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pH- AND ANION-DEPENDENT SALT MODIFICATIONS OF ALKALINE PHOSPHATASE FROM A SLIGHTLY HALOPHILIC *VIBRIO ALGINOLYTICUS*

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SUMMARY

1. The kinetics of the salt modification on the alkaline phosphatase from a slightly halophilic marine *Vibrio alginolyticus* was greatly influenced by pH. The pH-dependent variations in the type of salt modification were explained by the mechanism of single substrate–single modifier case described by Frieden.

2. At pH 7.5, the activity was affected by the species of anions and more chaotropic anions decreased the apparent K_m for *p*-nitrophenyl phosphate more effectively. But when compared in V/K_m, Cl^- was the most effective. The enzyme was not activated by SO_4^{2-}, which acted as a linear competitive inhibitor for the action of Cl^-. This anion dependence, however, was not observed at pH 10.3.

3. On the other hand, alkaline phosphatase from *Escherichia coli* showed no anion dependence and the type of salt modification was unaffected by pH.

4. It was suggested that the enzymes from halophilic bacteria are sensitive to anions and that the hydrophobic interactions in the enzyme molecules are more important than in those from non-halophiles, for the manifestation of their activities.

INTRODUCTION

Earlier studies in this laboratory have demonstrated that periplasmic 2′,3′-cyclic phosphodiesterase (3′-nucleotidase)[1,2] and membrane-bound 5′-nucleotidase[3] of a slightly halophilic marine *Vibrio alginolyticus* are modified by monovalent anions and that the order of effectiveness in activating 3′-nucleotidase and 5′-nucleotidase follows a lyotropic series: $Cl^- > Br^- > I^- > NO_3^-$. Since the corresponding enzymes from a non-halophilic *Escherichia coli* showed no response to monovalent anions, anion specificity was considered to be characteristic of the enzymes from marine bacteria. Recently we found that the alkaline phosphatase from *V. alginolyticus* is also affected by monovalent anions and that the type of salt modification varies depending upon pH. Alkaline phosphatases from *E. coli*[4,5] and animals[6] have been

reported to be activated by increasing ionic strength. To compare the effect of salts on the alkaline phosphatases isolated from non-halophilic and slightly halophilic bacteria, we studied the effect of salts on the enzyme from *V. alginolyticus* together with that from *E. coli*.

MATERIALS AND METHODS

Materials

p-Nitrophenyl phosphate was recrystallized from 87% ethanol, which contained less than 0.2% of inorganic phosphate. Alkaline phosphatase from *E. coli* (Type III) was purchased from Sigma, and $(NH_4)_2SO_4$ in the enzyme sample was removed by gel filtration before use. All other reagents were analytical grade.

Bacterial growth

To induce the formation of alkaline phosphatase, *V. alginolyticus* 138-2 was grown in a medium containing 0.2% Polypepton (Daigo-eiyo), 10 mM K_2SO_4, 5 mM $MgSO_4$, 0.2% glucose, 0.2% glutamate, 20 mM Tris–HCl (pH 7.4) and 3.0% NaCl. This medium contained 0.16 mM inorganic phosphate. The cells were harvested from a 10-l culture at the logarithmic phase of growth by centrifugation at 10 000 × *g* for 10 min and were washed twice with 1.0 M NaCl containing 50 mM Tris–HCl (pH 7.4).

Purification of alkaline phosphatase from V. alginolyticus

All procedures were performed at 0–4 °C and all the solutions used for enzyme purifications contained 10 mM Tris–HCl (pH 7.4) unless otherwise indicated.

