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Derepression of phosphomonoesterase and phosphodiesterase activities in *Aerobacter aerogenes*

Seeking a hydrolase for simple phosphodiesters, we have obtained by elective culture microorganisms capable of growth on dimethyl phosphate as sole phosphate source. Hydrolases for monoesters and diesters of phosphoric acid, present at low levels under normal growth conditions, appear abundantly in cells grown on dimethyl phosphate.

Barium salts of dimethyl phosphate and monomethyl phosphate were prepared, respectively, by hydrolysis of trimethyl phosphate[1] and by methylation of sodium phosphate[2]. Barium was removed with Dowex 50 (H^+). Neither preparation contained measurable inorganic phosphate[3]. Monomethyl phosphate, but no trace

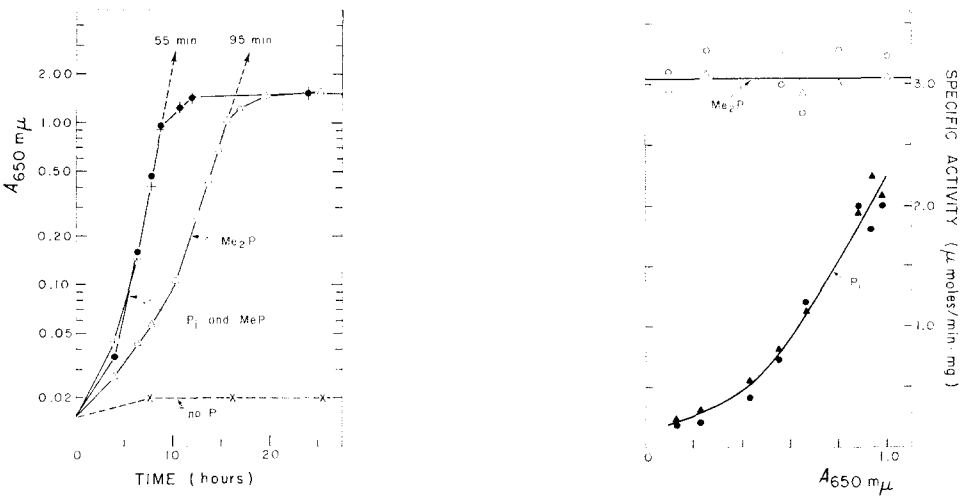

Fig. 1. Growth of shaken aerobic cultures of *A. aerogenes* at 37° on minimal salts–glucose medium, without added phosphate (×), with inorganic phosphate (●), with methyl phosphate (MeP) (+), and with dimethyl phosphate (Me_2P) (△), each at an initial concentration of $1 \cdot 10^{-4}$ M. Cultures were inoculated at time zero with cells grown to stationary phase on dimethyl phosphate.

Fig. 2. Specific activity of phosphomonoesterase (circles) and phosphodiesterase (triangles) during growth on inorganic phosphate (closed symbols) and on dimethyl phosphate (Me_2P) (open symbols); conditions as in Fig. 1. Cells grown on monomethyl phosphate (not shown) gave results similar to those grown on inorganic phosphate. Monoesterase was assayed on $8 \cdot 10^{-4}$ M *p*-nitrophenyl phosphate in 0.7 M Tris–HCl buffer, pH 8.0. Diesterase was assayed on $3 \cdot 10^{-4}$ M di-*p*-nitrophenyl phosphate in the same buffer with $1 \cdot 10^{-2}$ M magnesium chloride added. Specific activity is expressed in terms of wet weight of bacteria extracted, based on the finding that 1 g of bacteria gave an absorbance of 0.95 (1 cm, 650 mμ). Activity was measured at 25°.

of dimethyl phosphate, was completely hydrolyzed to inorganic phosphate by *Escherichia coli* alkaline phosphatase (Worthington Corporation). After hydrolysis in sealed tubes for 24 h at 110° in 6 M perchloric acid, both preparations gave the expected yield of inorganic phosphate. Elective cultures of sewage on a medium[4] supplemented with $2 \cdot 10^{-4}$ M dimethyl phosphate as sole phosphate source yielded

bacteria with fermentation patterns characteristic of Aerobacter. Investigations of type cultures showed that *Aerobacter aerogenes*, American Type Culture Collection No. 13048 (but neither *Aerobacter cloacae*, ATCC No. 13047, nor *E. coli*, ATCC No. 11775) grows well on this phosphate source.

