SHORT COMMUNICATIONS

Studies on Mg^{2+} - (Ca^{2+} -)activated Adenosine Triphosphatase from *Mycobacterium smegmatis* CDC 46

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(Received 8 November 1977)

INTRODUCTION

Adenosine triphosphatase (ATPase) activities have been demonstrated in many bacteria (Abrams & Smith, 1974). In most of these, ATPase activities are associated with the cell membrane structure. The enzyme is activated by Mg^{2+} or Ca^{2+} or both, is not inhibited by ouabain and is not usually stimulated by monovalent cations (Bonting, Caravaggio & Hawkins, 1962). The enzyme has been solubilized in some species, and its properties then differ from those of the membrane-bound enzyme (Harold & Baarda, 1968). This paper describes the partial characterization of ATPase of a fast-growing mycobacterial species, *Mycobacterium smegmatis* CDC 46.

METHODS

Chemicals. AMP, ADP, ATP, Atebrin, L-asparagine, Chlorpromazine, dithiothreitol, *N,N'*-dicyclohexylcarbodiimide (DCCD), 2,4-dinitrophenol, CTP, GTP, iodoacetic acid, ITP, β -mercaptoethanol, oligomycin, ouabain, *p*-chloromercuribenzoate (PCMB) and UTP were obtained from Sigma.

Organism, media and culture conditions. *Mycobacterium smegmatis* CDC 46 was grown at 37° C as surface cultures on Youmans & Karlson (1947) liquid medium in which magnesium citrate was replaced by magnesium carbonate and citric acid.

Preparation of cell-free extract. Bacteria in the late-exponential growth phase (4 d) were harvested and washed three times with cold glass-distilled water, suspending the bacteria using a magnetic stirrer and centrifuging at 5000 *g* for 15 min at 5° C. Cell suspensions (20 %, wet wt/v) in 0.05 M-Tris/HCl buffer (pH 7.4) were sonicated for 2 min in a MSE 100 W ultrasonic disintegrator at a frequency of 20 kHz, keeping the sample container in a beaker of ice. The sonicated material was centrifuged at 10000 *g* for 30 min at 5° C to obtain the cell-free extract.

Isolation of particles. The method followed was essentially that of Brodie (1959) with slight modifications. The cell-free extract was separated into soluble and particulate fractions by centrifugation at 144000 *g* for 1 h at 0 to 4° C in a Beckman model L2-65 B ultracentrifuge. Particles were washed with 0.05 M-Tris/HCl buffer (pH 7.4) and collected by centrifugation at 144000 *g* for 30 min. Washed particles suspended in the same buffer were designated the particulate fraction.

Enzyme assay. Adenosine triphosphatase (ATP phosphohydrolase; EC 3.6.1.3) activity was measured as the release of inorganic phosphate from ATP using the procedure of Evans (1969) with slight modifications. The assay mixture contained (in 1 ml): Tris/HCl buffer (pH 8.0), 100 mM; ATP, 5 mM; $MgCl_2$ or $CaCl_2$, 2 mM; and enzyme protein. The tubes were incubated at 37° C. After 30 min, the enzyme was inactivated by adding 1 ml of chilled 10% (w/v) trichloroacetic acid (TCA) and the tubes were kept in ice for 15 min. Precipitated proteins were removed by centrifugation. A control was run simultaneously, in which enzyme was added after TCA at the end of the incubation period. Inorganic phosphate was measured according to the procedure of Fiske & SubbaRow (1925). Protein was estimated by the method of Lowry *et al.* (1951).

Table 1. *Subcellular localization of adenosine triphosphatase in M. smegmatis CDC 46*

Bacteria were harvested in the late-exponential phase of growth and cell-free extract was prepared as described in Methods. Cell-free extract was centrifuged at 144000 g to obtain particulate and supernatant fractions. ATPase activity was measured as described in Methods; results are expressed as $\mu\text{mol P}_i$ liberated $(30 \text{ min})^{-1} (\text{mg protein})^{-1}$.

Fraction	ATPase activity	
	Mg ²⁺ -activated	Ca ²⁺ -activated
Cell-free extract	1.140	0.600
Supernatant	0.510	0.300
Particulate	0.900	0.570

RESULTS AND DISCUSSION

The preparation obtained from *M. smegmatis* CDC 46 contained Mg²⁺-(Ca²⁺-)activated adenosine triphosphatase (Table 1). More inorganic phosphorus was liberated in the presence of Mg²⁺ than with Ca²⁺. When neither of these ions was added to the reaction mixture, hydrolysis of ATP was negligible.

