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Extracellular Ca^{2+}-Dependent Inducible Alkaline Phosphatase from the Extremely Halophilic Archaebacterium *Haloarcula marismortui*

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When starved of inorganic phosphate, the extremely halophilic archaebacterium *Haloarcula marismortui* produces the enzyme alkaline phosphatase and secretes it to the medium. This inducible extracellular enzyme is a glycoprotein whose subunit molecular mass is 160 kDa, as estimated by sodium dodecyl sulfate-gel electrophoresis. The native form of the enzyme is heterogeneous and composed of multiple oligomeric forms. The enzymatic activity of the halophilic alkaline phosphatase is maximal at pH 8.5, and the enzyme is inhibited by phosphate. Unlike most alkaline phosphatases, the halobacterial enzyme requires Ca^{2+} and not Zn^{2+} ions for its activity. Both calcium ions (in the millimolar range) and NaCl (in the molar range) are required for the stability of the enzyme.

Members of the family *Halobacteriaceae* are archaebacteria that are adapted to growth in extremely saline environments. In order to overcome the osmotic stress imposed by the environment, these microorganisms maintain an internal salt concentration of 4 M KCl (1, 6). Therefore, all of the biochemical inventory of these organisms has been adapted to function at extremely high salt concentrations. Most of the halobacterial enzymes are soluble and functional at low water activity and become unstable and unfold when the salt concentration drops below 2 M KCl or NaCl (3, 19). Although many biochemical and biophysical studies have been performed on intracellular enzymes of halobacteria (7), very little is known about extracellular proteins of these organisms. Until now only two halobacterial extracellular proteins have been studied extensively: the cell surface protein (10, 18) and the flagellin (20), both from *Halobacterium halobium*. These two proteins are the only procaryotic glycoproteins for which the linkage units between the protein core and the carbohydrate moieties have been chemically identified. The genes coding for both proteins have been isolated and sequenced (5, 9).

In order to be able to gain a better understanding of the process of protein export in the extremely halophilic archaebacteria, an extracellular enzyme was sought. In this article we describe the purification and properties of the extracellular inducible alkaline phosphatase of *Haloarcula marismortui*.

MATERIALS AND METHODS

Chemicals. *o*-Phenanthroline, *p*-nitrophenylphosphate (disodium salt), DNase I, and the protein molecular mass standards tyroglobulin and bovine intestinal alkaline phosphatase were obtained from Sigma (St. Louis, Mo.). Sepharose 6B-CL was from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

Culture conditions for *H. marismortui*. *H. marismortui* (obtained from B. Z. Ginzburg, Hebrew University, Jerusalem, Israel [6]) was grown in rich medium containing 3.5 M NaCl, 150 mM $MgSO_4$, 5 mM KCl, 0.65 mM $MnCl_2$, 3.4 mM $CaCl_2$, 50 mM Tris hydrochloride (pH 7.2), and 0.5% Difco yeast extract.

Induction of alkaline phosphatase. Cells were shaken at 37°C in minimal medium containing 3.5 M NaCl, 150 mM $MgSO_4$, 5 mM KCl, 5 mM NH_4Cl, 0.45% glycerol, 0.05% sodium succinate, 3.4 mM $CaCl_2$, 50 mM Tris hydrochloride (pH 7.2), and trace elements according to the method of Mevarech and Werczberger (14).

Enzymatic assay. Unless otherwise mentioned, the assay mixture (200 μl) contained 1 mg of *p*-nitrophenylphosphate per ml in 4 M NaCl–1 M Tris hydrochloride (pH 9). The reaction was performed at 25°C and was started by adding 20 μl of the enzyme solution. Enzymatic activity was measured by monitoring the increase in the optical density at a wavelength of 410 nm. The assay was performed in microtiter plates (Nunclon; Nunc, Copenhagen, Denmark). The optical absorption was measured with an MR 600 microplate reader (Dynatech, Chantilly, Va.). Readings were taken every 30 s during the first 5 min of the reaction. When calf intestinal alkaline phosphatase was assayed, the test solution contained 1 mg of *p*-nitrophenylphosphate per ml in 1 M Tris hydrochloride (pH 9) either with or without 4 M NaCl as indicated.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed in 7.5% acrylamide gels with 5% stacking gels according to the method of Laemmli and Favre (8). The samples were desalted before electrophoresis by precipitation in 10% trichloroacetic acid at 0°C. The pellet was washed with ether, dried in air, and redissolved in 50 mM Tris hydrochloride (pH 8). The samples were prepared in 0.1 M dithiothreitol–1% SDS–60 mM Tris hydrochloride (pH 6.8)–2 mM EDTA–10% glycerol.

