THE EFFECT OF IONIC STRENGTH ON ALKALINE PHOSPHATASE

A.R. Glenn

Department of Biochemistry, University of Adelaide, Adelaide, South Australia, 5001, Australia.

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SUMMARY: The activity of purified alkaline phosphatase from B. subtilis 168 increases as the ionic strength increases. Maximal activity is found in 2.0 M $MgCl_2$ or $CaCl_2$. The change in activity is paralleled by changes in fluorescence emission, in sedimentation properties and in resistance to denaturing agents.

INTRODUCTION

It has been shown that alkaline phosphatase (E.C.3.1.3.1 orthophosphoric monoester phosphohydrolase) from Bacillus subtilis 168 is released from whole cells by high salt treatment[1,2]. Enzyme released in this manner has been purified to homogeneity and some of its properties described[2]. Despite the use of high salt solution in the purification of the enzyme, little or nothing is known about the effects of high ionic strength on the purified enzyme. In this paper it is reported that ionic strength affects not only enzyme solubility, but has profound effects on enzyme activity which are paralleled by changes in the physical properties of the enzyme and in its resistance to denaturing agents.

MATERIALS AND METHODS

Protein determinations were done by the method of Lowry, Rosebrough, Farr and Randall[3] using bovine plasma albumin as a standard. Enzyme Assay. Alkaline phosphatase activity was measured by the method described previously[2]. Purified enzyme was diluted in 10 mM tris HCl buffer pH 7.4 containing bovine serum albumin (1 mg/ml) since the enzyme undergoes dilution inactivation at low protein concentrations. Units are expressed as µM p-nitrophenol produced per min. at 30°

Enzyme Purification. Alkaline phosphatase was purified by ionic elution, dialysis and Sephadex G-100 chromatography as described previously[2].

Fluorescence spectroscopy. Fluorescence emission measurements were carried out at room temperature in a Hitachi Perkin-Elmer Model 203 spectrofluorometer.

Sedimentation. Solutions of the enzyme were centrifuged in a Spinco Model E Analytical Ultracentrifuge at 20° at 59,780 rev/min. with a bar angle of 60°. Sedimentation equilibrium was obtained at 15,220 rev/min. after 22 h.

RESULTS

Purified alkaline phosphatase became insoluble when dialysed against 10 mM tris HCl pH 7.4. Centrifugation of the dialysed enzyme at 100,000 x g for 2 hr. sedimented 95% of the activity. This activity could be solubilised by the addition of salt concentrations (e.g., KCl, $MgCl_2$, Na_2SO_4) in excess of 0.2 M. This was found to be the minimum level of salt to keep the enzyme in solution, in contrast to the purified phosphatase from Bacillus licheniformis which is soluble in low ionic strength buffers[4].

Preliminary experiments showed that increasing the molarity of the assay buffer (either diethanolamine.HCl pH 10, or tris-HCl pH 8) from 0.1 to 1.0 M gave a 20-30% stimulation in enzyme activity. Since the enzyme purification involved high concentrations of $MgCl_2$, the effect of $MgCl_2$ concentration on enzyme activity was examined. There was a ten-fold stimulation in phosphatase activity as the $MgCl_2$ concentration was increased up to 2.0 M. A similar stimulation of activity was also observed with $CaCl_2$ (Table 1). Sodium, potassium and lithium chlorides gave a 2-3 fold increase in enzyme activity and the corresponding nitrates a lower stimulation (Table 1). Increasing levels of NH_4Cl resulted

ACTIVITY OF ALKALINE PHOSPHATASE (μM/min.)

	MOLARITY							
SALT	0	0.1	0.25	0.5	0.75	1.0	1.5	2.0
$MgCl_2$	0.014	0.034	0.051	0.091	0.117	0.117	0.118	0.120
$CaCl_2$	0.012	0.032	0.064	0.084	0.156	0.150	0.18	0.18
NaCl	0.017	0.021	0.023	0.028	0.033	0.040	0.043	0.047
$NaNO_3$	0.018	0.022	0.025	0.027	0.029	0.031	0.033	0.035
KCl	0.015	0.021	0.023	0.028	0.030	0.036	0.037	0.040
KNO_3	0.020	0.023	0.026	0.027	0.029	0.030	0.030	0.031
LiCl	0.020	0.021	0.024	0.027	0.028	0.032	0.038	0.043
NH_4Cl	0.019	0.021	0.024	0.023	0.022	0.022	0.018	0.013
NH_4NO_3	0.017	0.017	0.023	0.019	0.023	0.023	0.026	0.028
Spermidine	0.011	0.013	0.018	0.016	0.0128	0.011	-	-

TABLE 1. The Effect of Salt Concentration on the Activity of Alkaline Phosphatase. Enzyme activity measured at 30° in diethanolanine HCl buffer pH 10 containing different salt concentrations.

in some inhibition of activity whereas NH_4NO_3 gave a small stimulation. The polyamine spermidine gave a 50% stimulation of activity at 250 mM but higher levels (up to 1.0 M) gave less stimulation. Experiments using NaCl over a wider concentration range showed that maximal enzyme activity was observed in 4.0 M NaCl (Fig. 1a).

