BBA 66185

ANION-ACTIVATED 5'-NUCLEOTIDASE IN CELL ENVELOPES OF A SLIGHTLY HALOPHILIC *VIBRIO ALGINOLYTICUS*

MAKI HAYASHI, TSUTOMU UNEMOTO, YOSHIMICHI KOZUKA AND MAKOTO HAYASHI
Laboratory of Microbiological Chemistry, Institute of Food Microbiology, Chiba University, Chiba-ken (Japan)

(Received June 26th, 1970)

SUMMARY

1. Unique properties of the 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) in cell envelopes of a slightly halophilic *Vibrio alginolyticus* were studied.

2. More than 70% of the 5'-nucleotidase activities were associated with the cell envelopes prepared by the osmotic lysis or by the lysis of penicillin spheroplasts.

3. The activities in the envelopes were increased by storage in a frozen state or by pre-incubation at an alkaline pH in the presence of monovalent cations. The order of effectiveness for the pre-activation was: $NH_4^+ \gg Li^+ > Na^+ > K^+ = Rb^+ = Cs^+$. Such pre-activation was not detected with the envelopes prepared in the absence of Mg^{2+} and Cl^-.

4. Both the unactivated and the activated enzymes hydrolyzed all 5'-ribonucleotides to the corresponding ribonucleosides and P_i. For the hydrolysis of these substrates, both Mg^{2+} and an appropriate anion were required for maximal activity. Among monovalent anions, Cl^- was the most effective, the order of effectiveness being $Cl^- = Br^- > I^- > NO_3^-$. Acetate and SO_4^{2-} showed no effect. No essential requirement for a particular species of monovalent cation was detected. From these results, the activities in the envelopes of *V. alginolyticus* were identified as a Mg^{2+}-dependent and anion-activated 5'-nucleotidase. Furthermore, Ca^{2+}-dependent and anion-activated ribonucleoside 5'-monophosphatase was also found in this envelope. This preparation was unable to hydrolyze UDPG, glucose 1- and 6-phosphates, 2'- and 3'-ribonucleotides, 2',3'-cyclic ribonucleotides, bis(*p*-nitrophenyl) phosphate, *p*-nitrophenyl phosphate or PP_i.

5. The activated enzyme was inhibited by high concentrations of salts such as 1.0 M each of LiCl and NaCl. The unactivated enzyme, however, was unaffected by these salts.

6. The significance of the Cl^- modifications on the enzyme activities isolated from slightly halophilic(marine) bacteria is discussed.

INTRODUCTION

In the previous paper[1], we described the presence of a cation-activated ATP-

hydrolyzing enzyme in cell envelopes of a slightly halophilic *Vibrio parahaemolyticus*. Recently, THOMPSON *et al.*[2] reported a similar enzyme in the envelopes of a marine bacterium. From the facts that these enzymes can hydrolyze ribonucleoside 5′-mono-, di- and triphosphates at similar rates and that Mg^{2+} and monovalent cations are required for maximal activity, they were characterized as Mg^{2+}-dependent and cation-activated 5′-nucleotidases[1,2]. Nonspecific monovalent cation activation has also been reported in ATPases of several terrestrial bacteria[3–7]. Recently, EVANS[8] reported that membrane-bound Mg^{2+}- and Ca^{2+}-ATPases of *Escherichia coli* were inhibited by monovalent cations. The roles of anions, however, have scarcely been investigated.

At the time of our previous report[1], we had been interested in the stimulatory effect of monovalent cations in relation to ion transport ($Na^+ + K^+$)-activated ATPases of mammalian systems[9]. Later, we discovered that anions play an important role in the modifications of the activities of 2′,3′-cyclic phosphodiesterase purified from a slightly halophilic *V. alginolyticus*[10,11]. In an attempt further to manifest the roles of anions, we re-investigated the effects of anions and cations on the 5′-nucleotidase activities in the cell envelopes of *V. alginolyticus*, and came to the conclusion that these activities should be characterized as due to Mg^{2+}-dependent and anion-activated 5′-nucleotidase.

Furthermore, it was found that the activities in the envelopes are increased during storage in a frozen state or by pre-incubation with NH_4Cl at an alkaline pH. The rate and extent of pre-activation were influenced by pH and by different kinds and concentrations of salt in the pre-incubation mixture.

This paper deals with the unique properties of the 5′-nucleotidase in the cell envelopes of *V. alginolyticus*, and the optimal conditions for the pre-activation of the enzyme.

MATERIALS AND METHODS

Bacterial growth

V. alginolyticus 138-2 was isolated from marine fish by Prof. K. Aiso of this Institute. Similar to *V. parahaemolyticus*, it is gram-negative and requires 0.5 M NaCl for optimal growth. It was grown and harvested by the method described previously[10].