Preparation of membranes. The cells were centrifuged for 3 min in an Eppendorf minicentrifuge, resuspended in a solution containing 4 M KCl, 50 mM $MgCl_2$, and 50 mM Tris hydrochloride (pH 7.2), and then frozen quickly in a dry ice-ethanol bath. The frozen cells were then thawed at 40°C, DNase I was added to a final concentration of 20 μg/ml, and the suspension was incubated for 1 h at 37°C. The membranes were pelleted in the minicentrifuge and washed with the solution described above. Both the pellet (the membrane

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fraction) and the first supernatant (the cytoplasmic fraction) were tested for alkaline phosphatase activity.

Determination of protein content. Protein content was determined according to the method of Lowry et al. (12) after the samples were dialyzed against distilled water.

Determination of sugar content. The sugar content was determined by a modification of the ferric cyanide method of Park and Johnson (15). Samples of the purified enzyme were dialyzed against distilled water and hydrolyzed in sealed tubes in 4 M HCl for 4 h at 100°C. The HCl was evaporated, and the samples were dissolved in distilled water.

Sucrose gradient centrifugation. The molecular mass of the native halophilic alkaline phosphatase was estimated by sucrose gradient ultracentrifugation according to a modified Martin and Ames method (13). Sucrose gradients of 5 to 20% (wt/vol) were prepared in a solution containing 3.5 M NaCl, 150 mM $MgSO_4$, and 50 mM Tris hydrochloride (pH 7.2). The enzyme (100 μl), dissolved in the same buffer, was layered on the gradient (4.5 ml) and centrifuged for 24 h at 38,000 rpm in a Beckman SW60 rotor at 4°C. Fractions of 50 μl each were collected and assayed for enzymatic activity at standard conditions. In order to obtain an estimation for the size of the native enzyme, a crude extract of *H. marismortui* was prepared and then was subjected to sucrose gradient centrifugation under the same conditions used for the alkaline phosphatase. The enzymatic activity of glutamate dehydrogenase, whose molecular mass was previously determined to be 213,000 Da (11), was assayed.

RESULTS

Induction of alkaline phosphatase. The alkaline phosphatase of *H. marismortui* is produced only when the cells are starved of phosphate. No activity can be detected when the phosphate concentration is higher than 0.1 mM, and the highest specific activity is obtained when the minimal medium is completely devoid of phosphate. When the cells are starved of phosphate, the production of the enzyme starts immediately and continues at a constant rate for at least 48 h (data not shown). In this period, the growth of the culture (determined by measuring the turbidity of the culture) levels off and eventually stops at a cell density twice as high as that in the beginning of the induction.

In order to localize the enzyme, the alkaline phosphatase activities of the membrane fraction, the cytoplasm, and the medium were assayed. Activity could be detected only in the membrane fraction and in the medium, indicating that alkaline phosphatase is secreted during phosphate starvation. Comparing the protein contents of the medium, the cell extracts, and the membranes of induced and uninduced cells by SDS-PAGE reveals a 160-kDa protein that appears only under starvation conditions (Fig. 1). This protein makes up over 90% of the total protein content in the supernatant.

Preparation of the enzyme. Alkaline phosphatase was prepared from the medium of induced cells. Cells were grown in rich medium to an optical density of 2 (measured at 550 nm) and then were centrifuged for 20 min at 10,000 rpm in a Sorvall SS34 rotor at 4°C. The pelleted cells were washed twice with minimal medium lacking phosphate and then diluted 1:4 into the same medium and incubated with shaking at 37°C.

