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Monomeric Alkaline Phosphatase of *Vibrio cholerae*

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Alkaline phosphatase has been purified to homogeneity from two strains of *Vibrio cholerae*. The enzymes from both strains are single polypeptides of molecular weight 60,000. Both of the enzymes have pH optima around 8.0 and can act on a variety of organic phosphate esters, glucose-1-phosphate being the best substrate. The enzymes are unable to hydrolyze ATP and AMP. Although they have identical K_m values, the two enzymes differ significantly in V_{max} with *p*-nitrophenyl phosphate as substrate. The enzymes from the two strains also differ in their sensitivity to EDTA, P_i, and metal ions and activities of the apoenzymes. Ca^{2+} reactivated the apoenzymes most.

In another report (16a), repression of the alkaline phosphatase (APase; EC 3.1.3.1) of *Vibrio cholerae*, a highly pathogenic gram-negative bacterium, has been shown in the presence of a high level of P_i. Derepression of the enzyme's synthesis occurred when the P_i content of the growth medium was reduced and was further stimulated in the presence of glucose. Whereas the enzyme from strain 569B of Inaba was stable, that from strain 154 of Ogawa was unstable in vivo (16a). The stability of the enzyme from cells of strain 569B in vivo was reduced during laboratory maintenance of these cells along with the reduction in toxinogenicity. However, animal passage of laboratory strains (3) increased the level of toxinogenicity, and the stability of the enzyme in vivo was restored (N. K. Roy, G. Das, T. S. Balganesh, S. N. Dey, R. K. Ghosh, and J. Das, J. Gen. Microbiol., in press). It has also been reported that the synthesis of this enzyme by cells of strain 569B was more sensitive to P_i than that of cells of strain 154 (16a). It thus appeared intriguing to examine the properties of APase purified from cells of strain 569B and 154.

The present report describes that although the enzyme from cells of strain 154 was unstable in vivo, the purified enzyme was stable. In contrast to APases from all other sources reported so far (7, 8, 16), which are dimers of identical subunits, the enzymes from both strains of *V. cholerae* are single polypeptides.

MATERIALS AND METHODS

Organisms and growth conditions. The *V. cholerae* Inaba 569B and Ogawa 154 strains used in this study were obtained from Cholera Research Center, Calcutta, India. The conditions for maintenance of these strains have been described previously (4).

Cells were grown in phosphate-depleted nutrient broth (25 μM P_i) containing 10 g of peptone (Difco Laboratories), 10 g of Lab-Lemco powder (Oxoid Ltd.), and 5 g of sodium chloride per liter of distilled water as described elsewhere (16a). LPG medium was low-phosphate medium containing 0.1% (wt/vol) glucose. Overnight cultures were used to inoculate LPG medium at an absorbancy at 540 nm (A_{540}) of 0.08 and allowed to grow at 37°C with shaking (180 rpm) to an A_{540} of 1.6. Cells were harvested by centrifugation at 10,000 × *g* for 10 min.

Assay of APase. APase activity was assayed by monitoring the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl phosphate (PNPP). The reaction mixture contained 1.0 M Tris-hydrochloride (pH 8.0), 2 mM PNPP, and the enzyme in a 2-ml volume. Incubation time was 15 min and 10 min for the enzyme from cells of strains 569B and 154, respectively. After incubation at 37°C, the reaction was terminated by the addition of 0.2 ml of 26% (wt/vol) K_2HPO_4, and the A_{410} was read in a Gilford model 250 spectrophotometer. One unit of enzyme is defined as the amount of enzyme which liberates 1 nmol of PNP per min at 37°C. The activity of the enzyme on other organic phosphate esters was measured by assaying the amount of P_i liberated by the method of Ernster et al. (6). The reaction mixture for this assay was 0.2 M glycine-NaOH (pH 9.6), 2 mM substrate, and the enzyme in a 2-ml volume. The reaction was stopped by the addition of 0.7 ml of ice-cold perchloric acid (70%). The stability of the enzyme during the assay was checked by the method of Selwyn (18).

Protein estimation. Protein was estimated colorimetrically by the method of Lowry et al. (13) or by absorbance by the method of Kalckar (9).