There was no evidence of a (Na⁺ + K⁺)-activated ATPase in this organism. Attempts to localize the enzyme revealed that the particulate fraction had a higher specific activity than the supernatant. The particulate fraction was therefore used as the source of enzyme for further studies. Incubation of the reaction mixture at 20, 25, 30, 35, 37 and 40 °C showed that in presence of either Mg²⁺ or Ca²⁺ maximum inorganic phosphorus was liberated at 37 °C. Enzyme from other bacterial sources has been shown to have an optimum temperature for incubation of 37 °C (Evans, 1969) or 30 °C (Higashi *et al.*, 1975). The optimum concentrations of Mg²⁺ and Ca²⁺ in the assay system were 2.5 and 3.0 mM, respectively.

Mg²⁺-activated ATPase was active over a broad range of pH values, with an optimum at approximately pH 8.8. The activity of Ca²⁺-activated ATPase increased sharply above pH 7.0 with an optimum at pH 9.2. The results reported in the present investigation are in close agreement with those reported for ATPase from other bacteria (Abrams, 1965; Evans, 1969).

To determine whether two independent ATP-hydrolysing systems were present in the preparation studied (one activated by Ca²⁺ and the other by Mg²⁺), the amounts of P_i released in reaction systems containing only Mg²⁺ or both Ca²⁺ and Mg²⁺ were compared. Similar amounts of P_i were released in these two systems and therefore the ATP-hydrolysing activities elicited by Ca²⁺ and Mg²⁺ were probably due to a single enzyme.

Substrate specificity. The different nucleotides were hydrolysed by the enzyme preparation in the order ATP > GTP > ITP > UTP > CTP > ADP; AMP was not hydrolysed. Experiments were carried out to find out whether these activities were attributable to a single enzyme. When each of the nucleotides was added to a reaction mixture in the presence of excess of ATP, there was no cumulative release of inorganic phosphorus indicating that the same enzyme hydrolysed all the different nucleotides. Hydrolysis of all the nucleotides was inhibited by PCMB to the same degree, further indicating the involvement of only one enzyme.

Thermostability. The enzyme preparations were pre-incubated for 15 min at different temperatures and residual ATPase activities were then measured. At all temperatures studied, the activities elicited by Ca²⁺ and Mg²⁺ were inactivated equally and there was no selective inhibition as reported in *Bacillus megaterium* (Greenawalt, Weibull & Low, 1962). There was only 20% loss of enzyme activity at 40 °C, but at higher temperatures (60 and 70 °C) a loss of 80% was observed.

Effect of inhibitors and activators. Various potential inhibitors and activators were tested for their effect on enzyme activity at concentrations in the range 0.05 to 1.0 mM. Dithionite, 2,4-dinitrophenol, dithiothreitol, β -mercaptoethanol, glutathione, cysteine, EDTA and

ouabain had no effect. Azide inhibited activity by 100% at 1 mM, whereas cyanide and fluoride inhibited it by only 60 to 70% at this concentration. Of the inhibitors of the respiratory chain that were tested, Atebrin exhibited 40% inhibition at 1 mM, but oligomycin and Chlorpromazine had no effect. The sulphhydryl inhibitors, PCMB, iodoacetic acid and mercuric chloride, inhibited activity by 40% at 1 mM.

N,N'-dicyclohexylcarbodiimide (DCCD) at 1 mM inhibited activity by almost 75%. Since the inhibition of Mg^{2+} - and Ca^{2+} -activated ATPase by DCCD in *S. faecalis* (Harold & Baarda, 1968) and *E. coli* (Evans, 1970) has been shown to be time-dependent, the ATPase preparations from *M. smegmatis* CDC 46 were pre-incubated with DCCD for different periods. Pre-incubation of enzyme preparations with DCCD for 5 min caused less inhibition than pre-incubation for 15 min.

Influence of Na^+ and K^+ ions. The effects of Na^+ and K^+ ions were studied by adding NaCl or KCl at different concentrations in the presence of either Mg^{2+} or Ca^{2+} . Activities of Mg^{2+} - and Ca^{2+} -activated ATPase were stimulated equally by Na^+ or K^+ up to a concentration of 60 mM. The pattern of stimulation by Na^+ or K^+ was the same for both Mg^{2+} - and Ca^{2+} -activated ATPase activities. Mixtures of Na^+ and K^+ showed no additive stimulating effect. A number of other microbial ATPases have been shown to be stimulated by Na^+ and/or K^+ ions (Abrams, McNamara & Johnson, 1960; Hayashi & Uchida, 1965).

Miss Gulshan Grover is grateful to the Indian Council of Medical Research, New Delhi, for the award of a Junior Research Fellowship. This investigation was supported in part by grants from the Indian Council of Medical Research, New Delhi, and the University Grants Commission, New Delhi.

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