Alkaline phosphatase was released from the cells by the osmotic shock procedure as described previously[1], except that the washed cells were first suspended in a medium containing 1.0 M NaCl, 0.5 M sucrose and 50 mM Tris–HCl (pH 7.4). The osmotic shock fluid thus obtained was concentrated by ultrafiltration in the Amicon apparatus fitted with a type G-20 T membrane (Nippon Shinkuu Polymer) and salts were removed by gel filtration. This fraction (I) was applied to DEAE-cellulose column (1.5 cm × 12 cm). The column was eluted by a linear gradient from 0.01 to 0.5 M NaCl in a total volume of 320 ml. The active fractions were eluted at a NaCl concentration of about 0.15 M. They were combined, concentrated and desalted by ultrafiltration. This fraction (II) was then applied to a DEAE-Sephadex column (0.9 cm × 3.5 cm), which had been equilibrated with 0.05 M NaCl. A linear NaCl gradient elution from 0.05 to 0.5 M in a total volume of 55 ml was carried out. The active fractions were eluted at about 0.2 M NaCl, which were combined and concentrated (Fraction III). This was further treated with Sephadex G-100 (2 cm × 40 cm). Results on the purification are summarized in Table I. As calculated from the crude extracts of the cells, the enzyme was purified 400-fold.

Enzyme assay

The hydrolytic activity of the enzyme was assayed at 37 °C by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate through the change in absorbance at 405 nm, in a Hitachi two-wavelength double-beam spectrophotometer 356 equipped with a thermostatically controlled chamber, using a 0.01 or 0.03 ab-

TABLE I

PURIFICATION OF ALKALINE PHOSPHATASE FROM *V. alginolyticus*

Enzyme activity was assayed in a reaction mixture containing 1.0 mM *p*-nitrophenyl phosphate, 0.2 M NaCl and 20 mM cyclohexylaminopropanesulfonic acid–NaOH (pH 9.5). Crude cell extracts prepared by osmotic lysis had a specific activity of 0.85 unit/mg protein.

Fraction	*Volume (ml)*	*Protein*		*Enzyme activity*	
		mg/ml	*mg*	*(units/ml)*	*(units/mg protein)*
I. Osmotic shock fluids, conc.	110	1.19	131	5.71	4.80
II. DEAE-cellulose fraction, conc.	16	0.58	9.3	20.0	34.5
III. DEAE-Sephadex fraction, conc.	2.5	1.32	3.3	89.9	68.1
IV. Sephadex G-100 fraction	10.8	0.032	0.35	10.9	340

sorbance scale. The reaction was started by adding 0.02–0.04 ml of enzyme solution in a total volume of 3.0 ml. The enzyme was diluted with 5 mM Tris–acetate (pH 7.4) containing 50 μg bovine albumin per ml before use. Buffers used were 20 mM veronal–HCl between pH 6.5 and 9.0 and 20 mM cyclohexylaminopropanesulfonic acid–NaOH between pH 9.0 and 10.3.

The molar extinction coefficient of *p*-nitrophenol was measured at each pH and the enzyme activity was expressed in μmoles of the substrate hydrolyzed in 1 min.

Salt solutions used were adjusted to the requisite pH before use. Protein was determined by the method of Lowry *et al.*[7] using bovine albumin as a standard.

RESULTS

Effect of pH on the salt modification of alkaline phosphatase from V. alginolyticus

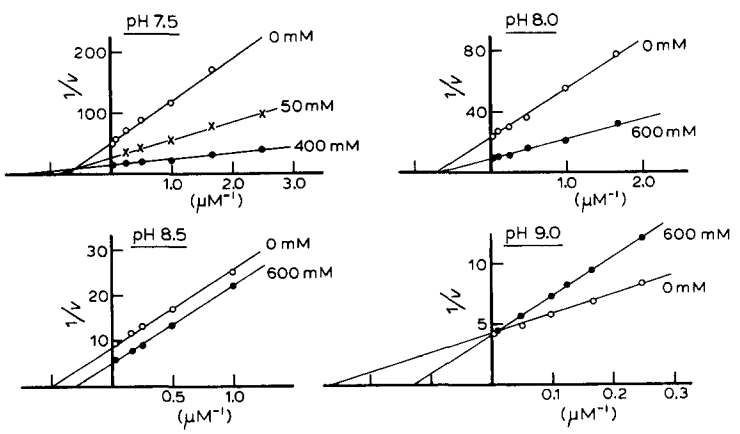

Fig. 1. The double reciprocal plots ($1/v$ *versus* $1/S$) in the presence and absence of NaCl with *p*-nitrophenyl phosphate as substrate using 20 mM veronal–HCl buffers. The concentrations of NaCl and pH were indicated in the figure. Initial velocity (v) was expressed in mmoles of the substrate hydrolyzed per min per mg protein. Note the difference in the scales on each graph.