Spheroplast formation. Cells were suspended to an A_{540} of 3.0 to 3.3 in 20% (wt/vol) sucrose buffered with 0.033 M Tris-hydrochloride (pH 8.0). The cell suspension was chilled to 0°C and treated with 0.1 volume of 0.1 M EDTA (pH 8.0) and 5 μg of lysozyme per ml suspension in succession. Mixing was achieved by gently rolling the suspension at 0°C. Spheroplast formation was monitored by the method of Malamy and Horecker (14), and it required about 8 to 10 min. After completion of spheroplast formation, the suspension

was centrifuged at 15,000 × g for 15 min, and the resulting supernatant (periplasmic supernatant) was collected. This was used as the starting material for enzyme purification.

Electrophoresis. Electrophoresis under nondenaturing conditions was carried out by the method of Davis (5). Enzyme protein (20 μg) was applied on a 7.5% polyacrylamide gel with a 5-mm 2.5% stacking gel on top, and electrophoresis was carried out in 38 mM glycine containing 5 mM Tris-hydrochloride (pH 8.3). Current was applied at 2.5 mA per tube until the tracking dye migrated at the bottom of the gel.

Electrophoresis under denaturing conditions was carried out in 10% acrylamide gels in siliconized tubes (8 by 0.6 cm) as described by Laemmli (10). The Tris buffer used for gel preparation contained 0.375 M Tris-hydrochloride (pH 8.8) and 0.1% sodium dodecyl sulfate. The gel buffer contained Tris buffer with 0.02% *N,N,N',N'*-tetramethylethylenediamine. The electrolyte buffer was 0.192 M glycine containing 0.025 M Tris-hydrochloride (pH 8.3). The protein samples were solubilized in 0.0625 M Tris-hydrochloride (pH 6.8) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Samples were incubated at 100°C for 2 min. After cooling, glycerol was added to a final concentration of 10%, and a small amount of bromophenol blue was added as the tracking dye. This mixture, containing about 20 μg of protein, was layered on the gel. Electrophoresis was carried out at 2.5 mA per tube until the tracking dye reached the bottom of gel.

Determination of molecular weight. Protein molecular weights were determined from their mobilities relative to protein standards as described by Weber and Osborn (19). The protein molecular weight standards used were bovine serum albumin (68,000), ovalbumin (43,000), trypsin (23,500), and lysozyme (13,600). Proteins were stained for 16 h in 0.125% Coomassie brilliant blue in a 1:5:5 mixture of acetic acid-water-methanol and destained in a solution of 5% methanol and 7.5% acetic acid.

Molecular weights were also determined by gel filtration in Sephadex G-200 column by the method of Andrews (1) with *Escherichia coli* APase (86,000), bovine serum albumin, and ovalbumin as molecular weight standards.

Preparation of antiserum. Antiserum against purified APase from cells of strain 569B was raised in rabbits. Enzyme protein (200 μg) emulsified with complete Freund adjuvant (Difco) in 1:1 (vol/vol) ratio was injected thrice subcutaneously on the abdomen at multiple sites at intervals of 7 days. The fourth and final dose of antigen was injected into the footpad without any adjuvant. One week after the final injection the animal was bled by cardiac puncture, and serum was separated. The serum was used as the source of antibody.

Transphosphorylation. Transphosphorylation activity of the enzymes was determined by simultaneous assay of PNP and P_i liberated from PNPP. The amount of missing phosphate was the amount of P_i transphosphorylated (20). The reaction in 2-ml samples of the incubation mixture was terminated by K_2HPO_4 for estimation of PNP and by 0.7 ml of ice-cold perchloric acid (70%) for estimation of P_i after the desired period of incubation of the enzyme. The phosphate standard controls were measured under identical conditions to eliminate the possibility of interference by Tris with P_i estimation.

RESULTS

Purification of APase from *V. cholerae* cells. Identical procedures were followed for purification of APase from cells of strains 569B and 154, except that the buffers used for the enzyme from cells of strain 154 contained 20 μM β-glycerophosphate to stabilize the enzyme in vivo (16a). The purification of the enzyme involved the following steps carried out at 4°C.