Forty-eight hours after the beginning of the induction, the cells were centrifuged as described above and the supernatant was concentrated 40- to 50-fold in a dialysis tube under vacuum. Solid CsCl was added (0.38 g/ml of enzyme solution) and the solution was centrifuged in a Beckman SW60 rotor at 50,000 rpm for 22 h at 25°C. The gradient was fractionated into 200-μl fractions. The enzymatic activity profile, the optical density at 280 nm, and the SDS-PAGE results of the various fractions are shown in Fig. 2. The only protein component of the peak is the 160-kDa protein identified earlier as the alkaline phosphatase (Fig. 2B). The density of the CsCl solution at the peak of the protein was 1.36 g/ml.

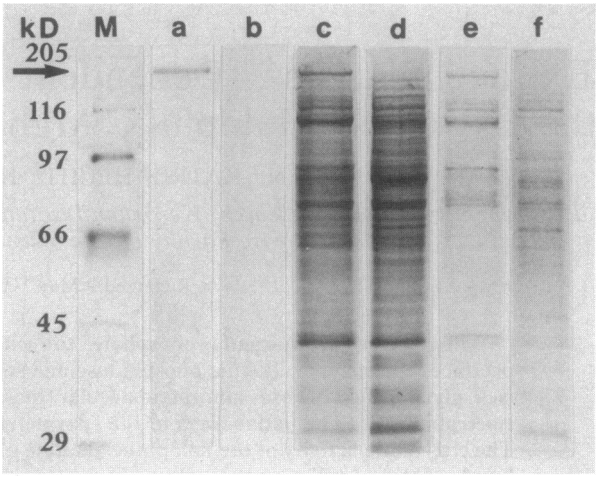

FIG. 1. SDS-PAGE of protein extracts isolated from *H. marismortui* supernatant of induced cells (lane a), supernatant of uninduced cells (lane b), induced whole cells (lane c), uninduced whole cells (lane d), membranes of induced cells (lane e), and membranes of uninduced cells (lane f). The arrow indicates the alkaline phosphatase protein band. Lane M, Molecular mass markers.

Sugar content. Quantitative estimation of the sugar content of the purified enzyme was performed as described in Materials and Methods. It was found that the sugar makes up 3% (wt/wt) of the protein. Additional evidence that the enzyme is a glycoprotein was obtained by in vivo labeling of the cells with radioactive glucosamine. In autoradiograms of SDS-PAGE gels of supernatants of induced and uninduced cells, a radioactive band corresponding to the alkaline phosphatase can be observed only in the supernatant of the induced cells (data not shown).

Molecular mass of the native enzyme. The molecular mass of the subunit of alkaline phosphatase, as determined by SDS-PAGE, was found to be 160 kDa. The degree of subunit aggregation of the native enzyme was estimated by two different methods: gel filtration and sedimentation through a sucrose gradient. The fractions from the CsCl gradient were pooled and dialyzed against a solution containing 3.5 M NaCl, 150 mM $MgSO_4$, 3.4 mM $CaCl_2$, and 50 mM Tris hydrochloride (pH 7.2). The enzyme was chromatographed on a Sepharose 6B column equilibrated with the same buffer. The elution profile of the alkaline phosphatase activity and the locations of the peaks of the standard protein markers are shown in Fig. 3. The alkaline phosphatase appears as a broad peak at a location corresponding to molecules having molecular masses of 650 to 850 kDa. The enzymatic activity profile of the sucrose gradient sedimentation analysis of the CsCl-purified enzyme is shown in Fig. 4. As can be seen, these results also indicate that the purified native enzyme is not a homogeneous multimeric enzyme.

Catalytic properties. (i) Effect of pH on enzymatic activity. The effect of pH on the enzymatic activity of the purified enzyme was tested at 4 M NaCl at various pHs. The reaction