Alkaline phosphatase from *V. alginolyticus* catalyzes a transphosphorylation reaction[8], which is also activated by the addition of salts. In these experiments, the effect of salts on the hydrolytic activity was examined in the absence of any phosphate acceptors, to avoid complications.

Fig. 1 indicates the effect of NaCl on the kinetics of the *p*-nitrophenyl phosphate hydrolysis at several pH values. The double reciprocal plots ($1/v$ *versus* $1/s$) in the presence and absence of NaCl were linear at all pH values examined. The linear plots obtained by varying the concentrations of NaCl had a common point, except in the case of pH 8.5. An example is indicated in the case of pH 7.5. A point of intersection, however, varied depending upon pH. At pH 7.5 and below, the reciprocal plots in the presence and absence of NaCl crossed at the negative substrate concentrations. Thus, the apparent K_m for the substrate was decreased and V was increased by increasing NaCl concentrations. At pH 8.0, NaCl increased V without appreciably affecting K_m. On the other hand, the reciprocal plots in the presence and absence of NaCl were almost parallel with each other at pH 8.5, where NaCl increased both K_m and V. At pH 9.0, the reciprocal plots crossed close to the $1/v$ axis. On close inspection of the results obtained between pH 9.0 and 10.3, the intersection was found to be at the positive substrate concentrations. Thus, NaCl increased K_m with only a slight increase in V at pH 9.0 and above, and the initial velocity was inhibited by NaCl at substrate concentrations below those of the intersection. These results clearly indicated that the kinetics of the NaCl modification are greatly influenced by pH. Although not shown here, the reciprocal plot over the entire range of substrate concentrations gave two intersecting lines at pH 7.5 in the absence, but not in the presence, of NaCl and the change in slope occurred at about 0.01 mM.

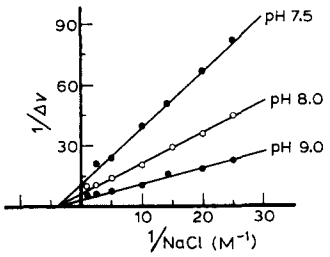

Fig. 2. Effect of NaCl on the enzyme activity at different pH values. The substrate concentration was 1.0 mM. Δv is the velocity corrected for the velocity without added NaCl and was expressed in mmoles/min per mg protein.

Fig. 2 indicates the effect of NaCl on the activity at different pH values in the presence of a saturated concentration of the substrate. The activation by NaCl was saturated at about 400 mM with a slight inhibition at high concentrations. The apparent K_m obtained from Fig. 2 was 230 mM and it was unaffected by pH.

Frieden[9] presented the useful method for the interpretation of the kinetic data in the single substrate–single modifier case. The modifications of the activity may be explained in the mechanism,

$$E + S \rightleftharpoons ES \quad ES \xrightarrow{k_5} E + P \tag{1}$$

$$E + M \rightleftharpoons EM \tag{2}$$

$$ES + M \rightleftharpoons EMS \quad EMS \overset{k_6}{\rightarrow} EM + P \tag{3}$$

$$EM + S \rightleftharpoons EMS \tag{4}$$

where S is the substrate and M is the modifier. The general form of the equation for the above mechanism derived by rapid equilibrium treatment is as follows:

$$\frac{v_0}{(E)_0} = \frac{k_5 \, (1 + k_6 \, M/k_5 \, K_3) \, / \, (1 + M/K_3)}{(1 + K_1/S) \, [(1 + M/K_2) \, / \, (1 + M/K_3)]}$$

where K_1–K_4 are the dissociation constants describing Steps 1–4, and k_5 and k_6 are the rate constants for the breakdown of the ES and EMS complex, respectively.

