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Repression of the Alkaline Phosphatase of *Vibrio cholerae*

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The synthesis of alkaline phosphatase by two strains of *Vibrio cholerae* belonging to the Inaba and Ogawa serotypes has been examined in relation to the phosphate concentration of the culture medium. The synthesis of the enzyme in both strains was repressed in cells grown in the presence of a high concentration of inorganic phosphate. Lowering the phosphate content of the growth medium led to a derepression of enzyme activity. The presence of glucose in low phosphate medium stimulated the degree of derepression. The synthesis of the enzyme by strain Inaba 569B was more sensitive to inorganic phosphate than that of strain Ogawa 154. The enzyme was presumably located in the periplasmic space since it was released when the organisms were converted to spheroplasts.

INTRODUCTION

Vibrio cholerae, the causative agent of cholera, is composed of several different biotypes. The classical strains associated with epidemic cholera can ferment mannose and sucrose, but not arabinose (Mukherjee, 1978). The *eltor* biotype differs from the classical strains in phage susceptibility and some biochemical reactions (Mukherjee, 1978; Finkelstein, 1973). However, *eltor* strains react with anti-*V. cholerae* antisera and taxonomic studies have shown that these two biotypes are of the same species (Citerella & Colwell, 1970; Colwell, 1970).

Interest in the study of *V. cholerae* has recently increased because of considerable progress in the understanding of the molecular mechanisms involved in the action of cholera toxin (Finkelstein *et al.*, 1974; Mekalanos *et al.*, 1978, 1979). Some important advances have been made in the study of the genetic regulation of toxin production (Baine *et al.*, 1978; Gerdes & Romig, 1975; Parker *et al.*, 1979) and transfection has been demonstrated in this system (Balganesh & Das, 1979).

A possible correlation between the amount of toxin produced and the physiology of classical vibrios has been predicted (Honda & Finkelstein, 1979). While strain Ogawa 154 is a mild toxinogenic strain of *V. cholerae*, strain Inaba 569B is a hyper-toxin-producing strain (Mukherjee, 1978; Finkelstein, 1973). We have studied the alkaline phosphatase (APase) from these two strains of *V. cholerae* to examine whether this enzyme might be used as an indicator of serotype or as a marker of toxicity. Synthesis of APase in micro-organisms can be regulated by varying the concentration of P_i in the growth medium: APase synthesis is repressed in medium in which the P_i content is high (Willsky & Malamy, 1976). It has recently been postulated that at least two regulatory genes might be controlling the structural gene for APase (Brickman & Beckwith, 1975; Kreuzer *et al.*, 1975). Several features of this enzyme and some unusual aspects concerning the regulation of its synthesis have made this protein intriguing for further study.

The results presented here show that the APases from *V. cholerae* strains 154 and 569B are periplasmic enzymes and are repressed when the organisms are grown in the presence of high concentrations of P_i.

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METHODS

Organisms and growth media. Vibrio cholerae Ogawa 154 and Inaba 569B strains were obtained from the Cholera Research Center, Calcutta, India. Cultures were stored in a lyophilized state or as frozen stocks at −40 °C in nutrient broth containing 15% (v/v) glycerol. Every 2 months cultures were passaged through guinea-pigs and stock cultures were prepared from single colonies after proper identification of the strain (Mukherjee, 1978).

Cultures were grown at 37 °C in nutrient broth containing 10 g bacteriological peptone (Oxoid), 10 g Lab-Lemco (meat extract) powder (Oxoid), and 5 g NaCl per litre distilled water. The pH of the medium was adjusted to 7·4. Cell growth was assayed by measuring turbidity at 540 nm (A_{540}) using a Gilford model 250 spectrophotometer (an A_{540} of 1·0 corresponded to $1{\cdot}2 \times 10^9$ cells ml^{-1}). The P_i content of the nutrient broth was estimated by the method of Ernster *et al.* (1950) and was found to be 2·5 mM. This medium is referred to as the high phosphate (HP) medium. The low phosphate (LP) medium was phosphate-depleted nutrient broth. To prepare this, magnesia mixture (40 ml) was added to 1 litre nutrient broth (Ghosh *et al.*, 1971) and kept at 4 °C overnight. The precipitate was removed by filtration and the pH was adjusted to 7·4. The P_i content of this medium was 25 μM. In some experiments, 0·1% (w/v) glucose was added to the low phosphate medium; this medium is referred to as LPG medium.