Preparation of cell envelopes

Since the enzyme activities in the envelopes are unstable in the absence of Mg^{2+} and Cl^-, Medium A (2 mM $MgCl_2$, 10 mM NaCl and 10 mM Tris–acetate (pH 7.2) at 5°) was used for washing and suspending the envelopes. For the lysis of cells, Medium B (as Medium A but $MgCl_2$ omitted) was used to facilitate osmotic lysis.

Osmotic lysis

The cells of *V. alginolyticus* were washed twice with 1.0 M NaCl containing 10 mM Tris–acetate (pH 7.2). The washed cells were osmotically lysed by being rapidly dispersed in Medium B at the concentration of 5 mg protein per ml. When sufficient lysis of the cells could not be attained, they were centrifuged for 20 min at 10 000 × *g* and the cell pellets were resuspended in Medium B. Absorbance of the cell suspension was measured at 500 nm after appropriate dilution with 1.0 M NaCl. After lysis of

more than 90% of the cells (determined from the decrease in absorbance), deoxyribonuclease (Sigma, stock No. DN-C) and $MgCl_2$ were added to give final concentrations of 5 μg/ml and 2 mM, respectively. The mixture was allowed to stand for 5 min at 20°, followed by centrifugation for 20 min at 10 000 × *g*. The pellets were washed twice with Medium A and were finally suspended in Medium A.

Pressure disruption and centrifugal fractionation

The washed cells were suspended in Medium A and passed through a French pressure cell at 500–600 kg/cm^2. The disrupted cells were centrifuged for 5 min at 3000 × *g* to remove unbroken cells. The supernatant fraction was further centrifuged for 60 min at 100 000 × *g*. The pellets were dispersed in Medium A with a teflon-glass homogenizer and recentrifuged for 60 min at 100 000 × *g*. The washed pellets were finally suspended in Medium A.

Spheroplast formation and its lysis

Spheroplasts of *V. alginolyticus* were prepared by the penicillin method as previously described[12]. The washed spheroplast pellets were suspended in Medium B. After lysis of the spheroplasts, the suspension was treated in the same manner as in osmotic lysis, and the washed lysates were finally suspended in Medium A.

Enzyme assay

The standard reaction mixture (1.0 ml) contained 100 mM $MgCl_2$, 50 mM Tris–acetate (pH 7.4 at 37°), 4.0 mM substrate and enzyme (30–80 μg of protein). After 10 min incubation at 37°, the reaction was stopped by adding 1.0 ml of 2.4 M $HClO_4$. The mixture was centrifuged, and an aliquot of the supernatant was analysed for P_i by the method of ALLEN[13] in a final volume of 3.0 ml. One unit of activity is defined as the amount that will hydrolyze 1.0 μmole of substrate in 1 min.

Protein was determined by the method of LOWRY *et al.*[14] using bovine albumin as a standard.

Pre-activation treatments of the envelopes

The mixture, containing 20 mM NH_4Cl, 50 mM Tris–acetate (pH 9.0 at 5°) and 500 μg of envelope protein per ml, was frozen at −16° and stored for 60 min. This was thawed at 20°, the envelopes were washed by centrifugation with Medium A, and were finally suspended in Medium A.

RESULTS

Localization of the enzyme activity in the cell envelopes of V. alginolyticus

Membrane-rich cell envelope fractions of *V. alginolyticus* were prepared by the three methods as described in MATERIALS AND METHODS. The envelopes prepared by the osmotic lysis still maintained a rod-shaped configuration and showed weak contrast as compared with the intact cells under phase-contrast microscopy. Since further washings of the lysed cells removed of none significant amount of protein nor of the enzyme activities, this fraction seems to be almost free from intracellular materials. This preparation was therefore used as the envelopes of this organism.

Table I shows the distribution of protein and the enzyme activity in the envelope

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITY IN CELL ENVELOPES AND CYTOPLASMIC FRACTIONS PREPARED BY OSMOTIC LYSIS AND A FRENCH PRESS

Assays were made under the standard conditions using ATP as a substrate. The amounts of protein and enzyme activity in each fraction are expressed in per cent of the original total amounts. Details for the method of cell fractionations are described in MATERIALS AND METHODS. Standard deviations were calculated from 6 separate experiments.

Method of preparation	*Fraction*	*Protein (% ± S.D.)*	*Enzyme activity (% ± S.D.)*	*Specific activity (units/mg protein ± S.D.)*
Osmotic lysis	Cytoplasm	74.0 ± 4.5	14.3 ± 3.3	0.10 ± 0.02
	Envelope	28.0 ± 3.5	74.7 ± 5.6	0.82 ± 0.11
French press	Cytoplasm	79.8 ± 4.9	42.5 ± 12.7	0.12 ± 0.05
	Envelope	18.8 ± 3.3	59.5 ± 10.1	0.68 ± 0.13

and the cytoplasmic fractions. About 75% of the total activity for ATP hydrolysis was recovered in the envelope fraction prepared by osmotic lysis. The recovery of the activity in each French press fraction fluctuated, as can be seen from the values of the standard deviation. This may be caused by a partial solubilization of the enzyme or by fragmentation of the envelopes to pieces too small to be sedimented by the present centrifugation. When the envelopes were prepared from the spheroplast lysate, the protein and the enzyme activity in this fraction amounted to 17 and 72% of the total, respectively. These results indicate that at least 70% of the total activity is associated with the envelopes. The remaining activity having a low specific activity was recovered in the cytoplasmic fraction.