TABLE II

SUMMARY OF KINETIC CONSTANTS AT DIFFERENT pH

pH	K_1 *(μM)*	K_2 *(M)*	K_3 *(M)*	K_4 *(μM)*	k_5 *(units/mg protein)*	k_6 *(units/mg protein)*	*Case*
7.5	1.29	0.44	0.23	0.67	19.2	66.7	$K_1/K_4 > k_5/k_6$, $K_1 > K_4$
8.0	1.39	0.22	0.23	1.43	43.3	105	$K_1/K_4 > k_5/k_6$, $K_1 \approx K_4$
8.5	2.00	0.14	0.23	3.23	116	192	$K_1/K_4 \approx k_5/k_6$, $K_1 < K_4$
9.0	3.70	0.11	0.23	8.00	234	239	$K_1/K_4 < k_5/k_6$, $K_1 < K_4$

When the mechanism is applied to the present results, the values of the kinetic constants are obtained from Figs 1 and 2 (see Table II). The value of K_2 was calculated from the relationship that in this mechanism, $K_1/K_4 = K_2/K_3$. It is apparent that the kinetic constants except for K_3 are greatly influenced by pH. The ratio of the Michaelis constant for the substrate in the absence of the saturated concentration of the modifier to that in its presence, *i.e.* K_1/K_4, increased with decreasing pH. On the other hand, the ratio of rate constant for the breakdown of ES to that of EMS, *i.e.* k_5/k_6, decreased with decreasing pH. The dissociation constant for NaCl to the free enzyme (K_2) decreased with increasing pH, whereas that to the ES complex (K_3) was unaffected by pH, indicating that the binding of the substrate to the enzyme makes the affinity of NaCl independent of pH. In the last column of Table II, the relationships between these kinetic constants are indicated. From these, pH-dependent variations in the type of NaCl modification may well be explained from changes in the kinetic constants, due to pH. Since the type of NaCl modification below pH 7.5 and above pH 9.0 is identical to that at pH 7.5 and at pH 9.0, respectively, the variations in the type of salt modification were observed only between pH 7.5 and 9.0.

Ion specificity and effect of anions on the kinetic constants

Since the modification of the activity by NaCl was markedly influenced by pH, the effect of salt was examined at both neutral and alkaline pH values. Although the optimum pH of this enzyme is 10.3, an activating effect of salt was manifested,

especially at neutral pH. Chloride salts of monovalent cations such as Li^+, Na^+, K^+ and Rb^+ could be substituted for one another except that, at pH 10.3, the presence of more than 0.2 M Li^+ caused enzyme inactivation.

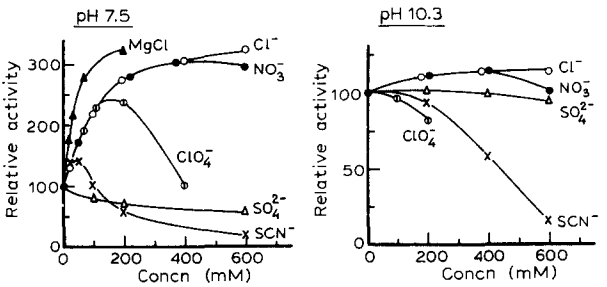

Fig. 3. Effects of several salts on the enzyme activity at pH 7.5 and 10.3. The substrate concentration was 1.0 mM at pH 7.5 and 2.0 mM at pH 10.3. Sodium salts of anions indicated in the figure were used. A value of 100 was assigned to the activity in the absence of salts which corresponded to 19.9 and 790 units/mg protein at pH 7.5 and 10.3, respectively.

Fig. 3 indicates the effect of several salts on the activity at pH 7.5 and 10.3 At pH 7.5, the enzyme was activated to the same extent by the sodium salts of monovalent anions within the salt concentrations exhibiting no inhibition. However, chaotropic anions such as ClO_4^- and SCN^- became inhibitory at high concentrations. The presence of above 0.4 M ClO_4^- inactivated the enzyme during assay. Therefore, the extent of enzyme activation was affected by the species of monovalent anions, the order of effectiveness in activation being $Cl^- \geqslant NO_3^- > ClO_4^- > SCN^-$. However, SO_4^{2-} showed no activation even at low concentrations. On the other hand, activation by monovalent anions was only slight at pH 10.3 and the inhibitory effect by SCN^- was also less than that at pH 7.5. SO_4^{2-} showed no significant effect on the activity and the presence of above 0.2 M ClO_4^- caused the enzyme inactivation.

