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EFFECT OF FREE MAGNESIUM AND SALTS ON THE INORGANIC PYROPHOSPHATASE PURIFIED FROM A SLIGHTLY HALOPHILIC *VIBRIO ALGINOLYTICUS*

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SUMMARY

1. Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) of a slightly halophilic marine *Vibrio alginolyticus* was purified 100-fold. This enzyme has an optimum at pH 8.7 and exhibits an absolute requirement for free Mg^{2+}. $MgPP_i^{2-}$ acts as a true substrate for this enzyme, but Mg_2PP_i is also able to serve as a substrate.

2. The enzyme is inactivated by preincubation with salts, which are dependent upon the species of monovalent anions; the order of their effectiveness follows a chaotropic series: ClO_4^-, $SCN > Br^- \geqslant NO_3^- > Cl^-$. The dissociation constant for the enzyme–chloride complex was estimated to be 150 mM. The enzyme is effectively protected from inactivation only when both Mg^{2+} and $MgPP_i^{2-}$ are coexistent, either one of which is ineffective separately.

3. The enzyme is inhibited by salts independently of the species of monovalent cations and anions. NaCl exhibits a parabolic non-competitive inhibition. The salt inhibition is prevented by increasing Mg^{2+}.

4. It is suggested that the inactivation and the inhibition of this enzyme by salt might be protected by the presence of high concentrations of Mg^{2+} under physiological conditions.

INTRODUCTION

Earlier studies in this laboratory have demonstrated that monovalent anions modify the activities of 2′,3′-cyclic phosphodiesterase (3′-nucleotidase)[1,2], 5′-nucleotidase[3], and alkaline phosphatase[4] isolated from a slightly halophilic marine *Vibrio alginolyticus*. The primary role of salts in the metabolism of marine bacteria was ascribed to their regulatory function of the enzyme activities. On the other hand, inorganic pyrophosphatase is well known to play an important role to drive the biosynthetic activities of the living cells[5]. Therefore, it is of interest to purify the inorganic pyrophosphatase from marine bacteria and to examine the effect of salts

on this enzyme. The enzyme from a non-halophilic *Escherichia coli* has been reported to be exceptionally stable[6]. However, we found that the enzyme from a marine *V. alginolyticus* is inactivated by preincubation with salts and also inhibited by their presence during the assay. The intracellular concentrations of Na^+, K^+ and Cl^- in this organism[7] are sufficiently high to cause inactivation and inhibition of this enzyme. To solve this contradiction, we examined the effect of salts and free Mg^{2+} on the enzyme purified from *V. alginolyticus* and it was found that this enzyme is not inactivated nor inhibited by salts in the presence of high concentrations of Mg^{2+}.

METHODS

Enzyme assay

The standard reaction mixture contained 2.0 mM Na_4PP_i, 4.0 mM $MgCl_2$, 50 mM Tris–BICINE [*N*,*N*-bis(2-hydroxyethyl)glycine] buffer (pH 8.7) and an appropriate amount of the purified enzyme in a total volume of 1.5 ml. The reaction was initiated by addition of the enzyme and, after 5 min at 37 °C, it was termiuated by adding 0.5 ml of 24% $HClO_4$. The mixture was rapidly immersed in an ice–water bath to prevent the spontaneous hydrolysis of PP_i in the acid medium. P_i released from PP_i was measured by a modified method of Allen[8] in a total volume of 3.0 ml.

The concentrations of the ion species in the standard reaction mixture calculated as described below are as follows: $MgPP_i^{2-}$ = 1.5 mM, Mg^{2+} = 1.5 mM and Mg_2PP_i = 0.5 mM. When the assay conditions are varied, the concentratoin of each component is given in the legends.

The enzyme was diluted with 10 mM Tris–BICINE buffer (pH 8.7) just before use and the amount of enzyme hydrolyzing less than 10% of the total substrate was applied. Each assay was performed in duplicate or triplicate. Under the assay conditions employed, the reaction rates are linear with time and enzyme concentration.

