Alkaline Phosphatases from *Mycobacterium smegmatis*

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Mycobacterium smegmatis formed an alkaline phosphomonoesterase (EC 3.1.3.1) and an alkaline phosphodiesterase (EC 3.1.4.1). The former was regulated by inorganic phosphate and was repressed in medium containing a high concentration of phosphate. The synthesis and the function of the alkaline phosphodiesterase were not controlled by inorganic phosphate.

INTRODUCTION

The alkaline phosphatases are regulated differently in different micro-organisms. In some, the alkaline phosphomonoesterase is synthesized only when the concentration of orthophosphate in the medium is limiting (Bhatti, 1974; Garen & Echols, 1962; Horiuchi, Horiuchi & Mizuno, 1959; Stewart & Stewart, 1971; Taniguchi & Tsugita, 1966; Torriani, 1960). In *Staphylococcus aureus* and in *Bacteroides ruminicola*, the enzyme is constitutive (Cheng & Costerton, 1973; Kuo & Blumenthal, 1961). *Bacillus subtilis* also forms a phosphodiesterase that, like its phosphomonoesterase, is repressible by orthophosphate (Taniguchi & Tsugita, 1966; Takeda & Tsugita, 1966). These enzymes have not been reported before in the mycobacteria. In view of their possible taxonomic importance, it was of interest to establish their occurrence, and to examine their regulation in such bacteria.

METHODS

Bacteria and growth conditions. *Mycobacterium smegmatis* H34495 was from the culture collection of the Institut Pasteur, Paris, and was kindly supplied by Henry Boisvert. Bacteria were grown in a modified Proskauer & Beck medium containing (g l⁻¹): ferric ammonium citrate, 0.05; citric acid, 2.0; glycerol, 2.0; potassium sulphate, 0.6; dipotassium phosphate, 4.0; sodium glutamate, 1.75; magnesium chloride, 1.2; and Tween 80, final concentration 0.05 % (w/v). The pH was adjusted to 6.8 to 7.0 before autoclaving. When required, the medium was prepared without phosphate and phosphate was added to the desired final concentration. Cultures were incubated in a New Brunswick R86 water-bath reciprocal shaker at 75 rev. min⁻¹ and at 37 °C.

Growth was estimated by monitoring the extinction of cultures at 650 nm using a Coleman Jr model A20 spectrophotometer (Coleman Instruments, New Jersey, U.S.A.). In the exponential phase of growth, 1.0 mg dry wt bacteria ml⁻¹ was equivalent to an extinction of 0.62 at 650 nm. When growth was allowed to continue beyond the exponential phase, clumping occurred and so the mass was estimated by weighing bacterial suspensions washed in distilled water and dried in an oven at 80 °C to constant weight.

Assay of alkaline phosphatase. The alkaline phosphatases were usually assayed in reaction mixtures containing washed bacteria (or enzyme protein) in 1.0 ml 0.05 M-Tris/HCl buffer, pH 8.0, containing 1 mM-MgSO₄, plus 1.0 ml 20 mM-*p*-nitrophenyl phosphate (NPP) or 10 mM-bis-*p*-nitrophenyl phosphate (bis-NPP) (both from Sigma). The reaction mixtures were incubated at 37 °C for the desired time, and 1.0 ml 1.0 M-Na₂CO₃ was then added. The amount of nitrophenol liberated was estimated spectrophotometrically at 410 nm using an appropriate calibration curve. Enzyme activities are expressed as pmol nitrophenol liberated per mg dry wt bacteria per hour.

Phosphorus estimations. Inorganic phosphorus (P_i) was assayed by the Fiske & Subbarow method (Dryer, Tammes & Routh, 1957).

Fig. 1

Fig. 2

Fig. 1. Effect of pH on alkaline phosphomonoesterase (○) and phosphodiesterase (●). Buffers of the indicated pH were prepared by mixing Tris and Tris hydrochloride. Enzyme activities are expressed as pmol nitrophenol liberated (mg dry wt)⁻¹ h⁻¹.

Fig. 2. Differential rate of synthesis of alkaline phosphomonoesterase (○) and phosphodiesterase (●). A culture was grown to an extinction at 650 nm of 0.1, and then samples were taken at intervals for 48 h. Each sample was filtered through a 0.8 μ m pore-size Millipore filter, and the enzyme activities were measured in the bacteria trapped in the filters (WS) and in the filtrate (DS). The initial and final concentrations of P_i in the medium were 25.7 and 20.0 mM respectively. Inset: growth curve of *M. smegmatis*. Enzyme activities are expressed as pmol nitrophenol liberated (mg dry wt)⁻¹ h⁻¹.

Preparation of extracts. Washed bacteria were added to about twice their volume of washed sand and then frozen. The frozen material was finely broken and then manually ground in a mortar in an ice bath until completely melted. Tris/HCl buffer, pH 8.0, containing 1 mM-MgSO₄ was added and the mixture was centrifuged at 5000g for 20 min. The supernatant was filtered through a 0.8 μ m pore-size Millipore filter, and the crude enzyme preparation was precipitated with (NH₄)₂SO₄ at 0 to 40 % saturation (fraction I) and 40 to 80 % saturation (fraction II).