Spheroplasts were formed from cells grown in LPG medium, and the periplasmic supernatant was collected as described above. More than 90% of the whole cell activity was recovered in the periplasmic supernatant. Periplasmic supernatants from 500-ml cultures were loaded on a DEAE-cellulose column (1.6 by 56 cm) equilibrated with buffer A (33 mM Tris-hydrochloride [pH 8.0], 0.1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, and 10% glycerol). The column was washed with the same buffer, and the enzyme activity was eluted with a linear 0 to 50 mM KCl gradient (Fig. 1). The enzyme from cells of strain 154 (Fig. 1b) eluted

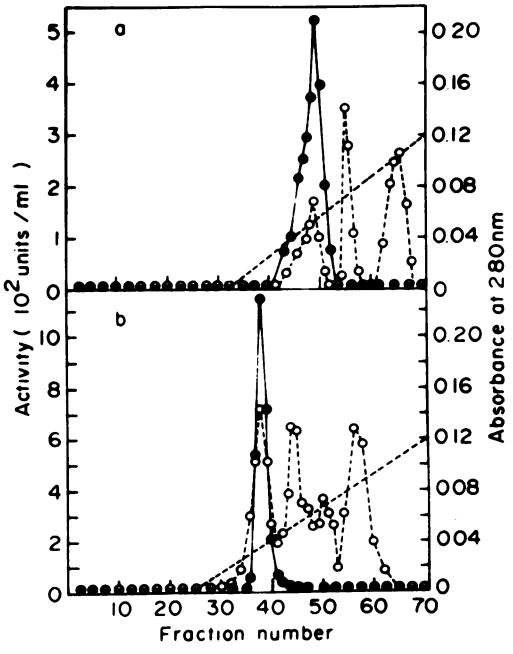

FIG. 1. Chromatography on DEAE-cellulose. Periplasmic supernatants from cell of strains 569B (a) and 154 (b) were applied on DEAE-cellulose columns equilibrated with buffer A and eluted with a linear gradient of 0 to 50 mM KCl (—). Fractions (8 ml) were collected and assayed for enzyme activity (●) and A_{280} (○) as described in the text.

TABLE 1. Purification of APase from *V. cholerae* cells of strain 569B and 154

Fractions	569B				154			
	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Recovery (%)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Recovery (%)
Periplasmic supernatant	53,800	134	401	100	34,102	58	589	100
DEAE-cellulose	49,980	5.67	8,800	93	27,450	4.6	6,000	81
Sephadex G-200	23,500	2.60	9,000	44	11,530	1.92	6,000	34

at a lower salt concentration (15 mM) relative to the enzyme from cells of strain 569B (23 mM) (Fig. 1a). Fractions containing APase activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 Diaflo membrane.

The concentrated APase activity was further purified by gel filtration on a Sephadex G-200 column (2 by 86 cm) equilibrated with buffer A. Only one symmetrical protein peak coincident with the enzyme activity was eluted. Similar results were obtained with the enzyme from cells of strain 154. The active fractions were pooled, concentrated, and stored at −20°C without any loss of activity. The purification steps are summarized in Table 1. Although the enzyme was pure after DEAE-cellulose chromatography, the gel filtration on Sephadex was carried out to determine the molecular weight of the native protein. The purification was about 22- and 10-fold for enzymes from cells of strains 569B and 154, respectively, estimated with respect to periplasmic supernatant.

Assessment of purity of APase. The following criteria were used to assess the purity of enzymes obtained from cells of strains 569B and 154.

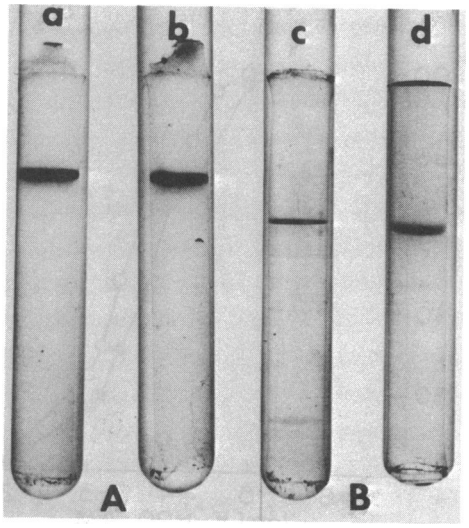

FIG. 2. Polyacrylamide gel electrophoresis under nondenaturing (A) and denaturing (B) conditions of enzyme from cells of strain 569B (a, c) and 154 (b, d).