On a molar basis, $MgCl_2$ was much more effective than NaCl at pH 7.5, but when compared with the ionic strength, both NaCl and $MgCl_2$ activated the enzyme to the same extent, indicating no particular effect of divalent cations on the activity.

Table III indicates the effect of anions on apparent K_m and V. Monovalent anions decreased K_m at pH 7.5, the order of effectiveness being $Cl^- < NO_3^- < SCN^-$. On the contrary, these anions increased K_m at pH 10.3 and only a slight difference was observed in their effectiveness. SO_4^{2-} increased K_m at both pH values. Since SO_4^{2-} is the most lyotropic among the anions examined, the effectiveness in decreasing K_m follows a chaotropic series of the anions at pH 7.5.

The effect of anions on V, however, could not be explained from their chaotropic series. At pH 7.5, V was increased by Cl^- and NO_3^-, but was decreased by SCN^- and SO_4^{2-}. These anions exhibited only a slight effect on V at pH 10.3. Since SCN^- decreases K_m at pH 7.5, the inhibitory effect of SCN^- is due to the inhibition of the rate of breakdown of the enzyme–substrate complex.

When the effects of anions were compared in V/K_m, which corresponds to the first-order rate constant at substrate concentrations sufficiently below the K_m, monovalent anions increased, but SO_4^{2-} decreased, V/K_m at pH 7.5. The order of effec-

TABLE III

EFFECT OF ANIONS ON APPARENT K_m AND V AT pH 7.5 AND 10.3

The salt concentration added was 0.2 M except that 66.7 mM Na_2SO_4 was used to adjust the ionic strength equivalent to that of the mono-monovalent salts. With 0.2 M Na_2SO_4, the effects on K_m and V were more evident than with 66.7 mM.

Salt added	*pH 7.5*			*pH 10.3*		
	K_m* *(μM)*	V* *(units/mg)*	V/K_m	K_m* *(μM)*	V* *(units/mg)*	V/K_m
None	1.23 ± 0.16	20.4 ± 1.95	16.6	227 ± 20.3	943 ± 34.2	4.15
NaCl	0.62 ± 0.08	59.8 ± 2.96	96.5	288 ± 23.6	1050 ± 46.5	3.65
$NaNO_3$	0.59 ± 0.03	56.9 ± 0.80	96.4	374 ± 31.7	1025 ± 41.7	2.74
NaSCN	0.23 ± 0.02	8.52 ± 0.54	37.0	418 ± 24.8	931 ± 30.4	2.22
Na_2SO_4	1.56 ± 0.10	13.5 ± 0.92	8.65	345 ± 12.0	949 ± 19.5	2.75

* Mean ± S.D. ($n = 4$).

tiveness in increasing V/K_m is as follows: $Cl^- \geqslant NO_3^- > SCN^- >$ no salt $> SO_4^{2-}$. These anions, however, decreased V/K_m at pH 10.3. From these, it is apparent that the effect of monovalent anions is manifested especially at neutral pH and that Cl^- is the most effective among monovalent anions for the activation.

SO_4^{2-} showed no activating effect on the enzyme and when compared with Cl^-, it inversely modified K_m and V at pH 7.5. Thus the effect of SO_4^{2-} on the activation due to Cl^- was examined. As indicated in Fig. 4, SO_4^{2-} acted competitively against Cl^- in a simple linear relationship. The inhibitor constant for SO_4^{2-} was 93 mM. Therefore, it is possible to consider that SO_4^{2-} affects the enzyme activity by acting at a site where Cl^- interacts, thereby preventing both the association of the substrate to the enzyme and the rate of breakdown of the enzyme-substrate complex.