Growth conditions. Cells were grown in 40 ml HP medium for 18 h with shaking at 37 °C and centrifuged at 5000 ***g*** for 10 min. The cell pellet was washed once and resuspended in 2·5 ml LP medium. This suspension was used to inoculate 100 ml LP medium in a 500 ml Erlenmeyer flask to give an initial A_{540} of 0·08. The suspension was incubated at 37 °C with shaking (180 rev. min^{-1}) and samples were removed at appropriate intervals for assaying enzyme activity either in the whole culture or in the cell pellet.

Spheroplast formation. Cells grown to an A_{540} of 1·2 were harvested by centrifugation (9000 ***g***, 5 min) and resuspended in 0·033 M-Tris/HCl buffer (pH 8·0) containing 20% (w/v) sucrose. The cell suspension was chilled to 0 °C and treated successively with 0·1 vol. 0·1 M-EDTA (pH 8·0) and 5 μg lysozyme ml^{-1}. Mixing was achieved by gently rolling the suspension at 0 °C. Spheroplast formation required about 8 to 10 min and was monitored by the method of Malamy and Horecker (1964).

Assay of alkaline phosphatase (APase). APase was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenylphosphate (PNPP). One unit of the enzyme activity is defined as the amount of enzyme that liberates 1 nmol *p*-nitrophenol min^{-1} under the conditions of assay. The reaction mixture contained 2 mM-*p*-nitrophenylphosphate, 1 mM-$MgCl_2$ and the enzyme in a final volume of 2 ml 1 M-Tris/HCl buffer (pH 8·0). The reaction was carried out at 37 °C for 15 min, and then terminated by adding 0·2 ml 26% (w/v) K_2HPO_4. Cell debris was removed by centrifugation at 9000***g*** for 5 min. The absorbance of the supernatant was read at 410 nm (A_{410}) on a Gilford model 250 spectrophotometer. The stability of the enzyme during assay was checked by the method of Selwyn (1965).

APase activity in the whole culture was assayed by adding 0·2 ml culture to the reaction mixture. For assaying activity in the cell pellet, the whole culture was centrifuged at 9000 ***g*** for 5 min and the pellet was resuspended in same volume of 10 mM-Tris/HCl buffer (pH 8·0) containing 1 mM-$MgCl_2$ and 1 mM-$CoCl_2$; 0·2 ml of this suspension was added to the reaction mixture as the enzyme source. The activity released into the medium was measured by assaying 0·2 ml of the 9000 ***g*** supernatant fraction.

RESULTS

Dependence of APase synthesis in V. cholerae on P_i content of growth medium

Synthesis of APase by *V. cholerae* in media containing low (LP, 25 μM) or high (HP, 2·5 mM) concentrations of P_i was examined. Growth of both strain Inaba 569B and strain Ogawa 154 was reduced by 30 to 40% in LP medium compared with growth in HP medium. The initial lag in growth in LP and LPG media relative to that in HP medium was longer for strain 154 than for strain 569B (data not shown). However, APase synthesis was totally repressed in both strains grown in HP medium. Derepression of APase activity was observed when cells were grown in LP medium, and this derepression was enhanced in LPG medium, i.e. LP medium containing 0·1% glucose (Fig. 1). Synthesis of the enzyme began after an initial lag of about 2 h in both LP and LPG media. While enzyme production increased linearly with growth in LP medium, in LPG medium the activity increased much more rapidly to a maximum in about 5 h. The maximum enzyme activity was about twofold higher in LPG medium than in LP medium (Fig. 1).