Fig. 1 shows the growth behavior of *A. aerogenes* on glucose–minimal salts (the medium above without peptone) in the absence of phosphate and in the presence of growth-limiting concentrations ($1 \cdot 10^{-4}$ M) of inorganic phosphate, methyl phosphate, and dimethyl phosphate. In separate experiments the final growth level was found to become independent of phosphate at concentrations above $3 \cdot 10^{-4}$ M under these conditions. Fig. 1 shows that in the absence of phosphate there was negligible growth. In the presence of inorganic phosphate or monomethyl phosphate growth was rapid, with a generation time of 55 min in both cases. In the presence of dimethyl phosphate the generation time was 95 min. The final level of growth was the same on all three phosphate sources, and no phosphate (less than a detectable $2 \cdot 10^{-6}$ M) remained in the centrifuged medium at stationary phase. With trimethyl phosphate (not shown) the growth response was similar to that obtained with dimethyl phosphate; however this compound was found to decompose spontaneously to dimethyl phosphate in the growth medium.

Treatment of the harvested cells with lysozyme and versene[5] released activities for hydrolysis of mono- and di-*p*-nitrophenyl esters of phosphoric acid. Recovery was similar to that obtained by grinding the cells with alumina. Neither activity was found in detectable amounts in the growth medium. Fig. 2 shows that cells grown on inorganic phosphate or monomethyl phosphate contained low specific activities of both enzymes early in growth, rising to much higher levels before the end of log phase. In contrast, cells grown on dimethyl phosphate maintained a high content of both enzymes throughout logarithmic growth. In separate experiments, the addition of inorganic phosphate to cultures growing on dimethyl phosphate resulted in a halt in the appearance of new enzyme activities of both types, although growth was accelerated.

The ratio between phosphomonoesterase and phosphodiesterase activities remained nearly constant throughout growth on all phosphate sources. The numerical similarity between the molar specific activities of the two enzymes (Fig. 2) is fortuitous. Under the conditions of the standard assays the ratio of substrate concentration to K_m (see below) was considerably higher for the monoesterase than for the diesterase.

Monoesterase and diesterase activities appear to reflect distinct proteins. Upon addition of ammonium sulfate to 80% saturation at 4°, diesterase activity was precipitated quantitatively from dilute cell extracts, leaving 65% of the monoesterase activity in solution. When the precipitate was redissolved in water, the diesterase activity was recovered. The enzymes also differed in sensitivity to heat, mercurials, and phosphate inhibition. Incubation of extracts for 10 minutes at 70° destroyed 85% of diesterase activity but only 10% of monoesterase activity. When the initial rates of hydrolysis of *p*-nitrophenyl substrates ($1 \cdot 10^{-3}$ M) were compared in the presence and absence of mersalyl ($1 \cdot 10^{-3}$ M), monoesterase activity was unaffected whereas diesterase activity was reduced to 24% of the value obtained in the absence of mercurial. The effect of substrate concentration on reaction rate was similar for the two enzymes with approximate K_m values of $1.7 \cdot 10^{-3}$ M for *p*-nitro-

phenyl phosphate and $1.3 \cdot 10^{-3}$ M for di-*p*-nitrophenyl phosphate under the usual assay conditions. However the monoesterase activity was the more strongly inhibited by inorganic phosphate, and was the only one of the two enzymes to be inhibited by monomethyl phosphate. K_i for inorganic phosphate was approx. $3 \cdot 10^{-4}$ M for the monoesterase and $5 \cdot 10^{-3}$ M for the diesterase. The initial rate of breakdown of *p*-nitrophenyl phosphate was strongly reduced by the presence of high concentrations of monomethyl phosphate as expected for an alternate substrate. The rate of breakdown of di-*p*-nitrophenyl phosphate was unaffected. The kinetic parameters for the two enzymes are such that initial rate measurements for diesterase were not appreciably affected by monoesterase action on the product.

Since the discovery of repression of alkaline phosphatase in *E. coli*[6,7], repression of enzymes by inorganic metabolites has been detected in several microorganisms; sulfate and phosphate have been found to repress hydrolases for their respective monoesters in *A. aerogenes*[8,9]. Coordinate repression by complex organic end-products has been recognized in several metabolic pathways[10]. In the present system an inorganic metabolite appears to produce coordinate repression of enzymes involved in its own production.

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Identification of the water radicals involved in X-ray inactivation of enzymes in solution and determination of their rate of interaction with the enzyme

The inactivation of enzymes irradiated in dilute aqueous solution is due to the action of water radicals ($OH^{\cdot}$, e^-_{aq}, O_2^- *etc.*). It is the purpose of the present paper to demonstrate that in cases where the enzyme inactivation is primarily due to one type of water radical, it is possible from competition experiments to identify this