Polyacrylamide gel electrophoresis under both denaturing and nondenaturing conditions gave single protein bands for both enzymes (Fig. 2).

Antibody raised against APase from cells of strain 569B gave a single precipitin band in Ouchterlony double-diffusion plates at all antigen concentrations used (Fig. 3). No spur formation was observed in the precipitin bands of enzymes from cells of strains 569B and 154 against the above antibody, suggesting identity between the two enzymes.

Molar extinction coefficient. The A_{280}/A_{260} ratio of about 1.7 of the enzyme preparations indicates absence of any nucleic acid contamination. The molar extinction coefficients of enzymes from cells of strains 569B and 154 were 6.7×10^4 and 6.0×10^4, respectively.

Stability. APase can be stored at −20°C for at least 2 months without any loss of activity. Although the enzyme from cells of strain 154 was unstable in vivo, the purified enzyme was stable even in the absence of substrate.

Molecular weight and subunit structure of *V. cholerae* APase. The molecular weights of APase from cells of strains 569B and 154 were determined by polyacrylamide gel electrophoresis and gel filtration techniques. Based on the migration of purified APase on sodium dodecyl sulfate-polyacrylamide gels, the monomer molecular weight was estimated as 60,000. The molecular weight of the native enzyme was estimated from the elution position on Sephadex G-200 gel filtration, and a value of about 60,000 was obtained. There was no significant difference between the molecular weights of the enzymes from cells of strains 569B and 154. Comparison of the values of the molecular weights obtained from gel electrophoresis and gel filtration shows that the enzymes from *V. cholerae* cells are monomeric proteins.

Effect of pH. Optimum pH for the hydrolysis of PNPP was determined at 37°C in 1 M Tris-hydrochloride and 0.2 M glycine-NaOH buffers. In Tris-hydrochloride buffer, the optimum pH for enzyme activity was 8.2. In glycine-NaOH buffer, the maximum enzyme activity was ob-

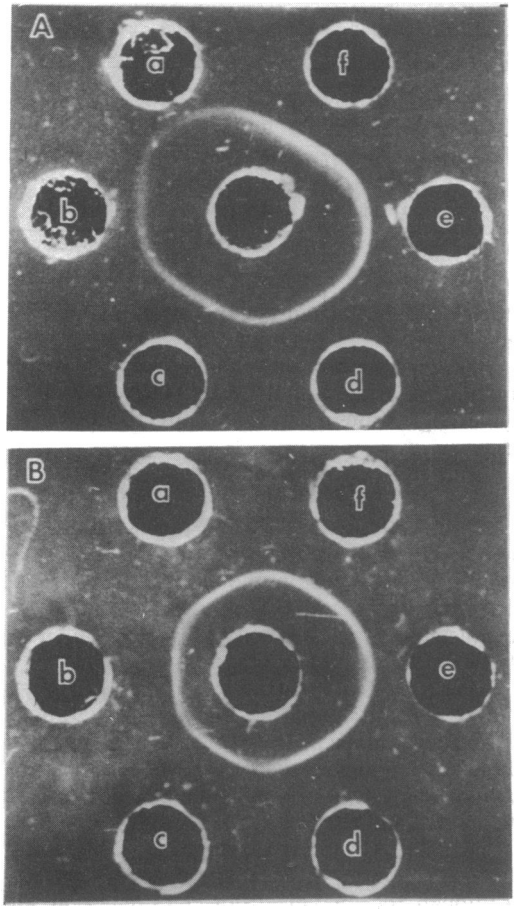

FIG. 3. Immunoprecipitation reaction of APase from cells of strains 569B and 154 with antisera raised against enzymes from cells of strain 569B. The central well of both A and B contained antiserum (40 μl). A, Samples of 0.16, 0.32, 0.63, 1.25, 2.5, and 5.0 μg of APase from cells of strain 569B were applied in the wells a to f respectively. B, Samples of 5-μg of enzyme protein from cells of strains 569B (a, c, e) and 154 (b, d, f) were applied.

served in a relatively wider range of pH (9.2 to 10.0). There was no appreciable difference between the pH optima of enzymes from cells of strains 569B and 154.

