NaCl with 3'-AMP as substrate. The reaction velocities at the various concentrations of 3'-AMP were measured using 20 mM Tris H_2SO_4 (pH 8.3) in the presence or absence of NaCl as indicated above. The amounts of enzyme used for the assay in the presence of 0, 10, 20, and 200 mM of NaCl were 0.5, 0.05, 0.05, and 0.025 μg of enzyme, respectively. v: μmole P_i/10 min per 0.05 μg of enzyme, except that in the absence of NaCl, v represents μmole P_i/10 min per 0.5 μg of enzyme. $[S]$: the concentration of 3'-AMP in mM.

Fig. 11. $[S]/v$ *versus* $[S]$ plots at different concentrations of NaCl with Urd-2',3'-*P* as substrate. The reaction velocities at the various concentrations of Urd-2',3'-*P* were measured using 20 mM Tris–H_2SO_4 (pH 8.6) and enzyme (0.1 μg/ml) in the presence or absence of NaCl as indicated above. v: μmole of Urd-2',3'-*P* hydrolyzed/min per 0.1 μg of enzyme. $[S]$: the concentration of Urd-2',3'-*P* in mM.

the modifications of the activities may be explained in the mechanism,

$$E + S \rightleftharpoons ES \qquad ES \xrightarrow{k_5} E + P \tag{1}$$
$$E + M \rightleftharpoons EM \tag{2}$$
$$ES + M \rightleftharpoons EMS \qquad EMS \xrightarrow{k_6} EM + P \tag{3}$$
$$EM + S \rightleftharpoons EMS \tag{4}$$

where S is the substrate and M is the modifier. FRIEDEN[16] reported the effect of the modifier on the kinetic parameters of single substrate enzymes. The general form of the equation for the above mechanism derived by the rapid equilibrium treatment is presented by him,

$$\frac{v_0}{(E)_0} = \frac{k_5(1 + k_6M/k_5K_3)/(1 + M/K_3)}{(1 + K_1/S)\,[(1 + M/K_2)/(1 + M/K_3)]}$$

where K_1 to K_4 are the dissociation constants describing Steps 1 to 4, and k_5 and k_6 are the rate constants for the breakdown of the ES and EMS complex, respectively. The results on the modifications of the activity for di-*p*-nitrophenyl phosphate (Fig. 9) corresponded to the case where $K_2/K_3 > k_5/k_6$, $K_2 > K_3$, and $k_6 > k_5$. Since

$M_{1/2}$ for the di-*p*-nitrophenyl phosphate hydrolysis is 20 mM (see Table II), the values of kinetic parameters obtained from Fig. 9 are as follows: $K_1 = 0.45$ mM, $K_2 = 45$ mM, $K_3 = 20$ mM, $K_4 = 0.20$ mM, $k_5 = 170$ units/mg protein, and $k_6 = 286$ units/mg protein. These values satisfy the above conditions. The negative substrate concentration at the point of intersection calculated from $K_1(K_3/K_2 - k_6/k_5)/(1 - k_6/k_5)$ is 0.82, and that obtained from Fig. 9 is 0.83, which is in good agreement with the calculated value.

The results on the hydrolysis of 3'-AMP (Fig. 10) corresponded to the case, where $K_2/K_3 > k_5/k_6$ and $K_2 = K_3$. The values obtained on the 3'-AMP hydrolysis are as follows: $K_2 = K_3 = 20$ mM, $K_1 = K_4 = 0.08$ mM, $k_5 = 11$ units/mg protein, and $k_6 = 170$ units/mg protein. The kinetic parameters on the hydrolyses of the other 3'-ribonucleotides may be obtained from Table II. Similarly, the results on the cyclic phosphodiesterase (Fig. 11) corresponded to the case, where $K_2/K_3 < k_5/k_6$, and $K_2 = K_3$. From Fig. 11, the following values are obtained. $K_2 = K_3 = 40$ mM, $K_1 = K_4 = 0.36$ mM, $k_5 = 1560$ units/mg protein and $k_6 = 600$ units/mg protein.

In this manner, the Cl^- modifications of the activities for several substrates may well be explained by the mechanism of single substrate–single modifier case. Since it is strongly suggested that these activities are associated with the same protein molecule and that Cl^- does not affect the size of the protein molecule, monomer–polymer interactions do not seem to play a major role in the regulation of the activities. The modifications may arise from the reversible conformational changes in the protein molecule. Since the cyclic phosphodiesterase and 3'-nucleotidase activities are inversely modified by Cl^-, it is evident that the active sites for each activity exist in the different sites of the same protein. The activation of the di-*p*-nitrophenyl phosphate hydrolysis by Cl^- may suggest the participation of the 3'-nucleotidase site in this reaction. However, which of the active sites actually catalyzes the hydrolysis of di-*p*-nitrophenyl phosphate is uncertain. The relatively complex results on the case of di-*p*-nitrophenyl phosphate may be caused by the fact that di-*p*-nitrophenyl phosphate is not a natural substrate but an artificial one. The relationship between the active sites for several substrates will be reported elsewhere.

The unique responses of this enzyme to Cl^- were only observed with the enzyme of *V. alginolyticus* and the corresponding enzymes of *E. coli* and *Serratia marcescens*[14] showed no response to Cl^-. Since *V. alginolyticus* requires 0.35–0.85 M NaCl for the optimal growth, this will be classified as a slightly halophilic bacterium according to LARSEN[17]. The specific responses of this enzyme to Cl^- will be a reflection of the halophilic nature of this organism. The importance of salts in the stabilization and activation of the enzymes of extreme halophiles has been reported by several investigators[17–19], but a regulatory function of Cl^- such as that described above has not been mentioned so far.

This enzyme may take part in the reutilization of nucleotides, as in the case of *E. coli* discussed by ANRAKU[1,2] and by MIZUNO and coll.[20,21]. However, the precise role of the Cl^- modifications of this enzyme in the metabolism of *V. alginolyticus* is uncertain at present. However, it is interesting to note that the two activities which reside in the same protein molecule and catalyze the successive hydrolyses of 2',3'-cyclic ribonucleotides are inversely modified by nonmetabolites such as Cl^-.

ACKNOWLEDGMENTS

This work was partly suported by a grant from the Japanese Ministry of Education. We are indebted to Miss F. HAYASE for excellent technical assistance in some experiments.

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