Pre-activation treatments of the enzyme in the envelopes of V. alginolyticus

When freshly prepared envelopes were freeze-stored in a medium containing 2 mM $MgCl_2$, 10 mM NaCl and 10 mM Tris–acetate (pH above 8.0), a gradual increase in activity was observed over a period of several days. Maximal activation was attained after 7–8 days at pH 9.0. On the other hand, the envelopes, prepared in the absence of Mg^{2+} and Cl^-, or thoroughly washed with 10 mM Tris–acetate before storage, exhibited no, or only a slight, increase in activity by such treatments. NaCl and alkaline pH were important for the pre-activation, and $MgCl_2$ for the stabilization, of the enzyme. Therefore, the effects of pH and several salts on the pre-activation were examined.

As indicated in Fig. 1a, when the freshly prepared envelopes were freeze-stored, the addition of NH_4Cl was the most effective in producing pre-activation, followed by LiCl. NaCl, KCl, RbCl and CsCl were less effective. Tris–HCl buffer by itself showed a slow activation at alkaline pH. Addition of $MgCl_2$ caused no increase in the activity. The activations, as observed at pH 9.0 by the addition of several salts, could not be detected at pH 7.2.

The activating effects of these salts were saturated at about 20 mM. It took a long time for the maximal activation except with NH_4Cl. Under such low concentrations of the salts, no activation was observed at 2°. The effective activation in the frozen state may be due to an increase in salt concentrations caused by freezing. Since the enzyme is more stable at lower temperature, the above-described pre-treatments

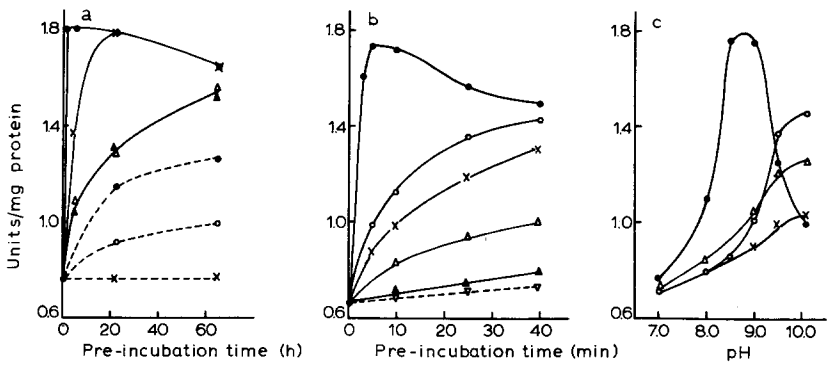

Fig. 1. Effect of several salts and pH on the pre-activation of the enzyme in the cell envelopes of *V. alginolyticus*. Envelopes were prepared by the method of osmotic lysis and used immediately. For the pre-activation, 300 μg protein per ml of the envelopes were used. The enzyme activity was then immediately measured under standard conditions using 30 μg of protein and AMP as a substrate. a. The mixture, containing 10 mM Tris–HCl (at 5°), enzyme and 20 mM each of the salts as indicated below, was rapidly frozen and stored at −16°. After standing for different time intervals, it was thawed at 20° and the activity was measured. Each salt was added at pH 9.0. ●—●, NH_4Cl; ×—×, LiCl; △—△, NaCl; ▲—▲, KCl, RbCl or CsCl; ●— — —●, pH 9.0; ○— — —○, pH 8.0; ×— — —×, pH 7.2. The latter three contained only the buffer of the indicated pH. b. The mixture, containing 50 mM Tris-acetate (pH 9.0 at 37°), 10 mM $MgCl_2$, enzyme and the salts (adjusted to pH 9.0 before use) as indicated below, was incubated at 37°. At intervals, it was rapidly cooled to 5° and the activity was measured. ●—●, 0.5 M NH_4Cl; ×—×, 0.5 M LiCl; ○—○, 0.1 M NH_4Cl; △—△, 0.5 M NaCl; ▲—▲, 0.5 M each of KCl, RbCl or CsCl; ▽— — —▽, without added salt. c. The conditions are the same as those described in b except for the concentrations of the salts and pH. After the mixture had stood for 5 min at 37°, the activity was measured. The salt solutions were adjusted to the required pH before use. ●—●, 0.5 M NH_4Cl; ○—○, 0.1 M NH_4Cl; △—△, 1.0 M LiCl; ×—×, 0.5 M LiCl.

were employed as a standard method of preparing the activated enzyme (see MATERIALS AND METHODS).