To determine the minimum concentration of P_i required to repress APase synthesis in *V. cholerae*, cells were grown in LP or LPG medium supplemented with various amounts of P_i

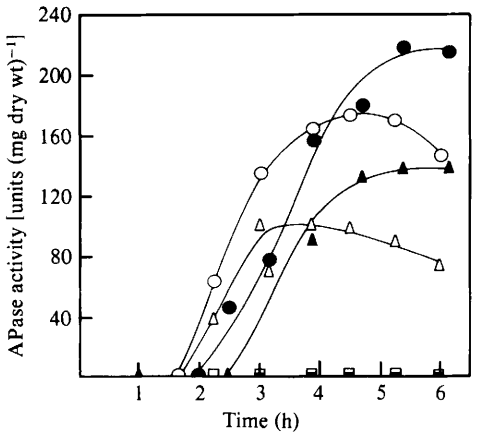

Fig. 1

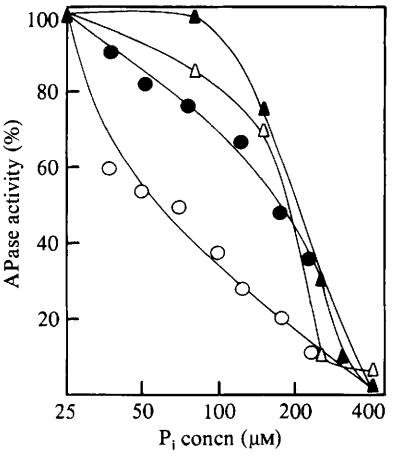

Fig. 2

Fig. 1. APase production by *V. cholerae* in high and low phosphate media. Samples (5 ml) were removed at various times during growth and assayed for enzyme production as described in Methods. APase activity in strains 569B (open symbols) and 154 (closed symbols) grown in HP medium (□, ■), in LP medium (△, ▲) and in LPG medium (○, ●).

Fig. 2. Effect of P_i concentration in the growth medium on APase synthesis by *V. cholerae*. Cells were grown to an A_{540} of 1·2 in LP and LPG media supplemented with different amounts of P_i and harvested by centrifugation (9000 *g*, 5 min). APase activity was assayed in the cell pellet. Activity in strain 154 grown in LP (△) and LPG (▲) media and in strain 569B grown in LP (○) and LPG (●) media [100% activity represents 80 and 120 units (mg dry wt)$^{-1}$ for strain 154 grown in LP and LPG medium, respectively, and 100 and 150 units (mg dry wt)$^{-1}$ for strain 569B grown in LP and LPG medium, respectively].

and the enzyme activity was assayed in the cell pellet (Fig. 2). Synthesis of the enzyme was more sensitive to repression by P_i in strain 569B than in strain 154. The presence of up to 100 μM-P_i had very little effect on APase synthesis in strain 154 grown in LPG medium and the activity was only 15% lower when grown in LP medium. In contrast, APase activity in strain 569B grown in LPG and LP media was decreased by more than 30% and 70%, respectively, in the presence of 100 μM-P_i. At higher concentrations of P_i, a progressive repression of APase synthesis was observed in both strains and total repression occurred at 400 μM (Fig. 2).

Distribution of APase in different subcellular fractions

To determine whether APase in *V. cholerae* cells was intracellular or was released into the medium, cells at different phases of growth in LP or LPG media were harvested and the enzyme activity was assayed in the cell pellet and in the culture supernatant. The total APase activity in the pellet and in the supernatant were compared with the activity in the same volume of the whole culture. About 90% of the whole culture APase activity was recovered in the pellet of strain 569B (Table 1). During the stationary phase of growth activity amounting to about 10% of the activity present in the cell pellet was released into the supernatant. Similar results were obtained for cells grown in LP or LPG medium. In contrast, with strain 154, not more than 70% of the whole culture activity was recovered in the cell pellet (Table 1). No measurable APase activity was found in the culture supernatant. Recovery of activity in the cell pellet of strain 154 was dependent on the age of the culture: the recovered activity increased to a maximum during the late-exponential phase of growth and then decreased. To

Table 1. *Distribution of APase activity in different subcellular fractions*

Cells were grown in LPG medium to an A_{540} of 1·2 and harvested by centrifugation (9000 **g**, 5 min). Spheroplasts were formed as described in Methods. Enzyme activity is expressed relative to that in the whole culture [120 and 150 units (mg dry wt)$^{-1}$ for strains 154 and 569B, respectively].