The activity was expressed in μmoles of the substrate hydrolyzed in 1 min.

Calculation of concentrations of the ion species

In solutions containing Mg^{2+} and PP_i, many ion species are under equilibria. Under our conditions of pH 8.7, where enzymatic activity is maximum, the protonated species such as $H_2PP_i^{2-}$ and $MgHPP_i^-$ are virtually absent and can be neglected. Therefore, the following equilibrium was taken into consideration. The

$$PP_i^{4-} \underset{K_1}{\rightleftharpoons} MgPP_i^{2-} \underset{K_2}{\rightleftharpoons} Mg_2PP_i$$

respective concentrations of Mg^{2+}, PP_i^{4-}, $MgPP_i^{2-}$ and Mg_2PP_i in any given mixture were calculated by the method of Klemme and Gest[9]. Since the enzyme studies were performed at 37 °C and an ionic strength of 0.05–0.2, the stability constants, $K_1 = 10^{5,41}\ M^{-1}$ and $K_2 = 10^{2,34}\ M^{-1}$, reported by Josse[6] were used for the calculations of the concentrations of each ion species.

Bacterial growth and preparation of crude extracts

V. alginolyticus 138-2 was grown under aeration at 37 °C en a medium described previously[1], and about 134 g wet wt of the cells in an exponential phase of growth was obtained from a 60-l culture.

Since the cells of *V. alginolyticus* are very fragile to a hypotonic environment[7], crude cell extracts were prepared by the method of osmotic lysis described previously[3] except for the addition of 2 mM thioglycol in the lysis medium. The lysed cell suspensions were centrifuged for 20 min at 10 000 × *g* and the supernatant was used as the crude cell extract.

Purification of inorganic pyrophosphatase from V. alginolyticus

All procedures were performed at 2–4 °C and the solution used for enzyme purifications contained 10 mM Tris–HCl buffer (pH 7.2), 2 mM thioglycol and 1 mM $MgCl_2$ (Medium A) unless otherwise indicated. Protein was determined by the method of Lowry *et al.*[10] using bovine albumin as a standard.

The crude cell extracts obtained from 134 g wet wt of the cells were fractionated by the stepwise addition of finely powdered $(NH_4)_2SO_4$ at pH 7.2. The fraction precipitated between 60 and 80% satn was collected by centrifugation for 20 min at 10 000 × *g* and dissolved in Medium A (Step 1). The protein concentration was diluted to 12 mg/ml with Medium A and the pH was adjusted to 4.3 by the dropwise addition of 0.5 M acetic acid. The precipitates formed were removed by centrifugation and discarded. The pH of the supernatant was again adjusted to pH 7.2 by adding 1.0 M Tris (Step 2). From 30 to 40 ml of this fraction was applied to Sephadex G-75 column (4.5 cm × 30 cm), and the column was eluted with Medium A. The active fractions were combined, and concentrated and desalted by ultrafiltration in an Amicon apparatus fitted with a type UM-10 membrane (Step 3). This fraction was applied to DEAE-Sephadex A-50 column (2.0 cm × 10 cm) equilibrated with Medium A containing 50 mM KCl. The column was eluted by a linear gradient from 0.05 to 0.4 M KCl in a total volume of 500 ml. Active fractions were eluted at 0.27 M KCl. They were combined, and concentrated and desalted by membrane ultrafiltration (Step 4). The 2nd DEAE-Sephadex column chromatography was carried out at the same conditions using a 1.6 cm × 10 cm column (Step 5). The concentrated fraction of Step 5 was applied to the Sephadex G-100 column (2.0 cm × 39 cm) and the column was eluted with Medium A. The active fraction was concentrated and stored at −20 °C (Step 6). This fraction was used throughout these experiments.

Results on the purification are summarized in Table I. As calculated from the

TABLE I

PURIFICATION OF INORGANIC PYROPHOSPHATASE FROM *V. alginolyticus*

Detailed procedures are described in Methods.