Michaelis constant. Purified enzymes were incubated with various concentrations of PNPP in 1 M Tris-hydrochloride (pH 8.2), and the velocity of the reaction was measured as the amount of PNP liberated. The Lineweaver-Burk (12) double-reciprocal plot in Fig. 4 shows that under these conditions the enzymes from cells of strains 569B and 154 had similar K_m values (0.22 mM). However, the enzymes from the two sources differed significantly in the value of V_{max} (9 and 6 μmol/min per mg of protein for

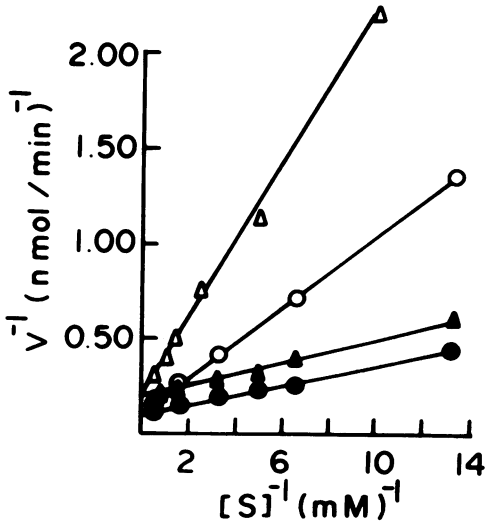

FIG. 4. Lineweaver-Burk plot for determination of K_m and K_i. The rates of liberation of PNP per min per μg (V) of enzyme from cell of strains 569B (●) and 154 (▲) were estimated in the presence of various concentrations of PNPP (S). For K_i, V was estimated in presence of 10^{-4} M P_i for the enzymes from cell of strains 569B (○) and 154 (△).

enzymes from cells of strains 569B and 154, respectively). K_m and V_{max} were also determined in glycine-NaOH buffer. Whereas the K_m values were identical (0.24 mM) for both the enzymes, the V_{max} values were different (4.0 and 2.8 μmol/min per mg of protein for enzymes from cells of strains 569B and 154, respectively).

P_i inhibition. The activities of both the en-

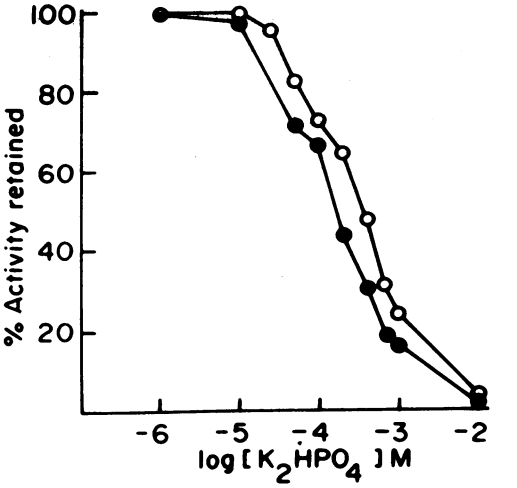

FIG. 5. Effect of P_i on APase activity from cells of strains 569B (●) and 154 (○).

TABLE 2. Effect of various divalent metal ions on the activity of APase of *V. cholerae*[a]

Metal ion	% APase activity restored	
	569B	154
None	20	1
Ca^{2+}	57	84
Co^{2+}	57	48
Ni^{2+}	50	51
Mn^{2+}	37	32
Mg^{2+}	35	3
Zn^{2+}	20	3

[a] Purified enzymes from cells of strains 569B and 154 were dialyzed against buffer A containing 5 mM EDTA to remove metal ions. The resulting apoenzyme was dialyzed against buffer A to remove EDTA. The activity of the dialyzed apoenzyme was assayed in the presence of 1 mM concentrations of various metal ions as described in the text. The results are expressed as percentage of activity restored compared with untreated enzymes. Reaction rates were linear up to 10 min.

zymes from cells of strains 569B and 154 were assayed in the presence of various concentrations of P_i. At 10^{-2} M P_i, complete inhibition of activity was observed for enzymes from both sources (Fig. 5). At lower concentrations, the enzyme from cells of strain 569B was more sensitive than that from cells of strain 154. To determine the value of K_i, the velocity of the enzyme reaction was measured in presence of a fixed concentration (10^{-4} M) of P_i. From the double-reciprocal plot (Fig. 4) the inhibition was found to be competitive. The values of K_i are similar for both enzymes (0.033 mM).