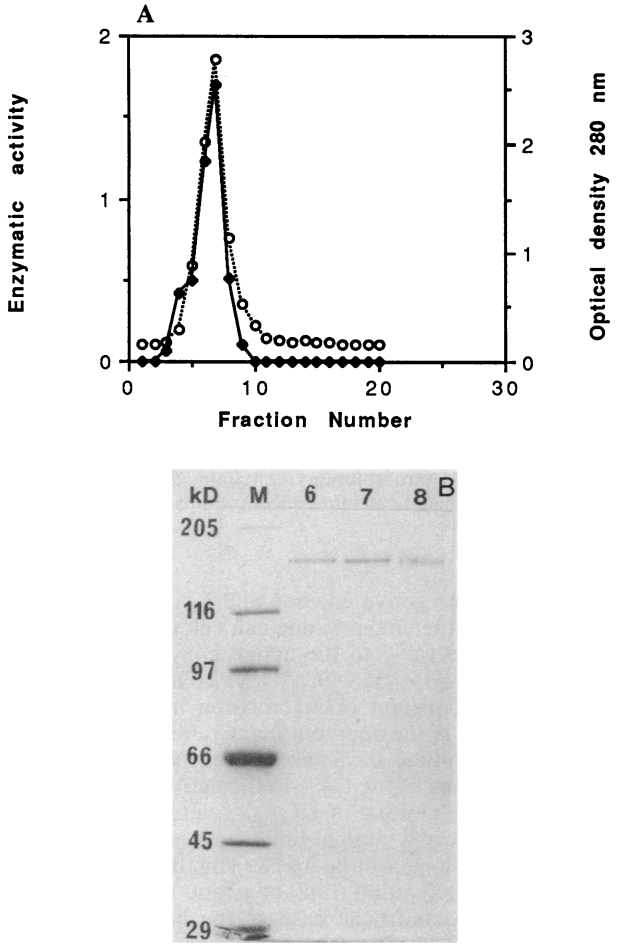

FIG. 2. Centrifugation of the alkaline phosphatase in a CsCl gradient. CsCl (0.38 g) was added for every milliliter of enzyme solution. Samples (4.5 ml) were centrifuged at 50,000 rpm in a Beckman SW60 rotor for 22 h at 25°C. After centrifugation, the gradient was fractionated into 200-μl fractions. (A) Aliquots were assayed for alkaline phosphatase activity (◆) and for optical density at 280 nm (○). (B) SDS-PAGE of the activity peak fractions (6 to 8) of the CsCl gradient shown above.

was stopped after 10 min by adding 5 M NaOH to a final concentration of 1 M, and the optical density was measured at 410 nm. The enzymatic activity was maximal at pH 8.5 (Fig. 5). No activity could be found below pH 7.

(ii) Effect of phosphate concentration on catalytic properties. The effect of the phosphate concentration on the catalytic properties of the enzyme was determined by comparing the Lineweaver-Burk plots of the enzymatic activities at different phosphate concentrations. The presence of phosphate in the assay affects the K_m of the reaction (the apparent binding constant of the substrate) but not the V_{max} (which is proportional to the turnover of the reaction). The K_m of *p*-nitrophenylphosphate in the absence of phosphate is 0.16 mM. This value increases with an increase in phosphate concentration. This mode of inhibition is typical of competitive inhibition. The K_i of phosphate can be calculated by the equation $K_{m(P)} = K_m(1 + [P_i]/K_i)$, in which K_m is the Michaelis constant in the absence of phosphate and $K_{m(P)}$ is the apparent K_m in the presence of phosphate. Table 1 summarizes the results of $K_{m(P)}$ measurement at different concentrations of phosphate, from which the K_i of phosphate was calculated to be 0.17 mM.

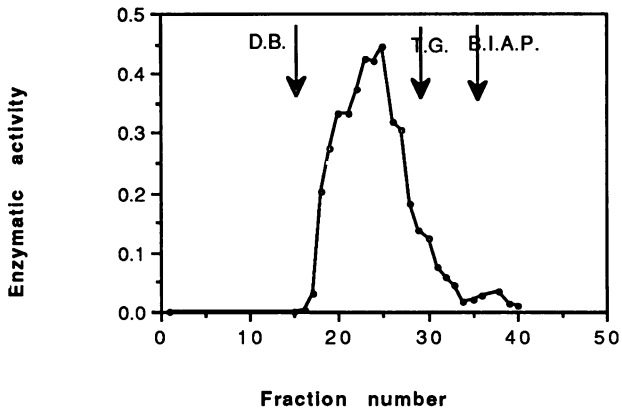

FIG. 3. Gel filtration chromatography of the alkaline phosphatase. CsCl-purified alkaline phosphatase was passed through a Sepharose 6B column (1 by 90 cm) equilibrated with 3.5 M NaCl–150 mM $MgSO_4$–5 mM KCl–5 mM NH_4Cl–3.4 mM $CaCl_2$–50 mM Tris hydrochloride (pH 7.5). Fractions (1.64 ml each) were collected and assayed for enzymatic activity. The column was calibrated with several macromolecular markers. The locations of the peak fractions of dextran blue (D.B.), tyroglobulin (T.G.; molecular mass, 670,000 Da), and bovine intestinal alkaline phosphatase (B.I.A.P.; molecular mass, 105,000 Da) are indicated.