Step	*Fraction*	*Volume (ml)*	*Protein (mg)*	*Total activity (units)*	*Spec. act. (units/mg protein)*	*Yield (%)*
	Cell extracts	695	6255	5004	0.80	100
1.	$(NH_4)_2SO_4$ fraction	81	1296	2981	2.30	59.6
2.	pH fraction	104	874	2402	2.75	48.0
3.	Sephadex G-75	42.7	248	1277	5.15	25.5
4.	DEAE-Sephadex	11.5	41.4	948	22.9	18.9
5.	2nd DEAE-Sephadex	5.6	12.9	730	56.6	14.6
6.	Sephadex G-100	1.5	3.45	286	82.9	5.7

crude cell extracts, the enzyme was purified 100-fold. Since this enzyme is unstable in the presence of salts, further purifications were not attempted at present.

RESULTS

Optimum pH

As indicated in Fig. 1, the inorganic pyrophosphatase of *V. alginolyticus* has an optimum pH of 8.7. No appreciable activity was observed at the pH range between 1.5 and 6.0.

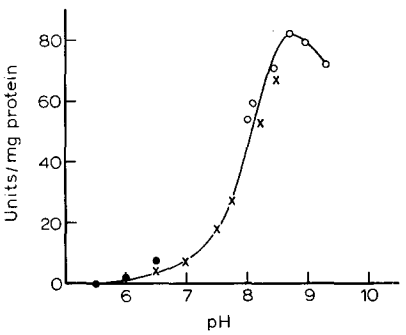

Fig. 1. Effect of pH on the activity of purified inorgainc pyrophosphatase. Enzyme activity was assayed under the standard condition except for buffers. ●—●, 50 mM acetate buffer; ×—×, 50 mM Tris–acetate buffer; ○—○, 50 mM Tris–BICINE buffer. Activity was expressed in units/mg protein.

Mg^{2+} requirement

To determine the Mg^{2+} requirement in the assay system, reaction rates were measured at fixed concentrations of total PP_i and varying concentrations of $MgCl_2$. As indicated in Fig. 2, the activity was observed only in the concentration range where $MgCl_2$ was in excess of total PP_i. The optimum activity was attained at the presence of 2.0 mM Na_4PP_i and 4.0 mM $MgCl_2$. The activity at high concentrations of $MgCl_2$ could not be determined due to the formation of aggregates during the assay. Therefore, the total PP_i was reduced to 0.4 mM and the activity was

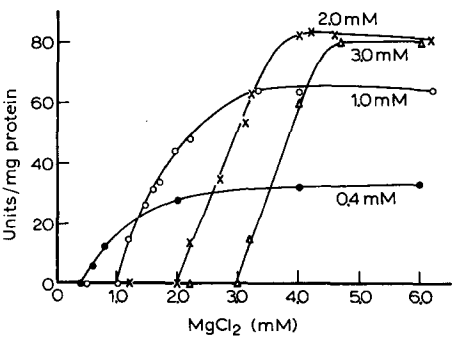

Fig. 2. Effect of $MgCl_2$ on the activity at the different levels of Na_4PP_i concentrations. The activity was assayed at various concentrations of $MgCl_2$ in the presence of fixed amount of Na_4PP_i indicated in the figure.