When the envelopes were pre-incubated at above 0°, much higher concentrations of salts were required. The rate of activation was also enhanced by increasing the temperature of pre-incubation. Figs. 1b and 1c indicate the effects of several salts and pH at 37°. Here, too, NH_4Cl was the most effective. Although not shown here, when the anions were added as ammonium salts at the concentration of 0.1 M and at pH 9.0, no significant differences among Cl^-, Br^-, I^- and acetate were observed. Thus, monovalent cations seem to be essential for the pre-activation. Fig. 1b shows the order of effectiveness to be $NH_4^+ \gg Li^+ > Na^+ > K^+ = Rb^+ = Cs^+$. Since the dissociation of ammonia is pH-dependent, the actual concentration of NH_4^+ in the pre-incubation mixture is lower than that of the added NH_4Cl at pH 9.0. When calculated from $pK_a = 8.89$ at 37°, 0.5 M NH_4Cl gives about 0.22 M NH_4^+. Thus, NH_4^+ exhibits very effective activation as compared with the same concentration of Li^+. In general, the activation occurred much faster at higher concentrations of each salt and at higher pH except for 0.5 M NH_4Cl at above pH 9.0. Excess ammonia seems to inhibit the activation of the enzyme.

Pre-incubation at 37° for 5 min with 0.2 M each of the ammonium derivatives monomethylamine, ethanolamine, Tris, dimethylamine, diethanolamine and trimethylamine, at pH 9.0, increased the activity by at most 20%. Under such con-

TABLE II

COMPARISON ON THE ACTIVITY RATIOS FOR AMP, ADP AND ATP BETWEEN THE UNACTIVATED AND THE ACTIVATED ENZYMES

Assays were made under the standard conditions using AMP, ADP or ATP as substrates. Pre-activation treatments of envelopes prepared by different methods were carried out as described in MATERIALS AND METHODS. Activities are expressed in units/mg protein. Numbers in parentheses are relative activities, where the activity for AMP is assigned the value 1.0.

Substrate	*Osmotic lysis*		*French press*		*Spheroplast lysis*	
	Unactivated	*Activated*	*Unactivated*	*Activated*	*Unactivated*	*Activated*
AMP	0.96 (1.0)	1.96 (1.0)	0.66 (1.0)	1.31 (1.0)	0.58 (1.0)	1.40 (1.0)
ADP	1.16 (1.21)	2.42 (1.23)	0.88 (1.33)	1.66 (1.27)	0.74 (1.28)	1.82 (1.30)
ATP	0.89 (0.93)	2.05 (1.05)	0.65 (0.98)	1.44 (1.10)	0.54 (0.93)	1.47 (1.05)

ditions, almost maximal activation was attained with 0.2 M NH_4Cl. Amino acids such as glycine and L-lysine acted with much the same effectiveness as NH_4Cl.

On the other hand, 0.5 M sucrose, 0.1–2.0 M urea, 0.1–0.5 M guanidine, 5–10 mM sodium dodecyl sulfate and 1–5% Triton X-100 showed no activation at any pH. The use of NaOH or KOH alone to produce a suitable pH also resulted in no pre-activation.

Properties of the 5′-nucleotidase in the envelopes of V. alginolyticus

The envelopes prepared by osmotic lysis were used as an enzyme preparation unless otherwise indicated. The method of osmotic lysis is the simplest procedure giving the envelopes with highest specific activity among the three methods (see Table II). Both the unactivated and the activated enzymes were used for the examination of enzymic properties.

Substrate specificity. The envelopes of *V. alginolyticus* hydrolyzed all 5′-ribonucleotides to the corresponding ribonucleosides and P_i. P_i released from 1 μmole each of AMP, ADP and ATP amounted to 1, 2 and 3 μmoles, respectively. In the course of ATP hydrolysis, neither ADP nor AMP could be detected in the reaction mixture, indicating no appreciable accumulation of an intermediate. In the standard assay conditions, the activity ratios for several substrates were as follows: AMP, 100 ADP, 121; ATP, 95; GMP, 34; GTP, 62; CMP, 62; CDP, 74; CTP, 56; UMP, 120; UTP, 60; TTP, 60; and ITP, 94. Table II indicates that the activity ratios for AMP, ADP and ATP were the same between the unactivated and the activated enzymes, *i.e.* the pre-activation treatments enhanced the activities for these substrates to the same extent.

This preparation was unable to hydrolyze UDPG, glucose 1- and 6-phosphates, 2′- and 3′-ribonucleotides, 2′,3′-cyclic ribonucleotides, bis(*p*-nitrophenyl) phosphate, *p*-nitrophenyl phosphate and PP_i, indicating no significant contamination with alkaline phosphatase (EC 3.1.3.1), 2′,3′-cyclic phosphodiesterase or inorganic pyrophosphatase (EC 3.6.1.1).