(iii) Effect of divalent cations on catalytic activity. *H. marismortui* alkaline phosphatase is completely inhibited by 3 mM EDTA. The inhibition can be reversed by adding Ca^{2+} ions but not by adding zinc, manganese, or magnesium ions. In order to demonstrate that Zn^{2+} ions, which are essential metal ions for most of the other alkaline phosphatases (4, 16), are not essential for the halobacterial enzyme, the effect of *o*-phenanthroline (a chelator that binds Zn^{2+} ions but not Ca^{2+} ions) on the enzymatic activity was determined. It was found that *o*-phenanthroline did not inhibit the halophilic enzyme even after prolonged incubation, whereas under the same conditions, bovine intestinal alkaline phosphatase was completely inhibited by this chelator. Thus, the *H. maris-*

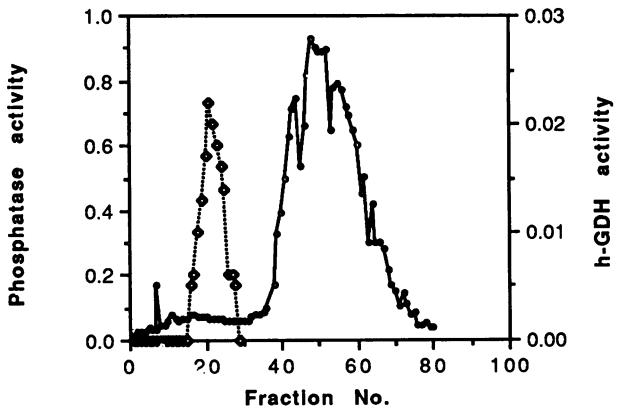

FIG. 4. Sedimentation profile of alkaline phosphatase in sucrose gradient. One hundred microliters of CsCl-purified alkaline phosphatase and 100 μl of halobacterial extract were layered onto two separate 4.5-ml sucrose gradients (5 to 20% [wt/vol] dissolved in 3.5 M NaCl–150 mM $MgSO_4$–3.4 mM $CaCl_2$–50 mM Tris hydrochloride [pH 7.5]). Centrifugation was performed in a Beckman SW60 rotor at 38,000 rpm for 24 h at 4°C. Fifty-microliter fractions were collected and assayed for enzymatic activity. Symbols: ●, alkaline phosphatase activity; ◇, glutamate dehydrogenase (h-GDH) activity.

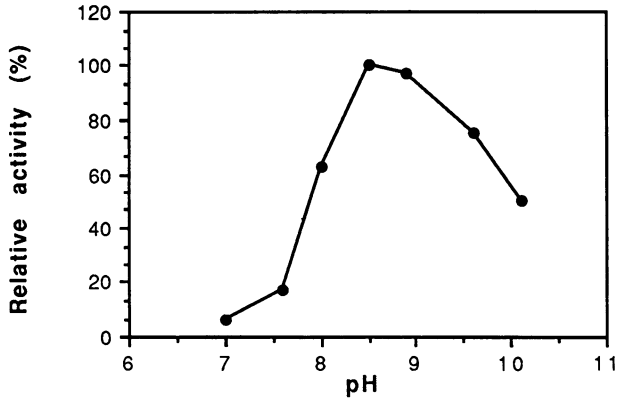

FIG. 5. Alkaline phosphatase activity at various pHs. Alkaline phosphatase stock solution was diluted 20-fold into 180 μl of a solution containing 4 M NaCl and 100 mM Tris hydrochloride at different pHs. The enzymatic reaction was started 10 min later by adding 20 μl of *p*-nitrophenylphosphate (10 mg/ml in 3.4 mM $CaCl_2$) and stopped after 10 min by adding 50 μl of 5 M NaOH. The amount of *p*-nitrophenol produced was determined at 410 nm.