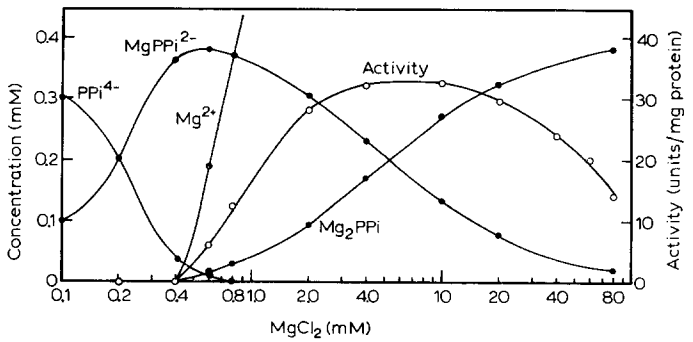

Fig. 3. Comparison of the variation in the concentration of ion species in the reaction mixture with the activity as a function of total $MgCl_2$. The reaction mixture contained 0.4 mM Na_4PP_i, 50 mM Tris–BICINE buffer (pH 8.7) and varying concentrations of $MgCl_2$ indicated above. The concentrations of each ion species were calculated as described in Methods.

measured in the wide range of $MgCl_2$ concentrations. Fig. 3 indicates the comparison of the variations in the concentration of Mg^{2+}, PP_i^{4-}, $MgPP_i^{2-}$ and Mg_2PP_i with the activity as a function of total $MgCl_2$. No activity was observed at 0.4 mM $MgCl_2$ and below. The activity was increased by increasing $MgCl_2$ and became nearly constant between 4.0 and 10 mM. A further increase in $MgCl_2$ gradually decreased the activity, where the concentration of $MgPP_i^{2-}$ decreased and that of Mg_2PP_i increased. These results suggested that (a) PP_i^{4-} or $MgPP_i^{2-}$ cannot serve as a substrate of this enzyme by itself or in combination; (b) Mg^{2+} is essential for the manifestation of activity; (c) $MgPP_i^{2-}$ acts as a true substrate of this enzyme in the presence of Mg^{2+}; (d) Mg_2PP_i also is possible to act as a substrate.

Effect of Mg^{2+} on the enzyme kinetics

It is impossible to vary the concentration of only one component of an equilibrium system at a constant level of all other components. Therefore, assuming that the substrate is $MgPP_i^{2-}$, the effect of Mg^{2+} was examined keeping the concentrations of Mg_2PP_i in low levels. Fig. 4a indicates the double reciprocal plot ($1/v$ *vs*

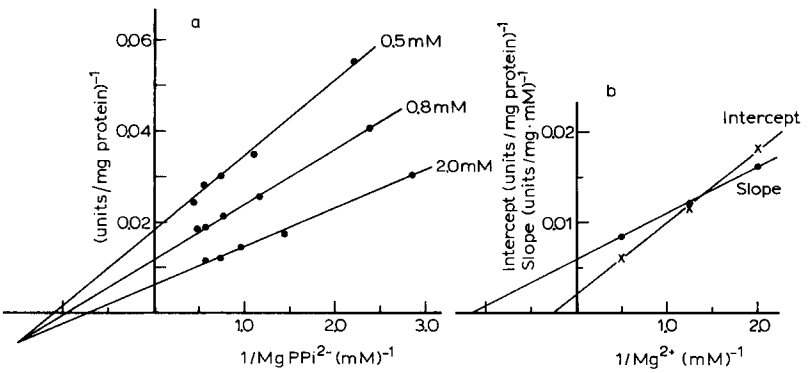

Fig. 4. Effect of free Mg^{2+} on the enzyme kinetics. (a). $1/v$ *vs* $1/MgPP_i^{2-}$ plots at fixed concentrations of Mg^{2+} indicated. The ratios of $MgPP_i^{2-}$ to Mg_2PP_i in the reaction mixture were kept within the range of 8.4–9.0, 5.3–5.8, and 2.2–2.3 in the presence of 0.5, 0.8, and 2.0 mM Mg^{2+}, respectively. (b). Secondary plot of the slopes and intercepts obtained from Fig. 4a against $1/Mg^{2+}$.

$1/MgPP_i^{2-}$) at fixed concentrations of Mg^{2+}. The plots were linear and had a common point. Although not shown here, at fixed concentrations of Mg^{2+} lower than 0.5 mM or higher than 2.0 mM, the double reciprocal plots did not intersect at a common point. This may be due to the effect of an ion species other than $MgPP_i^{2-}$. As indicated in Fig. 4b, secondary plots of the slopes and intercepts against $1/Mg^{2+}$ also gave a linear relationship under the conditions of our experiments.