EDTA inhibition. To examine the effect of EDTA on APases of cells from strains 569B and 154, activities were assayed in presence of various concentrations of EDTA. The enzyme from cells of strain 569B was more susceptible to EDTA, and its activity was completely inhibited at 0.03 mM. Under identical conditions the enzymes from cells of strain 154 retained 60% activity. Complete loss of enzyme activity from cells of strain 154 occurred at 0.07 mM EDTA.

Metal ion. To investigate the metal ion requirement of APases from cells of strains 154 and 569B, apoenzymes were prepared by dialyzing the enzyme against EDTA. No enzyme activity could be detected when the apoenzyme from cells of strain 154 was assayed. However, apoenzyme from cells of strain 569B retained about 20% activity. The apoenzymes from both sources were assayed for activity in the presence of 1 mM concentrations of various metal ions (Table 2). Ca^{2+} appeared to be the best metal ion for recovery of activity of the apoenzymes. Zn^{2+} had no effect on enzymes from *V. cholerae* cells. Co^{2+}, Mn^{2+}, and Ni^{2+} could restore the activity by 20 to 40%. Significant differences in sensitivity to Na^+, K^+, and Mg^{2+} ions were observed between enzymes from cells of strains 154 and 569B (Table 3). About 80% of the enzyme activity from cells of strain 154 was inhibited by 200 mM NaCl or 5 mM $MgCl_2$. On the other hand at these concentrations of sodium and magnesium ions the enzyme from cells of strain 569B was less affected. Whereas the enzyme from cells of strain 569B was not affected by up to 250 mM KCl, the activity of the enzyme from cells of strain 154 was about 60% inhibited.

Transphosphorylation. It is known that Tris can act as an acceptor for P_i liberated by APase from organic phosphate esters (20). This removal of P_i may result in increased hydrolysis of the substrate. Comparison of K_m and V_{max} values of the enzymes from cells of strains 569B and 154 (Fig. 4) suggested that the two enzymes might differ in their transphosphorylation capability. To investigate this possibility, the kinetics of transphosphorylation was examined. The results presented in Fig. 6 indicated that the two enzymes have different abilities to transfer phosphate. However, the percentage of transphosphorylation at all times was almost identical (about 50%) for the two enzymes. Thus the observed difference in V_{max} of the two enzymes reflects intrinsic properties of the enzymes rather than their difference in transphosphorylation capability.

Substrate utilization. Table 4 shows the relative activity of the purified enzyme(s) on several substrates relative to PNPP. The enzymes from

TABLE 3. Effect of K^+, Na^+, and Mg^{2+} ions on APase activity[a]

Salt	Concn (mM)	% APase activity retained	
		569B	154
NaCl	1	100	100
	5	95	79
	10		73
	20	94	60
	50	79	44
	100	78	28
	200	47	21
$MgCl_2$	0.1	100	100
	0.5	81	68
	2	57	36
	5	55	17
	10	42	13
	40	18	
KCl	0	100	100
	50	100	100
	100	100	74
	250	100	44

[a] APase activities were assayed in the presence of various concentrations of different ions as described in the text.

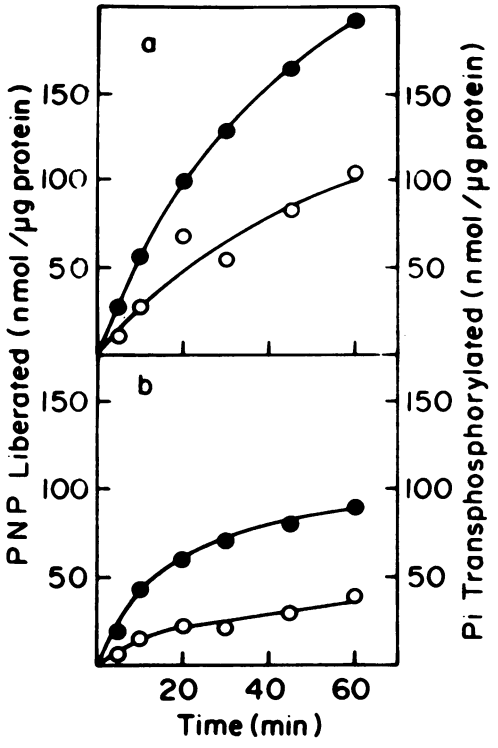

FIG. 6. Transphosphorylation by APase from cells of strains 569B (a) and 154 (b). Samples (1 µg) of the enzymes were incubated with 2 mM PNPP in 1 M Tris-hydrochloride (pH 8.0) at 37°C. PNP (●) and P_i liberated at various time intervals were measured. The amount of missing phosphate represents the P_i transphosphorylated (○).

cells of both strains were unable to hydrolyze ATP and AMP. The ability to hydrolyze P_i and glucose-1-phosphate was different for the two enzymes.