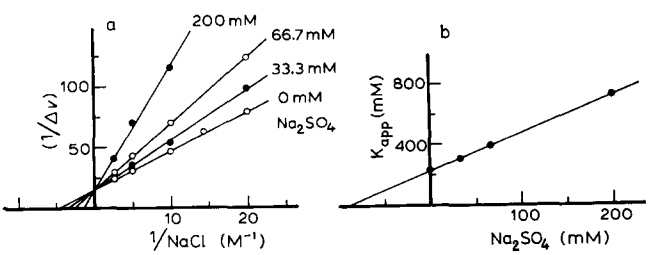

Fig. 4. Effect of Na_2SO_4 on the activating effect of NaCl. (a) The reaction was carried out in the presence or absence of Na_2SO_4 indicated in the figure, using 1.0 mM substrate and 20 mM veronal–HCl (pH 7.5). The expression of Δv is the same as in Fig. 2. (b) Apparent $K_m(K_{app})$ for NaCl obtained from Fig. (a) was plotted against Na_2SO_4 concentrations.

Comparison with alkaline phosphatase from E. coli

Fig. 5 indicates the effects of NaCl on the kinetics of the enzyme from *E. coli* at different pH values. The double reciprocal plots in the absence of NaCl gave two intersecting lines and the change in slope was obscured by the presence of NaCl. Such a substrate activation has been reported by Heppel *et al.*[4] and by Simpson and Vallee[10]. As mentioned earlier, the substrate activation was also observed with the

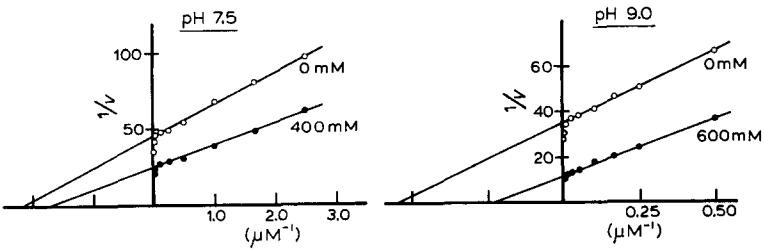

Fig. 5. $1/v$ *versus* $1/S$ plots in the presence and absence of NaCl at pH 7.5 and 9.0 for the enzyme from *E. coli*. The concentrations of NaCl and pH were indicated in the figure. The expression of v is the same as in Fig. 1.

enzyme from *V. alginolyticus* at pH 7.5 in the absence of NaCl. In contrast to the case of *V. alginolyticus*, the addition of NaCl increased both K_m and V between pH 7.5 and 10.3 and the type of salt modification was unaffected by pH (see also Fig. 6). But NaCl decreased V without appreciably affecting K_m at pH 6.5.

With respect to anion specificity, the addition of 0.6 M each of the sodium salt of Cl^-, NO_3^- and SO_4^{2-}, respectively, increased the activity 1.8-fold and ClO_4^-, 1.5-fold, at pH 7.5. The activity was inhibited 25% by 0.6 M NaSCN. At pH 10.3, 0.6 M each of these salts increased the activity 1.7-fold. Thus no significant anion specificity was observed for the activation of the *E. coli* enzyme except for the slight inhibition by SCN^- at neutral pH. Monovalent cations such as Li^+, Na^+, K^+ and Rb^+ could be substituted for one another and Li^+ showed no inhibition even at pH 10.3. It has been reported that the *E. coli* enzyme is nonspecifically activated by salts and that the activation is caused by their ionic strength[4,5].

Fig. 6 indicates the comparison between the two enzymes for the effect of NaCl on K_m and V/K_m at various pH values. In the case of *V. alginolyticus*, the addition

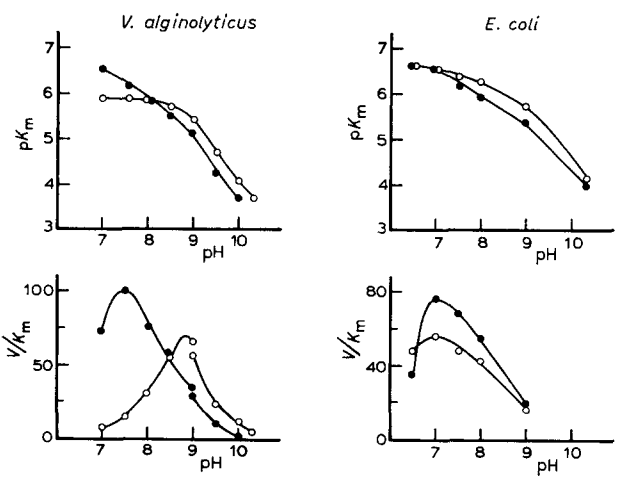

Fig. 6. Comparison between the enzymes from *V. alginolyticus* and *E. coli* in the effect of NaCl and pH on K_m and V/K_m. pK_m represents $-\log K_m$ in M and V, in units/mg protein. NaCl was used at the optimum concentrations, that is, 0.6 M at pH 8.0 and above, 0.4 M at pH 7.0 and 7.5 and 0.2 M at pH 6.5. ○———○, in the absence of NaCl; ●———●, in the presence of NaCl.