The results of Fig. 4 may be explained by the single substrate–single modifier case described by Frieden[11]. The general mechanism is written as follows:

$$\begin{array}{ccccc} & & E & \overset{K_1}{\rightleftharpoons} & ES \xrightarrow{k_5} E + P \\ & & K_2 \uparrow\downarrow & K_3 & \uparrow\downarrow k_6 \\ & & ME & \underset{K_4}{\rightleftharpoons} & MES \longrightarrow ME + P \end{array}$$

where S is the substrate, M is the modifier, K_1–K_4 are the dissociation constants describing each step, k_5 and k_6 are the rate constants for the breakdown of the ES and MES complexes, respectively. Since this enzyme exhibits no activity in the absence of Mg^{2+}, k_5 is virtually zero. The general form of equation for the above mechanism derived by Frieden[11] is simplified as follows:

$$\frac{1}{v} = \frac{1 + K_3/M}{V} + \frac{K_4(1 + K_2/M)}{V \cdot S}$$

where V represents the maximum velocity. K_2 and K_3 can be estimated from the horizontal intercepts, and K_4 from the vertical intercepts, of the secondary plots (Fig. 4b). By this procedure, K_2, K_3 and K_4 were estimated to be 0.83, 4.0 and 3.1 mM, respectively. Since K_2 is much smaller than K_3, the affinity of Mg^{2+} for the free enzyme is larger than that for the enzyme–substrate complex. These kinetic constants, however, are valid only when the substrate is $MgPP_i^{2-}$ and Mg_2PP_i exhibits practically no effect on the initial velocity of the enzyme. It is impossible to control the concentrations of Mg_2PP_i and Mg^{2+} keeping the concentrations of $MgPP_i^{2-}$ in low levels. Therefore, the kinetic analysis on the hydrolysis of Mg_2PP_i could not be studied.

Inactivation of inorganic pyrophosphatase by preincubation with salts

The inorganic pyrophosphatase of *V. alginolyticus* is very unstable in the presence of salts. Table II indicates the effect of salts on the inactivation of the

TABLE II

EFFECT OF SALTS ON THE INACTIVATION OF INORGANIC PYROPHOSPHATASE BY PREINCUBATION

The preincubation mixture (0.5 ml) containing 10 mM Tris–BICINE buffer (pH 8.7), 3.8 μg of the purified enzyme and 100 mM each of salts described below was incubated for 5 min at 37 °C. After that, 50 μl of the mixture was assayed under the standard condition. The activity was expressed as percent of that without preincubation. Since the enzyme solution was diluted 30-fold in the assay mixture, the effect of salts introduced with the enzyme solution was neglected.

	Salt added								
	None	*LiCl*	*NaCl*	*KCl*	*RbCl*	*$NaNO_3$*	*NaBr*	*NaSCN*	*$NaClO_4$*
Relative activity	100	43	40	35	41	23	19	4	3

purified enzyme by preincubation for 5 min at 37 °C. The extent of inactivation was not affected by the species of monovalent cations, but was greatly influenced by the species of monovalent anions. Chaotropic anions such as SCN^- and ClO_4^- strongly inactivated the enzyme during preincubation, the order of effectiveness being ClO_4^-, $SCN^- > Br^- \geqslant NO_3^- > Cl^-$. Among them, Cl^- was the least effective for the enzyme inactivation.