The Michaelis constants of the enzyme in low (no added NaCl) and high (4.0 M NaCl) ionic strength buffer (1 M diethanolamine HCl pH 10) were determined using p-nitrophenyl phosphate as substrate over the range 0.1 to 0.005 mM. The K_m for enzyme in low ionic strength was 2.8×10^{-5} M and 5.0×10^{-5} M for enzyme in high ionic strength buffer. V_{max} was found to double as the NaCl concentration was increased to 4.0 M (Fig. 1b).

Conformational changes in proteins induced by a change in environment have been investigated using the fluorescent probe 1-anilino naphthalene 8 sulphonate (ANS). The binding of ANS to

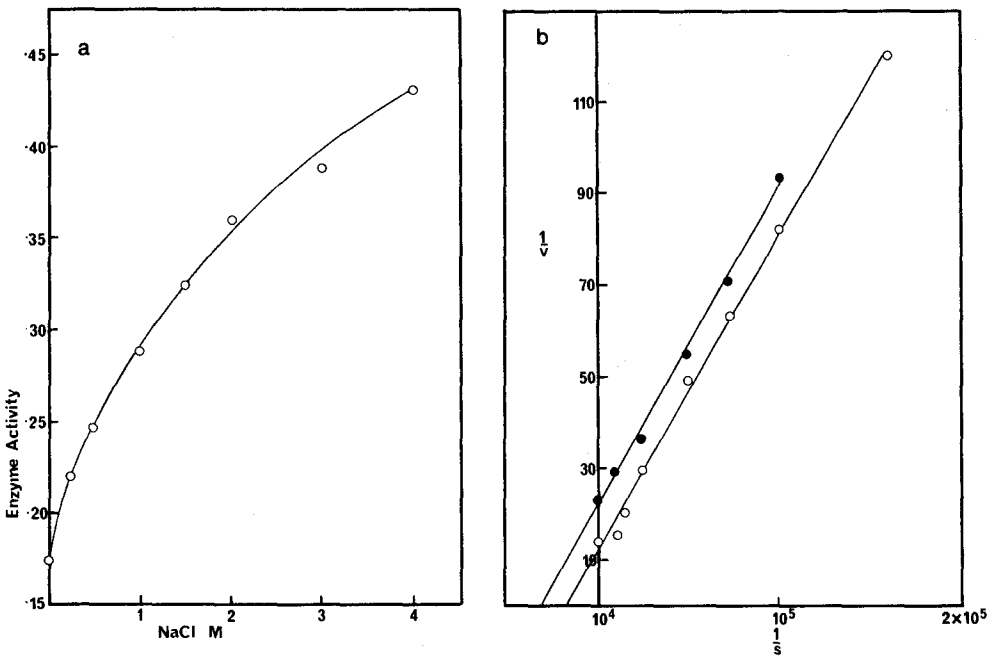

Fig. 1a. The Effect of NaCl Concentration on the Activity of Alkaline Phosphatase.

Fig. 1b. Lineweaver-Burk plot of alkaline phosphatase in "Low" (no added NaCl) and "high" (4.0 M NaCl) diethanolanine HCl buffer pH 10. Low (●); high (○).

alkaline phosphatase was accompanied by a considerable fluorescence enhancement; the excitation maximum was at 395 nm and the emission peak at 475 nm. ANS or enzyme alone under these conditions gave only a small emission (Fig.2a). Titration of the phosphatase (0.125 mg/ml) in "high" (1.0 M) and "low" (0.2 M) $MgCl_2$ with increasing amounts of fluorescent probe showed that the enzyme in "high" $MgCl_2$ gave a higher fluorescence intensity (Fig.3b). Corrections have been made for the emission of "high" and "low" salt buffers plus ANS and for the dilution caused by the addition of ANS.