These results indicate that the envelopes of *V. alginolyticus* contain a phosphohydrolase specific to ribonucleoside 5′-mono-, di- and triphosphates.

Optimal pH. As indicated in Fig. 2, the optimal pHs for AMP and ATP hydro-

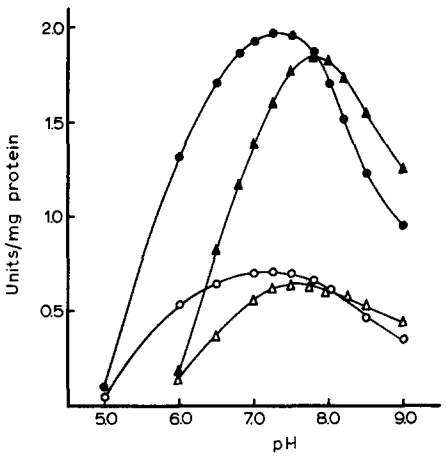

Fig. 2. pH–activity curves for the unactivated and the activated enzymes using AMP and ATP as substrates. Assays were made under standard conditions except that acetate buffer was used at pH 5.0 and 6.0, and Tris–acetate buffer between pH 6.5 and 9.0. All buffers were prepared at 37°. Pre-activation treatments of the enzyme were carried out as described in MATERIALS AND METHODS. Unactivated enzyme: ○—○, AMP as a substrate; △—△, ATP as a substrate. Activated enzyme: ●—●, AMP; ▲—▲, ATP.

lyses were 7.3 and 7.5, respectively. With the activated enzyme, the optimal pH for AMP hydrolysis was the same, while that for ATP was slightly more alkaline (pH 7.8). STETTEN AND BURNETT[15] reported a similar shift of the pH optimum in the alkaline direction with the activated enzymes of rat-liver microsomal glucose-6-phosphatase and the related enzymes.

Ionic requirements. This preparation showed no activity in the absence of divalent cations and an appropriate anion. Fig. 3 illustrates the effect of Mg^{2+} and anions using AMP as a substrate. As shown in Fig. 3a, maximal activation was obtained

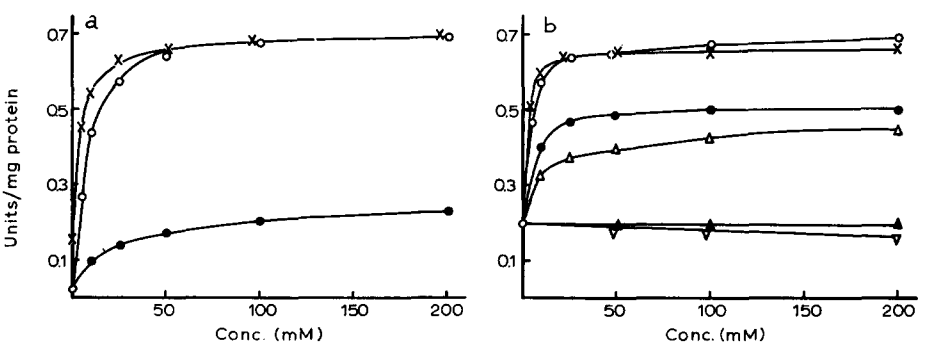

Fig. 3. Effect of Mg^{2+} and several anions on the hydrolysis of AMP. The reaction mixture (1.0 ml) contained 4.0 mM Tris–AMP, 50 mM Tris–acetate (pH 7.4 at 37°), 80 μg of the unactivated enzyme and the various amounts of the salts indicated below. The enzyme was washed twice by centrifugation with 10 mM Tris–acetate (pH 7.2) to remove Mg^{2+} and Cl^- in the enzyme solution and was immediately used to prevent irreversible inactivation. a. ●—●, magnesium acetate; ○—○, $MgCl_2$; ×—×, 200 mM KCl + magnesium acetate. b. All the reaction mixtures contained 100 mM magnesium acetate in addition to the salts indicated. ○—○, KCl; ×—×, KBr; ●—●, KI; △—△, KNO_3; ▲—▲, potassium acetate; ▽—▽, K_2SO_4

by the addition of 100 mM $MgCl_2$. When magnesium acetate was used, however, the activation was lower than that due to $MgCl_2$. In the presence of 200 mM KCl, an activation curve similar to that for $MgCl_2$ was obtained. Thus, both Mg^{2+} and Cl^- are required for maximal activity.

Fig. 3b shows the effects of several anions in the presence of saturated concentrations of Mg^{2+}. Among monovalent anions, Cl^- was the most effective, the order of effectiveness being $Cl^- = Br^- > I^- > NO_3^-$. Acetate and SO_4^{2-} showed no effect on the activity.