Fig. 5a indicates the enzyme inactivation during preincubation in the presence of several concentrations of NaCl. The time-course of inactivation was a single first-

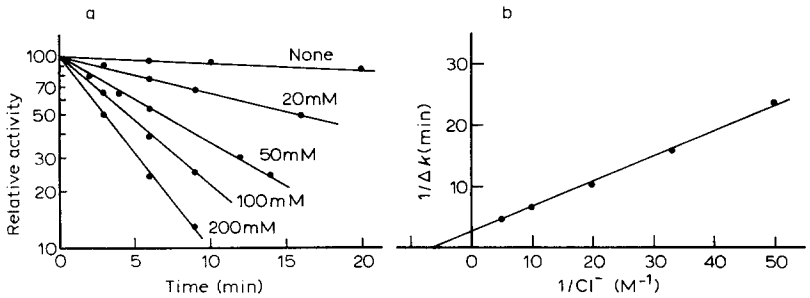

Fig. 5. Effect of NaCl on the enzyme inactivation. (a). Inactivation rate curves in the presence and absence of different concentrations of NaCl. (b). $1/\Delta k$ *vs* $1/Cl^-$ plot.

order reaction and the rate constant was dependent upon the NaCl concentrations. The mechanism of inactivation by salts can be written as follows:

$$\begin{array}{ccc} E & \overset{K_A}{\rightleftharpoons} & EA \\ \downarrow k_0 & & \downarrow k_a \\ D & & D \end{array}$$

where E is the enzyme, A is the anion, EA is the binary complex of the enzyme and anion, D is the inactive denatured form of the enzyme, K_A is the dissociation constant for A, k_0 and k_a are the first-order rate constants with which E and EA are inactivated. By similar procedures as described by Südi[12], the following equation can be derived:

$$\frac{1}{\Delta k} = \frac{1}{k_a - k_0} + \frac{K_A}{k_a - k_0} \cdot \frac{1}{A}$$

where Δk is the difference between the first-order rate constants of inactivation measured in the presence and absence of anion. Fig. 5b indicates the double-reciprocal plot ($1/\Delta k$ *vs* $1/Cl^-$), which is linear and gives the kinetic constants, $k_a = 0.379\ min^{-1}$ and $K_A = 150$ mM. Since k_0 is $0.009\ min^{-1}$, the first-order rate constant for the degradation of enzyme at 37 °C was increased 42-fold by the presence of saturated concentrations of Cl^-.

Effect of PP_i and Mg^{2+} on the inactivation of pyrophosphatase

Table III indicates the effect of PP_i and Mg^{2+} on the enzyme inactivation in the presence or absence of NaCl. The concentration of each ion species in the preincubation mixture is indicated in the table. The absence of NaCl, PP_i^{4-} and Mg^{2+} slightyl enhanced the inactivation. The presence of NaCl, PP_i^{4-} promoted, but Mg^{2+} pre-

TABLE III

EFFECT OF PP_i AND Mg^{2+} ON THE INACTIVATION OF PYROPHOSPHATASE IN THE PRESENCE OR ABSENCE OF NaCl

The preincubation mixture contained 1.00 mM Na_4PP_i and $MgCl_2$ to give the concentration of each ion species indicated in the table in the presence or absence of 100 mM NaCl. The other details are the same as described in Table II.

Substance added (mM)	*Concentration of ion species (mM)*				*Relative activity*	
	PP_i^{4-}	$MgPP_i^{2-}$	Mg_2PP_i	Mg^{2+}	*Without NaCl*	*With NaCl*
None	0	0	0	0	96	47
$MgCl_2$ (1.00)	0	0	0	1.00	83	54
$MgCl_2$ (10.0)	0	0	0	10.0	54	59
Na_4PP_i	1.00	0	0	0	72	29
Na_4PP_i + $MgCl_2$ (1.00)	0.06	0.93	0.01	0.05	96	42
Na_4PP_i + $MgCl_2$ (3.31)*	0	0.69	0.31	2.00	100	96

* In this case, the hydrolysis of PP_i proceeded during the preincubation. Therefore, the enzyme blank was incubated simultaneously with the standard assay and the activity was corrected for the enzyme blank.

vented, the inactivation due to NaCl. $MgPP_i^{2-}$ showed no significant effect with or without NaCl. On the other hand, the presence of both $MgPP_i^{2-}$ and Mg^{2+} almost completely prevented the inactivation by NaCl. These results indicate that the enzyme is protected from salt inactivation only when the substrate and the cofactor are coexistent, either one of them being ineffective separately. The presence of both ion species inevitably produces Mg_2PP_i in the preincubation mixture.