Sedimentation velocity experiments carried out at different salt concentrations have revealed reproducible differences in the $S_{20}W$ values of the enzyme in different salt concentrations. Enzyme

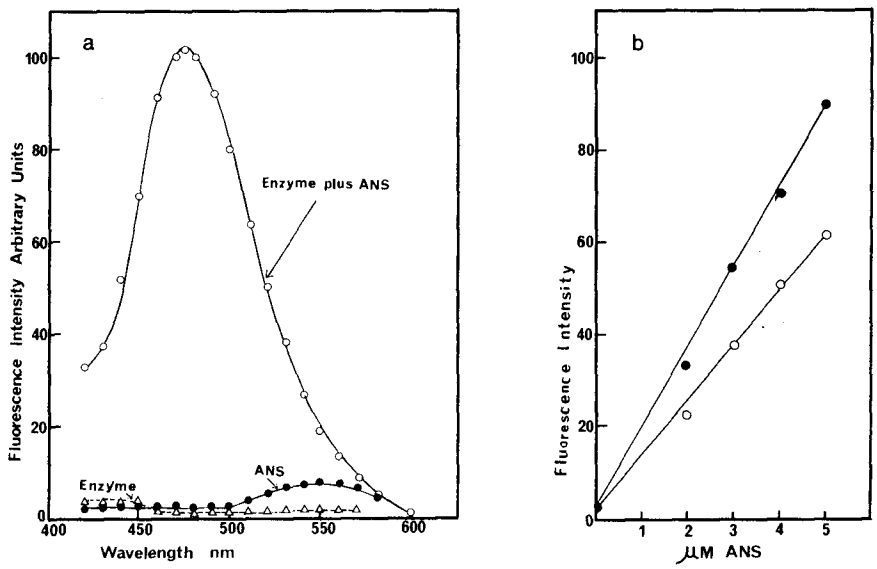

Fig. 2a. Emission spectrum of alkaline phosphatase in the presence of ANS. The complete mixture contained alkaline phosphatase (7.1 μM) ANS (10 M) in 200 mM $MgCl_2$ 10 mM tris-HCl pH 7.4. Excitation was at 295 nm for the emission spectrum. The concentration of $MgCl_2$ did not affect the emission peak.

Fig. 2b. Titration of alkaline phosphatase with ANS. Enzyme solution (0.125 mg/ml) in 0.2 M $MgCl_2$ or 1.0 M $MgCl_2$ was titrated with increasing amounts of fluorescent probe and the fluoresence intensity measured. Enzyme in 1.0 M $MgCl_2$ (●). Enzyme in 0.2 M $MgCl_2$ (○).

(5.3 mg/ml) was dialysed against either 0.2 or 0.5 M $MgCl_2$ 10mM tris-HCl pH 7.4. The $S_{20}W$ of alkaline phosphatase in 0.2 M $MgCl_2$ was 4.95S and 4.5S in 0.5 M $MgCl_2$. Sedimentation equilibrium of phosphatase (5.3 mg/ml) at 15,220 rev/min for 22 hr. in 0.2 M $MgCl_2$ tris HCl buffer gave an apparent molecular weight of 75,500 whereas in 0.5 M a molecular weight of 70,000 was recorded.

A further series of experiments were carried out to determine the effect of ionic strength on the resistance of the enzyme to denaturing agents. Trypsin (100 μg/ml) was added to solutions of alkaline phosphatase in 0.2 and 0.5 M $MgCl_2$ 100 mM tris HCl pH 7.5 at 30°.

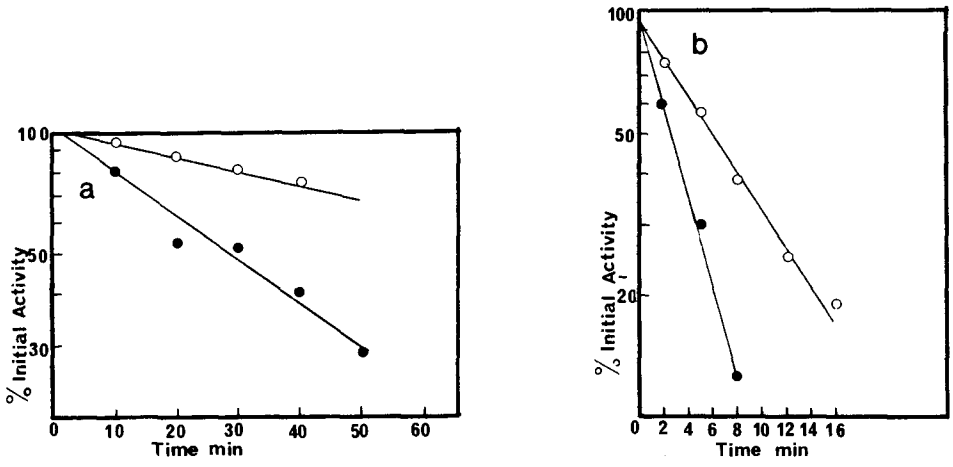

Fig. 3a. The effect of $MgCl_2$ concentration on the denaturation of alkaline phosphatase by trypsin. Enzyme in 100 mM tris-HCl buffer pH 7.5 at 30° was digested by trypsin (100 µg/ml). Enzyme activity in 0.2 M $MgCl_2$ (○); enzyme activity in 0.5 M $MgCl_2$ (●).