Table III indicates that when 200 mM each of monovalent cations were added as chloride salts in the presence of 100 mM Mg^{2+}, no significant differences in the

TABLE III

EFFECT OF MONOVALENT CATIONS ON THE HYDROLYSIS OF AMP

The reaction mixture contained 4.0 mM Tris–AMP, 50 mM Tris–acetate (pH 7.4), 80 μg protein of the unactivated enzyme and 200 mM each of chloride salts of monovalent cations in the presence of 5 mM $MgCl_2$ or 100 mM magnesium acetate as indicated. This enzyme showed the specific activity of 0.77 unit/mg protein in the standard conditions. Activities are expressed in units/mg protein.

Salt added	*In the presence of*	
	5 mM $MgCl_2$	*100 mM magnesium acetate*
None	0.30	0.17
LiCl	0.46	0.80
NaCl	0.47	0.76
KCl	0.52	0.78
RbCl	0.52	0.78
CsCl	0.52	0.76
NH_4Cl	0.53	0.77

activity were observed among Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+. Therefore, no one particular species of monovalent cation was required for the reaction. But, in the presence of 5 mM $MgCl_2$, the activity obtained by the addition of 200 mM each of LiCl or NaCl was slightly lower than that by KCl, RbCl, CsCl or NH_4Cl. COSTERTON *et al.*[16] and DE VOE AND OGINSKY[17,18] reported that Li^+ and Na^+, but not K^+, interact in an antagonistic manner with Mg^{2+} in the cell envelopes of marine bacteria. If such an antagonism does exist towards this enzyme, a slight inhibitory effect by Li^+ and Na^+, especially at low concentrations of Mg^{2+}, may well be explained as a result of competition between these monovalent cations and Mg^{2+}.

When the activated enzyme was used, the same patterns of ionic requirements as described above were obtained.

Table IV illustrates the effects of divalent cations in the presence or absence of saturated concentrations of Mg^{2+} or Cl^-. Mn^{2+} and Co^{2+} partially substituted for Mg^{2+}, and the activity was further stimulated by Cl^-. But they are slightly inhibitory in the presence of 100 mM $MgCl_2$.

Ca^{2+} acted as an activator for AMP hydrolysis but not for ATP. The activating effect of Ca^{2+} was restricted only to the hydrolysis of ribonucleoside 5′-monophos-

TABLE IV

EFFECT OF DIVALENT CATIONS AND Cl^- ON THE HYDROLYSIS OF AMP AND ATP USING THE UNACTIVATED AND THE ACTIVATED ENZYMES

The reaction mixture contained 4.0 mM substrate, the unactivated or the activated enzyme, 50 mM Tris–acetate (pH 7.4) and divalent cations in the presence or absence of 200 mM KCl or 100 mM $MgCl_2$. Optimal concentrations of divalent cations were added as acetate salts. The enzyme was washed prior to use as indicated in Fig. 3. The values are expressed in percent of the activity observed in the presence of 100 mM $MgCl_2$.

Substrate	*Divalent cation added as acetate*	*(mM)*	*Unactivated enzyme*			*Activated enzyme*		
			No addition	*KCl (200 mM)*	*$MgCl_2$ (100 mM)*	*No addition*	*KCl (200 mM)*	*$MgCl_2$ (100 mM)*
AMP	None		3	23	100	4	24	100
	Mg^{2+}	100	27	103	105	22	97	100
	Mn^{2+}	5	26	71	85	19	52	69
	Co^{2+}	5	13	54	79	13	60	84
	Ca^{2+}	25	28	69	120	18	34	96
ATP	None		0	0	100	1	1	100
	Mg^{2+}	100	24	91	91	21	79	82
	Mn^{2+}	5	19	52	63	12	36	55
	Co^{2+}	5	5	21	60	9	21	62
	Ca^{2+}	25	1	4	98	3	6	82

phates. Ca^{2+}-dependent activity was also further stimulated by Cl^-. The activity ratios for ribonucleoside 5′-monophosphates in the optimal conditions (25 mM $CaCl_2$, 100 mM KCl and 50 mM Tris–acetate, pH 7.5) were as follows: AMP, 1.0; UMP, 1.35; CMP, 0.68; and GMP, 0.59. Under such conditions, ADP, ATP, 2′- and 3′-ribonucleotides, glucose 1- and 6-phosphates, bis(*p*-nitrophenyl) phosphate and *p*-nitrophenyl phosphate were not hydrolyzed. These results predict that the envelopes of *V. alginolyticus* contain a Ca^{2+}-dependent and Cl^--activated nucleoside 5′-monophosphatase. Further characterization of this enzyme will be described elsewhere.

Table IV, also shows that, on the addition of 200 mM KCl alone, both the unactivated and the activated enzymes exhibited about 23% of the maximal activity for AMP hydrolysis. This may be due to the presence of a small amount of bound Mg^{2+} in the envelopes (see ref. 18). The activity for ATP, however, was almost negligible in the absence of the added Mg^{2+}. This was caused by the inhibitory effects of free ATP. Indeed, the activity for AMP in the presence of KCl alone was inhibited by 4.0 mM of Tris–ATP. Thus, $MgATP^{2-}$ seems to be a true substrate of this enzyme.