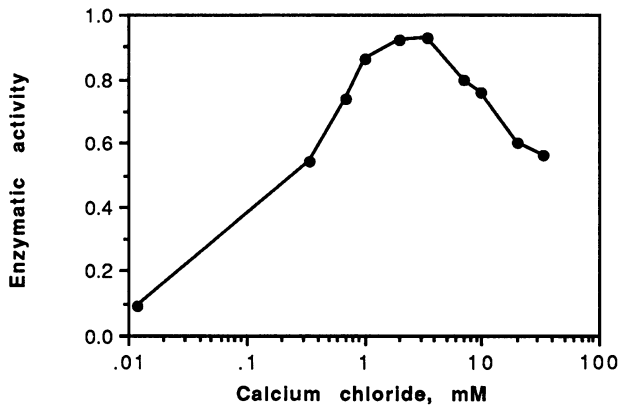

FIG. 6. Effect of calcium concentration on the enzymatic activity of *H. marismortui*. Enzymatic activity was measured by diluting the enzyme 20-fold into solutions containing the substrate, 3 M NaCl, and the indicated calcium concentrations in 1 M Tris hydrochloride (pH 9).

mortui enzyme requires Ca^{2+} and does not require Zn^{2+} for its catalytic activity. The optimal Ca^{2+} concentration for enzymatic activity is 3.4 mM (Fig. 6).

(iv) Effect of NaCl on the properties of *H. marismortui* alkaline phosphatase. Halobacterial alkaline phosphatase shows behavior characteristic of a halophilic enzyme, namely, its stability and activity increase with an increase in the salt concentration. The effect of NaCl and KCl concentrations on the enzymatic activity is shown in Fig. 7. The enzyme is more active in NaCl (its "native" salt) than in KCl. The stability of the active enzyme was determined by measuring the enzymatic activity after exposure of the enzyme for 24 h at 20°C to various NaCl and Ca^{2+} concentrations. The activity was measured under the standard assay conditions. The enzyme requires Ca^{2+} ions in addition to NaCl (Fig. 8). At 3.5 mM Ca^{2+}, the enzyme is unstable at low NaCl concentrations but its stability increases with the salt concentration. At 32 mM CA^{2+}, the enzyme is rather stable even at low NaCl concentrations. Interestingly, even at 4 M NaCl, the stability of the enzyme depends on the Ca^{2+} concentration. When the enzyme is exposed to low Ca^{2+} concentrations at 4 M NaCl, it loses activity in first-order kinetics (data not shown). The dependence of the kinetic rate constant on the Ca^{2+} concentration is shown in Fig. 9. Assuming the model

$$E_a - Ca^{2+} \rightleftharpoons E_a + Ca^{2+} \rightarrow E_i$$

TABLE 1. Inhibition of alkaline phosphatase by phosphate[a]

P_i concn (mM)	K_m (mM)	K_i (mM)
0.00	0.16	NA[b]
0.50	0.59	0.19
1.00	1.25	0.15
1.50	1.67	0.16
2.00	2.00	0.17
2.50	2.50	0.17

[a] Activity was measured in 4 M NaCl–1 M Tris hydrochloride (pH 9.0) containing various amounts of phosphate. The substrate concentration was varied between 0.05 and 1.00 mM. Michaelis constants were calculated by using Lineweaver-Burk plots.

[b] NA, Not applicable.

in which E_a is the active enzyme and E_i is the irreversibly inactive form of the enzyme, one can calculate the constant of binding of the Ca^{2+} to the active enzyme by using the equation $1/k_{app[Ca]} = (1 + [Ca^{2+}]/K)/k_i$, in which k_i is the first-order rate constant of inactivation in the absence of calcium, $k_{app[Ca]}$ is the apparent inactivation constant in the presence of the indicated calcium concentration, and K is the constant of binding of the Ca^{2+} to the active enzyme. If this model is correct, plotting of $1/k_{app[Ca]}$ versus Ca^{2+} concentration will result in a straight line whose y intercept will be $1/k_i$ and whose slope will be $1/k_iK$. This linear relationship between the inactivation rate constant and the calcium concentration exists in the case of the halophilic alkaline phosphatase (Fig. 9). The calculated values for k_i and K are 0.05 min^{-1} and 8.6 μM, respectively.