Fraction	APase activity (%)	
	strain 154	strain 569B
Whole culture	100	100
Cell pellet	67	91
Spheroplasts	ND	11
Periplasmic supernatant*	40	80

ND, No detectable activity.
* Activity in the supernatant after spheroplast formation.

examine whether the incomplete recovery of activity in the cell pellet was due to some cofactor(s) released into the supernatant, in some experiments the supernatant was added to the reaction mixture. No increase in recovery was observed (data not shown). Toluene treatment or sonication of the cell suspension did not result in increased recovery of the activity from strain 154.

More than 60% of the activity in the cell pellet was recovered in the supernatant after spheroplast formation from strain 154 (Table 1) and no enzyme activity was detected in the spheroplasts. With strain 569B, more than 85% of the activity in the cell pellet was released in the supernatant after spheroplast formation and about 10% was retained in the spheroplasts (Table 1). These results suggest that the enzymes of both strains are located in the periplasmic space. Incomplete recovery of APase activity in the cell pellet of strain 154 was not due to any protease degradation. However, the activity of the enzyme was stable under the assay condition used in this study as determined by the criterion described by Selwyn (1965) (data not shown).

When *V. cholerae* APase activity in culture grown in LPG medium was assayed in the standard reaction mixture supplemented with various concentrations of NaCl and $MgCl_2$, a gradual decrease in the enzyme activity was observed with increasing concentration of these salts. About 80% inhibition of APase activity occurred in presence of 250 mM-NaCl in both strains. Inhibition of enzyme activity was more pronounced in the presence of Mg^{2+}: 60–80% inhibition of activity was observed in presence of 20 mM-$MgCl_2$. Biochemical studies on purified enzymes are in progress.

DISCUSSION

The present study describes repression of APase activity in strains of classical vibrios belonging to the two serotypes Inaba and Ogawa (Mukherjee, 1978) which differ in their toxicity. The limiting concentration of P_i resulting in the repression of APase synthesis varies for different organisms. Low concentrations (10 μM) of P_i repress the synthesis of this enzyme in *E. coli* (Torriani, 1960), yet up to about 500 μM is required in some *Bacillus* species (Dobozy & Hammer, 1969). While APase synthesis in strain 154 was minimally affected by the presence of up to 100 μM-P_i, the expression of the enzyme of strain 569B was reduced by more than 30% at this concentration.

The stimulation of APase derepression observed in the presence of glucose has not been demonstrated in any other Gram-negative organism. The mechanisms involved in APase repression or derepression by carbon sources have not been adequately explained. A possible role of gluconeogenic metabolism has been postulated from studies on APases of Gram-positive organisms (Hydrean *et al.*, 1977).

The APase of *V. cholerae* can be released on spheroplast formation by lysozyme and EDTA. Although histochemical evidence is not available, it appears from the present study

that the enzyme is presumably located in the periplasmic space. However, spheroplasts formed by treatment with penicillin did not release the enzyme in the spheroplast supernatant. A similar observation was reported from studies on *E. coli* APase (Malamy & Horecker, 1964).

Subculture of strain 596B in the laboratory reduces its toxicity. It has been observed that *in vivo* stability of APase was reduced along with the toxicity of the strain. However, the stability of the enzyme from strain 154 was not affected by laboratory subculturing (unpublished observations). In addition, the capability of strain 569B to repair damage to DNA induced by ultraviolet light (Das *et al.*, 1981) was also reduced with the reduction of toxicity. These functions can be restored by animal passage of these cells (Das *et al.*, 1981; and unpublished observations). The APase thus might be useful as a marker of toxicity.

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