Inhibition of pyrophosphatase by salts

Table IV indicates the effect of salts on the enzyme activity. As described above, the enzyme is not inactivated by salts in the presence of $MgPP_i^{2-}$ and Mg^{2+}. The hydrolysis of PP_i proceeded linearly with time in the presence of salts. Thus, it is apparent that the decrease in activity observed by salts is not caused by the enzyme inactivation during the assay. The inhibition by salts was dependent upon

TABLE IV

EFFECT OF SALTS ON THE ENZYME ACTIVITY

The inhibitory effect of salts was assayed in the reaction mixture containing 1.0 mM Na_4PP_i, 3.3 mM $MgCl_2$, 50 mM Tris–BICINE buffer (pH 8.7), enzyme and salt described below. The concentration of each ion species in the reaction mixture is as follows: $MgPP_i^{2-}$, 0.69 mM; Mg^{2+} 2.0 mM; and Mg_2PP_i, 0.31 mM. The activity was expressed in percent of that without added salts.

Salt added	*Activity in the presence of*			*Reaction order* (n)*
	50 mM	*100 mM*	*200 mM*	
LiCl	60	30	11	1.83
NaCl	78	53	26	1.67
KCl	81	58	33	1.60
RbCl	81	54	36	1.47
NaBr	74	52	27	1.51
$NaNO_3$	74	49	26	1.57
$NaClO_4$	77	45	21	1.85

* See text.

their concentrations and no significant difference was observed among the salts except for the case of LiCl. Even $NaClO_4$, which causes strong inactivation (see Table II), exhibited the same extent of inhibition as NaCl. Therefore, salt inhibition may be considered to be independent of the species of monovalent cations and anions.

Fig. 6 indicates the effect of NaCl on the enzyme kinetics at a fixed concentration of Mg^{2+}. NaCl decreased the apparent V without affecting the K_m for $MgPP_i^{2-}$. If NaCl competes with Mg^{2+} at a site of Mg^{2+} binding, the apparent K_m for $MgPP_i^{2-}$

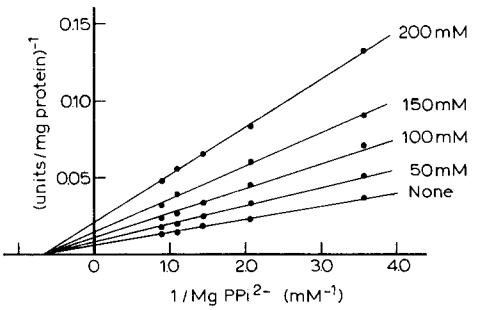

Fig. 6. Effect of NaCl on the enzyme activity. The double reciprocal plots ($1/v$ *vs* $1/MgPP_i^{2-}$) in the presence and absence of NaCl. The concentration of Mg^{2+} was fixed at 2.00 mM.

will be expected to be varied by NaCl from the results of Fig. 4. Therefore, it is unlikely that NaCl inhibits the activity by competing with $MgPP_i^{2-}$ or Mg^{2+} at their respective binding site. The secondary plot of the vertical intercepts against the concentrations of NaCl exhibited a parabolic non-competitive inhibition. When the type of inhibition by salts is non-competitive, the reaction order of each salt can be obtained from the equation:

$$\log[(v_0 - v_i)/v_i] = n \log A - \log K$$

where v_0 and v_i are the reaction velocities in the absence and presence of salt, respectively, A is the concentration of salt and K is the apparent dissociation constant for the enzyme–salt complex. The plots of $\log[(v_0 - v_i)/v_i]$ *versus* $\log A$ were linear and the reaction order (n) for each salt was obtained from each slope. As indicated in Table IV, the n values are well over 1.0 and close to 2.0 with each salt.