of NaCl decreased K_m below pH 8.0 and increased V/K_m especially at neutral pH, indicating the specific effect of NaCl in increasing the efficiency of the enzyme at neutral pH. With the enzyme from *E. coli*, NaCl increased K_m and the optimum pH for V/K_m was unaffected by the presence of NaCl.

DISCUSSION

It was shown that the kinetics of the salt modification on the alkaline phosphatase from a marine *V. alginolyticus* are greatly influenced by pH. Assuming that salt modifies the activity by binding to a site other than the active site of the enzyme, the variations in the type of salt modification were well explained by the mechanism of single substrate-single modifier case proposed by Frieden[9]. As indicated in Table II, all the kinetic constants except for K_3 were influenced by pH and the type of salt modification at a definite pH was determined by the relative values of these kinetic constants. Based on this mechanism, a remarkable activation by NaCl especially at neutral pH may be understood by considering that the substrate is more tightly bound to the enzyme in the presence of NaCl than in its absence ($K_1 > K_4$) and that k_6 is much larger than k_5. On the other hand, at alkaline pH, the presence of NaCl decreases the affinity of the substrate to the enzyme ($K_1 < K_4$) and there is no significant difference between k_5 and k_6, thus rendering NaCl ineffective for the activation. Indeed, NaCl inhibited the activity at unsaturated substrate concentrations at pH 9.0 and above. Recently, the mechanism of the reaction of *E. coli* alkaline phosphatase has been extensively studied[11-14]. Reid and Wilson[11] and Halford[13] postulated two enzyme conformations differing in the affinity to the substrate in their mechanisms and the effect of high ionic strength was considered to affect the equilibrium between, or the reaction pathway through, the two enzyme conformations. Since the mechanism used in the present experiments is a rapid equilibrium treatment, with k_5 and k_6 representing rate-determining steps, what steps in the course of the reaction are actually modified by NaCl remains unexplained.

With respect to ion specificity, monovalent cations such as Li^+, Na^+, K^+ and Rb^+ could be substituted for one another. But the activation of the enzyme was affected by the species of monovalent anions. The anionic effects on macromolecular systems have been studied by several investigators[15-19], and a wide variety of salt effects on biological systems has been found to give rise to the same sequence observed by Hofmeister[20]. Chaotropic anions such as SCN^- and ClO_4^- are considered to favor the transfer of apolar groups to water and weaken the hydrophobic interactions in the macromolecules. Therefore, it is conceivable that the alkaline phosphatase of *V. alginolyticus* is influenced by monovalent anions through the effects on the hydrophobic interactions in the enzyme molecule. More chaotropic anions decreased the apparent K_m more effectively. Thus, the binding of the substrate to the enzyme may be enhanced by weakning the hydrophobic interactions around the active site of the enzyme. But these chaotropic anions do not increase the enzyme activity. When the effect of anions was compared in V/K_m, Cl^- was the most effective. Since V/K_m corresponds to the first-order rate constant at very low concentrations of the substrate, it becomes clear that the physiological efficiency of the enzyme is most effectively increased by Cl^-. Inability of SO_4^{2-}, which is more lyotropic than Cl^-, to activate the enzyme suggested that lyotropic anions do not always activate

the enzyme and there is an optimum anion for the most effective maintenance of the enzyme structure. In fact, SO_4^{2-} acted as a competitive inhibitor for the action of Cl^-.