DISCUSSION

The growth of free-living organisms depends on the availability of phosphate in the medium, and therefore special

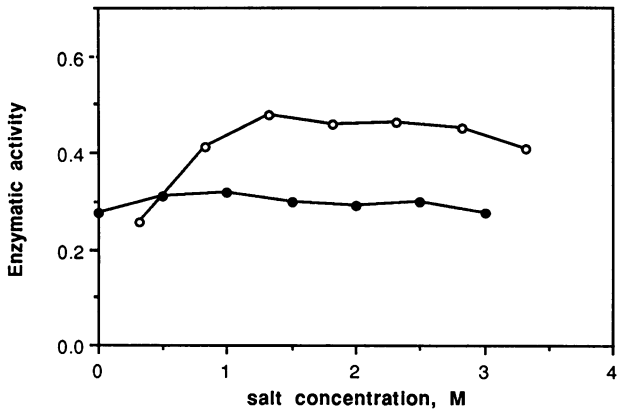

FIG. 7. Effect of NaCl (○) and KCl (●) concentrations on the enzymatic activity of *H. marismortui* alkaline phosphatase. Enzymatic activity was measured by diluting the enzyme 10-fold into solutions containing the substrate, 3 mM Ca^{2+}, at various NaCl or KCl concentrations in 1 M Tris hydrochloride (pH 9). Changes in the optical density at 410 nm were measured 1 min after the beginning of the reaction.

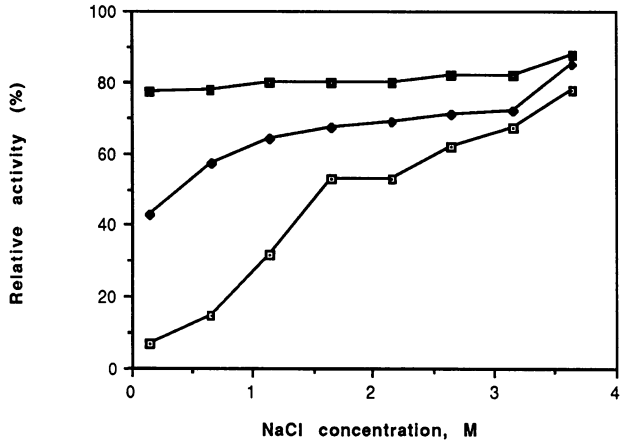

FIG. 8. Effect of NaCl and $CaCl_2$ on the stability of *H. marismortui* alkaline phosphatase. The enzyme was exposed for 24 h to the indicated NaCl and $CaCl_2$ concentrations at 20°C and then assayed under standard conditions. Symbols: ■, 32 mM $CaCl_2$; ◆, 10 mM $CaCl_2$; ⊡, 3.5 mM $CaCl_2$.

mechanisms were developed to cope with conditions of phosphate starvation. The best studied system is the *pho* regulon of *Escherichia coli* (17). One of the genes that is induced upon phosphate starvation is the *phoA* gene, which codes for the enzyme alkaline phosphatase. The induced enzyme is secreted to the periplasmic space and used to cleave phosphoester bonds of organic phosphates that are otherwise inaccessible to the cell. When cells of *H. marismortui* are exposed to a medium that lacks phosphate, the bacteria stop growing and the synthesis of alkaline phosphatase is immediately induced. A short time after induction, the activity can be detected in the membrane fraction and is followed by release of the active enzyme to the medium. When the protein components of the medium of an induced culture are examined by SDS-PAGE, a unique polypeptide of 160 kDa can be observed. This polypeptide can also be detected in the membrane fraction of induced culture but not in that of uninduced culture.