TABLE V

EFFECT OF PP_i AND Mg^{2+} ON THE INHIBITION BY NaCl

The reaction mixture containing 0.4 mM Na_4PP_i and $MgCl_2$ to give the concentrations of each ion species described below was assayed in the presence and absence of 100 mM NaCl. The activity in the presence of NaCl was expressed as a per cent of that in its absence. For the activity without added NaCl, refer to Fig. 3.

Concentration of ion species (mM)			*Activity (%)*
$MgPP_i^{2-}$	Mg_2PP_i	Mg^{2+}	
0.33	0.07	1.01	32
0.28	0.12	2.00	49
0.24	0.16	3.00	59
0.13	0.27	9.33	84
0.077	0.323	19.27	85
0.042	0.358	39.24	86

Effect of PP_i and Mg^{2+} on the inhibitory effect of NaCl

Table V indicates the effect of PP_i and Mg^{2+} on the inhibitory effect of NaCl. The inhibitory effect of NaCl was decreased by increasing Mg^{2+}. When the experiments were carried out by using 1.00 mM Na_4PP_i, similar results as in Table V were obtained. These results indicate that this enzyme is not ,ühibited in the presence of high concentrations of Mg^{2+} or under the conditions where Mg_2PP_i predominteas over $MgPP_i^{2-}$.

DISCUSSION

Inorganic pyrophosphatase purified from a slightly halophilic marine *V. alginolyticus* exhibits an absolute requirement for free Mg^{2+}. The kinetic results obtained were well explained by a model where $MgPP_i^{2-}$ is a true substrate and Mg^{2+} is an essential cofactor. Therefore, it is apparent that Mg^{2+} plays two roles in this enzyme. The importance of free Mg^{2+} in the activity of inorganic pyrophosphatase has been reported for the enzymes isolated from yeast[13–15], and spinach chloroplasts[16]. With the enzymes from yeast[13], human erythrocytes[17] and from spinach chloroplasts[18], Mg_2PP_i has been reported to be hydrolyzed, although at a low rate. Since this enzyme also hydrolyzes PP_i under the conditions where Mg_2PP_i predominates over $MgPP_i^{2-}$, Mg_2PP_i is possible to act as a substrate.

The enzyme from *V. alginolyticus* was found to be inactivated and inhibited by salts. The inactivation was dependent upon the species of monovalent anions, whereas the inhibition was non-specific to the species of monovalent cations and anions. Therefore, the mechanisms of inactivation and inhibition by salts are quite different from each other. The inactivation kinetics were well explained by a model that anions promote inactivation by binding to the enzyme. We previously demonstrated that the enzymes from a marine *V. alginolyticus* are sensitive to monovalent anion[1–4], suggesting the importance of hydrophobic interactions in the structure of these enzymes[4]. The strong inactivation caused by chaotropic anions such as SCN^- and ClO_4^- may be due to the effect on the hydrophobic interactions in the enzyme molecule. On the other hand, the inhibitory effect of salts is not influenced by the ion species and salts exhibit a parabolic non-competitive inhibition. From the kinetic data, it was suggested that salts interact with the enzyme at the two sites per active site to inhibit the activity.

We previously measured the intracellular concentrations of Na^+, K^+ and Cl^- in *V. alginolyticus*, which amount to 430, 420 and 240 mM, respectively, in the cells of exponential phase of growth[7]. Therefore, the enzyme of this organism might be inactivated and inhibited by these intracellular ions. However, since the intracellular Mg^{2+} concentration of *V. alginolyticus* is well over 20 mM (ref. 7), the inhibition by the intracellular monovalent ions may be effectively prevented. The inactivation may also be prevented if the PP_i concentration in the cells is high enough.

ACKNOWLEDGEMENTS

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