Fig. 3b. The effect of $MgCl_2$ concentration. The denaturation of alkaline phosphatase by SDS. SDS (0.1% final concentration) was added to alkaline phosphatase in 10 mM tris buffer pH 7.4 containing 0.2 M or 0.5 M $MgCl_2$. Enzyme activity in 0.2 M $MgCl_2$ (○) enzyme activity in 0.5 M.$MgCl_2$ (●).

Samples (0.1 ml) were removed at intervals and used to assay for residua activity. Separate control experiments showed that the activity of the trypsin preparation was not affected by the different salt concentrations. The phosphatase in 0.2 M Mg^{2+} was much more stable than the enzyme in 0.5 M $MgCl_2$ (Fig.3a). A similar but less marked difference was noted during inactivation of the enzyme by the anionic detergent sodium dodecyl sulphate (SDS). It was found that the enzyme was more rapidly denatured in 0.5 M $MgCl_2$ than in 0.2 M $MgCl_2$ (Fig.3b).

When the heat stability of the phosphatase was examined at 60° in 10 mM tris buffer pH 7.4 containing bovine plasma albumin (5 mg/ml), the half life was 2 min. There was a rapid loss of activity followed by a second phase of a slower loss of activity. The reason for this biphasic loss of activity is unknown. When $MgCl_2$ (10 mM or 100 mM) was added to the enzyme solution there was

a considerable stabilization ($t^1/2$ of 20 min and 32 min respectively), and at a concentration of 500 mM $MgCl_2$ less than 15% of the original activity was lost (Fig.4). Attempts to protect the enzyme from heat

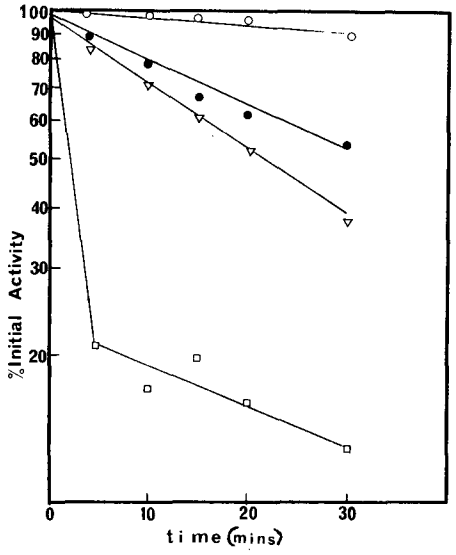

Fig. 4. The effect of $MgCl_2$ concentration on heat stability of alkaline phosphatase at 60°. Enzyme in 10 mM tris HCl pH 7.4; the protein concentration was adjusted to 5.0 mg/ml by the addition of bovine serum albumin. Samples were taken at intervals cooled in ice and assayed for residual enzyme activity. No Mg^{2+} (□), 10 mM Mg^{2+} (▽), 100 mM Mg^{2+} (●); 500 mM Mg^{2+} (○).

denaturation by ionic strength alone were completely unsuccessful, even when very high concentrations (up to 4.0 M NaCl) were used. A similar protective effect of Mg^{2+} has been reported for E.coli alkaline phosphatase[5].

DISCUSSION

The alkaline phosphatase from B. subtilis 168 is remarkably halotolerant. The highest enzyme activities are recorded in salt concentrations at which many enzymes from true halophiles are losing activity[6]. At the present time, however, there are no

grounds for believing that this salt tolerance has any physiological significance. One possible explanation of this effect is that the salt functions by altering electrostatic forces between ionized groups in the enzyme allowing the protein to take up an enzymically more active conformation.

As enzyme activity increases with increasing ionic strength, there appear to be parallel changes in some of the physical properties of the enzyme. Fluoresence emission in the presence of ANS is affected, suggesting conformational differences in the enzyme in different ionic environments. Similarly, sedimentation velocity and equilibrium studies indicate that the enzyme changes shape with differing ionic environments.

The denaturing studies with trypsin and sodium dodecyl sulphate suggest that in high salt concentrations the enzyme is in a more open conformation. The data is consistent with the enzyme unfolding as the ionic strength is increased, resulting in increased activity and changes in physical properties.

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