Inhibition of the activated enzyme by high concentrations of salts. The activated enzyme was inhibited by the addition of high concentrations of salts in the assay mixture. For example, by the addition of 1.0 M each of chloride salts of Li^+, Na^+, Rb^+, Cs^+, K^+ and NH_4^+, the activity for ATP was inhibited by 55, 35, 13, 12, 7 and 6%, respectively. Except for NH_4^+, the monovalent cations effective for the preactivation were also the effective inhibitors for the activated enzyme. With respect to monovalent anions, when 1.0 M each of potassium salts of I^-, NO_3^-, acetate, Br^- and Cl^- were added, the activity for ATP was inhibited by 38, 32, 24, 18 and 7%, respectively. Similar patterns of inhibition were also observed for AMP hydrolysis.

However, pre-treatment of the activated enzyme with such high concentrations of salts caused no decrease in the enzymic activity.

On the other hand, the unactivated enzyme was not inhibited by these salts. As might be expected from Fig. 1c, a slight stimulation was observed. When 1.0 M NH_4Cl was added in the assay mixture, a 2-fold increase in activity was observed. In this experiment, it was found that the rate of hydrolysis increased with time when measured at 2-min intervals. Thus, the apparent stimulation is caused by the activation of the enzyme during the incubation period (10 min) of the standard assay and not by the actual increase in the initial velocity of the hydrolysis.

DISCUSSION

Based on our previous findings that anions act as a modifier of 2′,3′-cyclic phosphodiesterase purified from a slightly halophilic *V. alginolyticus*[10,11], we re-investigated a possible role of anions on the 5′-nucleotidase of this bacterium, which we have previously described as a cation-activated enzyme[1]. This paper explains that this enzyme requires both Mg^{2+} and an appropriate anion for maximal activity. Among monovalent anions, Cl^- was the most effective, followed by Br^-, I^- and NO_3^-. The present enzyme preparation is a crude envelope fraction, and there is no conclusive evidence whether the hydrolytic activity is due to one or more enzymes. However, aside from a Ca^{2+}-dependent ribonucleoside 5′-monophosphatase, the same patterns of ionic requirements and properties were observed for the hydrolysis of a variety of 5′-ribonucleotides. Therefore, the activities in the envelopes of *V. alginolyticus* catalyzing the hydrolysis of 5′-ribonucleotides may well be characterized as a Mg^{2+}-dependent and anion-activated 5′-nucleotidase.

Recently, THOMPSON *et al.*[2] described a similar enzyme in the cell envelopes of a marine bacterium which they identified as a cation-activated 5′-nucleotidase. These and our previous[1] experiments, however, were intended to demonstrate the stimulatory effects of monovalent cations, and the effects of anions were out of consideration. In contrast to the present results, DRAPEAU AND MACLEOD[19] reported that, using isolated membranes of two species of marine bacteria, the Mg^{2+}–ATP ratio for maximal activity is 1:1 and that the activating effects by the chloride salts of four monovalent cations can also be replaced by their sulfate salts. In their experiments, 100 mM Tris–HCl (pH 9.0) was included in the standard assay mixture; hence no definite explanation on the role of Cl^- can be made. Since the wide specificity of the present enzyme for 5′-ribonucleotides coincides with those of the enzymes found in the envelopes of marine bacteria[2,19], these enzymes could belong to the same category. Whether such Cl^- requirements as demonstrated in this paper are common to all these enzymes must await further studies.

On the localization of the 5′-nucleotidase, more than 70% of the enzyme was recovered in the cell envelopes. In the previous paper[10], we reported that the 5′-nucleotidase of *V. alginolyticus* could not be released into the medium by a procedure of osmotic shock. Thus, this enzyme resides in the envelopes firmly bound to their structure, in contrast to the situation in *E. coli*[20] and other Enterobacteriaceae[21]. Since the total recovery of the activity is almost quantitative, there can be no such intrinsic protein inhibitor as reported in *E. coli*[22]. The precise location of this enzyme in the envelopes is under investigation with electron microscopy.

Another characteristic property was the pre-activation of the enzyme by treatment with NH_4Cl at alkaline pH. A similar activation was described by STETTEN *et al.*[23,24] on the microsomal enzymes. They reported that exposure of the rat-liver microsomes to 0.1 M NH_4Cl (pH 9.8) for 15 min at 30°, or contact for many hours at 0°, resulted in maximal elevation *in vitro* of the microsomal glucose-6-phosphatase (EC 3.1.3.9) and related enzymic activities. OH^- has been shown to produce an increase in the activities[23]. But both monovalent cations and alkaline pH were essential for the pre-activation of the present enzyme. Furthermore, the instantaneous activation of the microsomal enzymes by treatment with deoxycholate or Triton X-100 could not be detected with this enzyme. Thus, there are several differences in the mechanism of activation. In another experiment, this enzyme was found to be solubilized with Triton X-100. The enzyme detached from the envelopes was also activated by the same pre-treatment. Thus, the activation is not the result of solubility changes in the envelope, but it seems to be the result of conformational changes in the enzyme protein itself.