RESULTS

Preliminary observations

Bacteria harvested during the stationary phase of growth (2- to 3-week-old cultures) hydrolysed NPP and bis-NPP without a lag. These observations suggested that *M. smegmatis* formed a phosphomonoesterase (mono-AP) and a phosphodiesterase (di-AP). Most of the mono-AP in cell-free extracts was precipitated by ammonium sulphate at 0 to 40 % saturation and most of the di-AP was precipitated at 40 to 80 % saturation, indicating that the hydrolysis of NPP and bis-NPP was caused by different enzymes.

Properties of the enzymes

The optimum pH for mono-AP was about 7.8 and for di-AP was about 8.4 (Fig. 1). The amount of nitrophenol released was proportional to the concentration of NPP up to about 5 mM, and to the concentration of bis-NPP up to about 7.5 mM, as well as to the cell concentration in the reaction mixtures.

Table 1. Utilization of P_i by *M. smegmatis*

P_i concn (mM)		Bacterial dry wt (mg ml ⁻¹)	P_i utilized (μ mol mg ⁻¹)
Initial	Final*		
200	46.7	5.2	29.48
160	44.0	4.5	25.78
120	42.0	3.8	20.53
80	31.0	3.4	14.41
40	24.3	2.7	5.81
20	11.7	1.6	5.19

* After 10 days incubation at 37 °C.

Fig. 3. Effect of P_i on the synthesis and expression of alkaline phosphomonoesterase. Bacteria grown in high- P_i medium were harvested by centrifugation, washed, divided into two portions and added to (●) P_i -free medium or (○) high- P_i (20 mM) medium. Samples were taken at the indicated intervals, the bacteria were washed and the enzyme activity was measured in the presence of 100 μ g streptomycin ml⁻¹. Enzyme activities are expressed as pmol nitrophenol liberated (mg dry wt)⁻¹ h⁻¹.

The K_m values of the partially purified enzymes were about 0.7 mM and 0.01 mM for NPP and bis-NPP, respectively. Mono-AP was inhibited by P_i ($K_i = 0.3$ mM), but di-AP was not sensitive to P_i at the concentration used in these experiments (10 mM). However, di-AP was about 30 % inhibited by 100 mM- P_i . The ratio K_i/K_m was 0.43 for mono-AP and 0.92 for di-AP.

Effect of P_i on growth and enzyme synthesis

To study the effect of P_i on growth and on synthesis of the phosphatases, bacteria were inoculated into media containing between 1 and 200 mM- P_i . The cultures were incubated for 10 days at 37 °C, and the amount of growth, the final concentration of P_i and the enzyme activities were then estimated. There was no growth at P_i concentrations below 15 mM and P_i was in excess at above about 40 mM. At the end of the experiment (10 days incubation) unused P_i remained in the medium (Table 1). The activity of mono-AP was highest in the medium containing the lowest final concentration of P_i , whereas the di-AP activity was independent of the P_i concentration. When the enzyme activities were measured in bacteria harvested at different stages of the growth curve, mono-AP was virtually absent at all times (Fig. 2). The increase in the differential rate of synthesis of di-AP at cell densities above 1.0 was possibly due to increased permeability, and some cell lysis that usually occurs when bacterial cultures reach the stationary phase of growth.

Regulation of mono-AP

The above observations clearly indicated that di-AP was synthesized constitutively and that its activity was not inhibited by P_i . On the other hand mono-AP was inhibited by P_i , and since no activity was detected in washed bacteria grown in high- P_i medium it was possible that the synthesis of the enzyme was repressed. To examine the regulation of mono-AP,

bacteria grown in high- P_i medium were washed and divided between two vials containing P_i -free medium and high- P_i medium respectively. The vials were incubated in a reciprocal shaker and at intervals (see Fig. 3) samples were withdrawn and mono-AP activity was measured. Mono-AP activity began to be expressed about 50 min after the bacteria had been transferred to P_i -free medium (Fig. 3). This was not caused by the removal of P_i when the harvested bacteria were washed to perform the assay since bacteria in the second vial (containing high- P_i medium) showed no activity.

DISCUSSION

Mycobacterium smegmatis synthesized two non-specific alkaline phosphatases. Evidence that the hydrolysis of NPP and bis-NPP was caused by distinct enzymes was provided by the observations that the activities exhibited distinct optimum pH values, had distinct K_m values, were affected differently by P_i , were regulated differently, and could be partially separated by ammonium sulphate precipitation.

Alkaline phosphomonoesterase (optimum pH about 7.8) was not found in the bacteria grown in high P_i -medium (initial concentration 20 to 25 mM), but was fully expressed in bacteria allowed to reach the stationary growth phase (grown for 2 to 3 weeks). The enzyme activity was dependent on the concentration of P_i in the medium at the time when the bacteria were harvested. When the bacteria were grown in high- P_i medium, washed to remove P_i and resuspended in P_i -free medium, synthesis of mono-AP began after a lag of about 50 min. Thus, as in *E. coli* (Garen & Garen, 1963; Torriani, 1960) and other microorganisms, the mono-AP from *M. smegmatis* was regulated by the concentration of P_i in the medium.

Throughout this investigation, di-AP activity was detected irrespective of the presence of P_i in the growth medium. We, therefore, concluded that di-AP (optimum pH about 8.4) was constitutive and was not controlled by orthophosphate.

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