The fact that anion dependence is not observed at alkaline pH may be related to the ionic state of the enzyme molecule. Recently, Sluyterman and de Graaf[21] indicated that the inhibitory effects by monovalent anions are observed only on the acidic side of the optimum pH with native papain but not with succinyl-papain. Since positive charges in the molecule are much greater in neutral than in alkaline pH, it is possible to consider that the enzyme may become more susceptible to the species of anions at neutral pH.

In contrast to the case of *V. alginolyticus*, the enzyme from *E. coli* showed no anion dependence and the type of salt modification was unaffected by pH. Even SO_4^{2-} acted as an activator with this enzyme, indicating non-specific activation by salts. Thus, the enzyme from non-halophilic bacteria seems to have a structure relatively insensitive to the action of lyotropic and chaotropic anions.

Lanyi and Stevenson[22] pointed out the importance of hydrophobic interactions in the activity and stability of menadione reductase from extreme halophiles. Two mechanisms for the action of salts, namely a charge-shielding effect at low concentrations and a salting-out effect at high concentrations have been discussed in cytochrome oxidase[23] and in threonine deaminase[24]. Earlier studies in this laboratory have demonstrated the anion dependence of the activation of 3′-nucleotidase and 5′-nucleotidase from marine *V. alginolyticus*. These results suggested a possibility that the enzymes from halophilic bacteria are sensitive to anions, thereby rendering the hydrophobic interactions in the molecule more important than in the enzymes from non-halophiles, for the manifestation of their activities.

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REFERENCES

1 Unemoto, T. and Hayashi, M. (1969) *Biochim. Biophys. Acta* 171, 89–102
2 Unemoto, T., Takahashi, F. and Hayashi, M. (1969) *Biochim. Biophys. Acta* 185, 134–142
3 Hayashi, M., Unemoto, T., Kozuka, Y. and Hayashi, M. (1970) *Biochim. Biophys. Acta* 220, 244–255
4 Heppel, L. A., Harkness, D. R. and Hilmoe, R. J. (1962) *J. Biol. Chem.* 237, 841–846
5 Plocke, D. J. and Vallee, B. L. (1962) *Biochemistry* 1, 1039–1043
6 Fernley, H. N. and Walker, P. G. (1965) *Biochem. J.* 97, 95–103
7 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
8 Wilson, I. B., Dayan, J. and Cyr, K. (1964) *J. Biol. Chem.* 239, 4182–4185
9 Frieden, C. (1964) *J. Biol. Chem.* 239, 3522–3531
10 Simpson, R. T. and Vallee, B. L. (1970) *Biochemistry* 9, 953–958
11 Reid, T. W. and Wilson, I. B. (1971) *Biochemistry* 10, 380–387
12 Lazdunski, M., Petitclerc, C., Chappelet, D. and Lazdunski, C. (1971) *Eur. J. Biochem.* 20, 124–139
13 Halford, S. E. (1972) *Biochem. J.* 126, 727–738
14 Halford, S. E., Lennette, D. A. and Schlesinger, M. J. (1972) *J. Biol. Chem.* 247, 2095–2101
15 von Hippel, P. H. and Schleich, T. (1969) *Acc. Chem. Res.* 2, 257–265
16 von Hippel, P. H. and Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N. and Fasman, G. D., eds), pp. 417–574, Marcel Dekker, New York

17 Hatefi, Y. and Hanstein, W. G. (1969) *Proc. Natl. Acad. Sci. U.S.* 62, 1129–1136
18 Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 351–436, McGraw-Hill, New York
19 Dandliker, W. B. and de Saussure, V. A. (1971) in *The Chemistry of Biosurfaces* (Hair, M. L., ed.), Vol. 1, pp. 1–43, Marcel Dekker, New York
20 Hofmeister, F. (1888) *Arch. Exp. Pathol. Pharmakol.* 24, 247
21 Sluyterman, L. A. AE. and de Graaf, M. J. M. (1972) *Biochim. Biophys. Acta* 258, 554–561
22 Lanyi, J. K. and Stevenson, J. (1970) *J. Biol. Chem.* 245, 4074–4080
23 Lieberman, M. M. and Lanyi, J. K. (1971) *Biochim. Biophys. Acta* 245, 21–23
24 Lieberman, M. M. and Lanyi, J. K. (1972) *Biochemistry* 11, 211–216