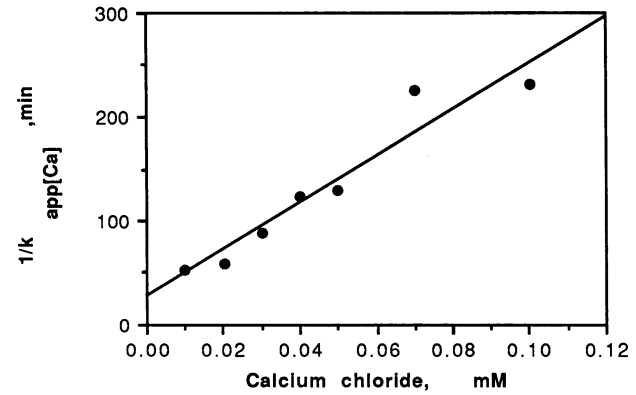

FIG. 9. Determination of the constant of binding between Ca^{2+} and the enzyme at 4 M NaCl. The inactivation rates of the alkaline phosphatase were determined by incubating the enzyme at 20°C in 4 M NaCl–1 M Tris hydrochloride (pH 9.0) with various amounts of Ca^{2+}. At different time intervals, samples were tested for alkaline phosphatase activity under standard assay conditions. A semilogarithmic plot of the logarithm of the enzymatic activity versus time resulted in a straight line (data not shown) from which the rate constants for inactivation were determined.

Since a single polypeptide subunit was observed in the medium of induced culture, the purification protocol was designed to free this protein from other polymer components of the medium. The main contaminant was a high-molecular-weight polysaccharide that upon concentration of the medium produces gelatinous material. By using CsCl density gradient ultracentrifugation, it was possible to separate the protein from the polysaccharide component that sediments to the bottom of the tube. This purification step was particularly important, since it made the determination of the sugar content in the protein preparation possible. It was found that, similar to the other extracellular halobacterial proteins, the alkaline phosphatase is a glycoprotein.

The size of the native enzyme was estimated by gel filtration and ultracentrifugation. The results of the gel filtration show a broad activity peak in the molecular mass range of 650 to 850 kDa. In the sucrose gradient centrifugation the peak of activity is partially resolved to several peaks. This resolution is very reproducible and is also supported by a sedimentation velocity experiment performed in the analytical ultracentrifuge (unpublished observation). The molecular mass of the alkaline phosphatase is greater than that of the halophilic glutamate dehydrogenase (213 kDa [11]). It seems, therefore, that the active enzyme exists in multiple oligomeric forms.

The high-molecular-weight alkaline phosphatase of *H. marismortui* resembles the extracellular alkaline phosphatase isolated from mammalian cartilage (2). The resemblance is not limited to size and to the fact that both enzymes are extracellular glycoproteins but is also apparent in that both enzymes are very unusual in their requirement for Ca^{2+} and not Zn^{2+}. So far, these two enzymes are the only calcium-dependent alkaline phosphatases known. In the case of the *H. marismortui* enzyme, the calcium ions are required both for the activity of the enzyme and for its stability. Whereas most of the halobacterial enzymes are very stable at monovalent ion concentrations of 2 M and higher, the halobacterial alkaline phosphatase requires calcium ions for stability even at 4 M NaCl. From the dependence of the inactivation constant on the calcium concentration, it was possible to calculate the binding constant of the calcium ions to the enzyme. This binding constant (8.6 μM) is about 500-fold lower than the calcium concentration that is required for maximum activity (3.4 mM). It seems, therefore, that there are at least two binding sites for calcium.

The activity of *H. marismortui* alkaline phosphatase is subject to product inhibition by phosphate. The K_i of phosphate is 0.17 mM, while a K_m of 0.16 mM was measured for *p*-nitrophenylphosphate. It seems, therefore, that the affinities of the phosphate and the phosphoester are very similar.

The purification of *H. marismortui* alkaline phosphatase should facilitate the isolation of its coding gene. This gene can be a very useful tool for the analysis of gene expression in halobacteria. First, the promoter of the gene is very tightly regulated by phosphate and therefore can be used to regulate the expression of other genes in the same way that the *lacZ* and λ p_L promoters are used to regulate recombinant fusions in *E. coli*. On the other hand, the alkaline phosphatase structural gene can be fused to other promoters and serve as an indicator gene with which to study the activity of promoters whose normal gene products are difficult to assay. Finally, since the alkaline phosphatase is an extracellular protein, it can be used to study the secretion process in halobacteria and its leader sequence can be used to export proteins produced by recombinant DNA techniques. Work in this direction is in progress in our laboratories.

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