DRAPEAU *et al.*[25], WONG *et al.*[26] and MACLEOD AND ONOFREY[27] have reported on the requirements of Na^+ and Cl^- in the nutrition and metabolism of marine bacteria, the former to permit the transport of metabolites into the cells, and the latter for maximal rate of growth. It was also shown that, among tricarboxylic acid cycle enzymes, aconitase and isocitrate dehydrogenase function better in media of appropriate ionic strength[28]. In this and the previous papers[10,11], we pointed out that Cl^- acts as modifier for the enzymes isolated from a slightly halophilic *V. alginolyticus*. Although it grows well at 37°, this bacterium is similar to marine bacteria in the ionic requirements for growth. The primary role of Cl^- in the metabolism of these bacteria may be ascribed to its regulatory functions for the enzyme activities. At present, the two enzymes we examined are those associated with the envelopes: further studies with other enzymes will be necessary to verify the present hypothesis.

ACKNOWLEDGEMENT

This work was partly supported by a grant from the Japanese Ministry of Education.

REFERENCES

1 M. HAYASHI AND R. UCHIDA, *Biochim. Biophys. Acta*, 110 (1965) 207.
2 J. THOMPSON, M. L. GREEN AND F. C. HAPPOLD, *J. Bacteriol.*, 99 (1969) 834.
3 J. W. GREENAWALT, C. WEIBULL AND H. LÖW, *J. Biol. Chem.*, 237 (1962) 853.
4 R. GROSS AND N. W. COLES, *J. Bacteriol.*, 95 (1968) 1322.
5 J. C. M. HAFKENSCHEID AND S. L. BONTING, *Biochim. Biophys. Acta*, 151 (1968) 204.
6 TH. GÜNTHER AND F. DORN, *Z. Naturforsch.*, 21b (1966) 1076.
7 J. C. BURNHAM AND G. J. HAGEAGE, JR., *J. Bacteriol.*, 93 (1967) 191.
8 D. J. EVANS, JR., *J. Bacteriol.*, 100 (1969) 914.
9 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
10 T. UNEMOTO AND M. HAYASHI, *Biochim. Biophys. Acta*, 171 (1969) 89.
11 T. UNEMOTO, F. TAKAHASHI AND M. HAYASHI, *Biochim. Biophys. Acta*, 185 (1969) 134.
12 T. UNEMOTO, MAKI HAYASHI, K. MIYAKI AND MAKOTO HAYASHI, *Biochim. Biophys. Acta*, 110 (1965) 319.
13 R. J. L. ALLEN, *Biochem. J.*, 34B (1940) 858.
14 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
15 M. R. STETTEN AND F. F. BURNETT, *Biochim. Biophys. Acta*, 132 (1967) 138.

16 J. W. COSTERTON, C. FORSBERG, T. I. MATULA, F. L. A. BUCKMIRE AND R. A. MACLEOD, *J. Bacteriol.*, 94 (1967) 1764.
17 I. W. DE VOE AND E. L. OGINSKY, *J. Bacteriol.*, 98 (1969) 1355.
18 I. W. DE VOE AND E. L. OGINSKY, *J. Bacteriol.*, 98 (1969) 1368.
19 G. R. DRAPEAU AND R. A. MACLEOD, *J. Bacteriol.*, 85 (1963) 1413.
20 H. C. NEU AND L. A. HEPPEL, *J. Biol. Chem.*, 240 (1965) 3685.
21 H. C. NEU AND J. CHOU, *J. Bacteriol.*, 94 (1967) 1934.
22 H. C. NEU, *J. Biol. Chem.*, 242 (1967) 3905.
23 M. R. STETTEN AND F. F. BURNETT, *Biochim. Biophys. Acta*, 128 (1966) 344.
24 M. R. STETTEN, S. MALAMED AND M. FEDERMAN, *Biochim. Biophys. Acta*, 193 (1969) 260.
25 G. R. DRAPEAU, T. I. MATULA AND R. A. MACLEOD, *J. Bacteriol.*, 92 (1966) 63.
26 P. T. S. WONG, J. THOMPSON AND R. A. MACLEOD, *J. Biol. Chem.*, 244 (1969) 1016.
27 R. A. MACLEOD AND E. ONOFREY, *Can. J. Microbiol.*, 3 (1957) 753.
28 R. A. MACLEOD AND A. HORI, *J. Bacteriol.*, 80 (1960) 464.