Effect of antibody of APase activity. In the presence of low concentrations of antibody the activity of the enzyme from cells of strain 569B was stimulated by about 30% (Fig. 7). Under identical conditions no change in the activity of the enzyme from cells of strain 154 was observed. This result suggested that the positions of the antigenic determinants of the enzymes from the two strains might be different.

DISCUSSION

The results presented in this report describe the purification and properties of APase from two different strains of *V. cholerae*. The enzymes from cells of strains 569B and 154, being periplasmic, offered a simpler purification procedure (Table 1). The enzymes from these two strains differ significantly in several aspects

TABLE 4. Hydrolysis of different substrates by purified APase of *V. cholerae*[a]

Substrate	P_i liberated relative to that by PNPP with enzyme from strain:	
	569B	154
PNPP	1 (87)[b]	1 (94)
NaPPi	5	2.3
β-Gl-P	0.52	0.6
ATP	ND	ND
AMP	ND	ND
G-6-P	0.67	0.8
G-1-P	5	3.3

[a] Abbreviations: NaPPi, sodium pyrophosphate; β-Gl-P, β-glycerophosphate; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; ND, not detectable.

[b] Numbers within parenthesis are the units of enzyme used.

(Fig. 4 through 7 and Tables 2 and 3). Comparison of the hydrolysis of sodium pyrophosphate and glucose-1-phosphate relative to PNPP by purified enzymes and whole cell lysates of cells from strains 569B and 154 (data not shown) suggests that cells of strain 154 might possess separate inorganic pyrophosphatase and glucose-1-phosphatase activities which are eliminated during the purification steps.

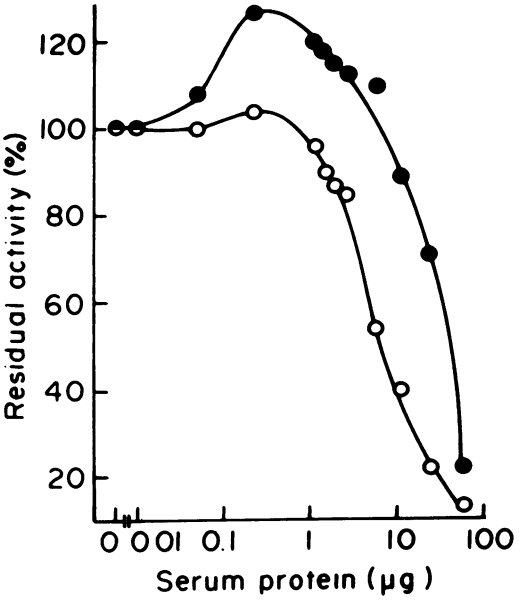

FIG. 7. Effect of APase antibody on the enzyme activity from cells of strains 569B (●) and 154 (○). Samples (1 µg) of enzyme were incubated with various amounts of antiserum raised against APase from cells of strain 569B at 0°C for 60 min, and the residual activity was assayed as described in the text.

The monomeric nature of APase of *V. cholerae* appears intriguing from the point of view of the mechanism of action of the enzyme. APases from all other sources studied so far are dimeric proteins exhibiting negative cooperativity (11), and the monomeric units are inactive at least in *E. coli* (17). Furthermore, whereas the *E. coli* enzyme can be assayed in the presence of 1 M NaCl and requires Mg^{2+} ions for activity (2, 15), and membrane-bound APase of *Bacillus subtilis* is extracted by 1 M magnesium acetate (8), the enzymes from *V. cholerae* cells were inhibited at very low concentrations of these metal ions (Table 3). These properties of *V. cholerae* APase make this protein interesting for further study.

ACKNOWLEDGMENTS

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