Adenylate Cyclase in *Mycobacterium smegmatis*

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INTRODUCTION

The intracellular concentration of cyclic AMP depends on the relative activities of adenylate cyclase and phosphodiesterase. Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] catalyses the formation of cyclic AMP and pyrophosphate from ATP. Cyclic AMP and phosphodiesterase have been identified in *Mycobacterium smegmatis* (Lee, 1977, 1978) and the present paper describes some properties of the adenylate cyclase in this bacterium.

METHODS

Culture and harvest of micro-organisms. *Mycobacterium smegmatis* (NCTC 523) was cultured as previously described (Lee, 1977, 1978). Harvested bacteria were stored at -80°C .

Preparation of extracts. Frozen bacteria (2.1 g wet wt) were thawed, suspended in 4 ml 0.25 M-Tris/HCl buffer (pH 7.3) and ultrasonically disintegrated at 0°C at 10 kHz for 80 s with a 100 W instrument. The homogenate was centrifuged at 4000 g for 10 min at 4°C to remove unbroken cells. The supernatant fraction was then centrifuged at 43 500 g for 20 min at 4°C . The supernatant was discarded and the pellet was suspended in 4 ml 0.25 M-Tris/HCl buffer (pH 7.3) with a glass homogenizer. This suspension contained all the adenylate cyclase activity and was stable for at least 4 d at -80°C . Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Determination of K_m for adenylate cyclase was done with extracts prepared in 0.25 M-Tris/HCl buffer (pH 9.0).

Assay of adenylate cyclase. The assay mixture (total volume 150 μl) contained: 12.5 μmol Tris/HCl buffer (pH 9.0), 2.7 μmol MgSO_4 , ATP (various concentrations) and extract (0.4 mg protein). The mixture was incubated for an appropriate time (3 to 5 min) at 37°C and the reaction was stopped by adding 100 μl of cold 0.25 M- ZnSO_4 followed by 100 μl of cold 0.25 M- $\text{Ba}(\text{OH})_2$. The tubes were mixed and centrifuged at 10 000 g for 20 min at 4°C . A sample of the supernatant (150 μl) was applied to a column (2.5 $\times$ 0.5 cm) of anion exchange resin (Bio-Rad, AG1-X2, 200 to 400 mesh). The column was washed with 5 ml water and cyclic AMP was eluted with 2 ml HCl (pH 1.5). A sample of the effluent was neutralized with 0.5 M-NaOH and assayed for cyclic AMP. In some experiments, including those on the effects of metal ions on adenylate cyclase activity, cyclic AMP was assayed directly in the supernatant after precipitation with ZnSO_4 and $\text{Ba}(\text{OH})_2$. Both methods gave the same results. In all experiments less than 9 % of the substrate was utilized and formation of cyclic AMP was linear with time and enzyme concentration. The identity of cyclic AMP was confirmed by digestion with phosphodiesterase and adsorption with charcoal.

The modified version of the assay was also used in studies on the effect of metabolites (and other compounds) on adenylate cyclase activity. In these experiments, the mixture (total volume 200 μl) contained: 12.5 μmol Tris/HCl buffer (pH 7.3), 0.3 μmol ATP, 4.0 μmol MnCl_2 , 0.2 μmol metabolite (or other compound) and extract (0.53 mg protein). The reaction mixture was incubated at 37°C for 4 min and the reaction was stopped by adding 1 ml of cold 0.25 M- ZnSO_4 followed by 1 ml of cold 0.25 M- $\text{Ba}(\text{OH})_2$. After centrifuging at 10 000 g for 20 min at 4°C , 10 μl of the supernatant were assayed for cyclic AMP. Enzyme activity in the absence of added compounds was 3.02 nmol cyclic AMP formed min^{-1} (mg protein) $^{-1}$. Results are the average of triplicate determinations. All compounds were prepared just before use by dissolving in 0.06 M-Tris/HCl buffer (pH 7.3). Suitable controls showed that the reaction mixture was maintained at pH 7.3 and the added compounds did not affect the assay for cyclic AMP.

Measurement of cyclic AMP. Cyclic AMP was assayed by the competitive protein binding procedure (Gilman, 1970).

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Table 1. *Effect of cations at their optimum concentrations on adenylate cyclase activity*

Activity was assayed at pH 7.3 by the modified procedure described in Methods. Adenylate cyclase activity in the absence of added cation (taken as 100 %) was 2.8 nmol cyclic AMP formed min^{-1} (mg protein) $^{-1}$. The various cations (used as chlorides) did not affect the assay for cyclic AMP.

Cation	Optimum concn (mM)	Activity (% of control)
Mn ²⁺	8.5	6113
Co ²⁺	6.67	4267
Fe ²⁺	6.67	2591
Ca ²⁺	6.67	560
Cd ²⁺	1.33	404
Zn ²⁺	1.33	383
Ni ²⁺	6.67	358
Ba ²⁺	13.3	283
Mg ²⁺	3.0	275
Cu ²⁺	0.13	258
Fe ³⁺	1.33	136
Na ⁺	13.3	129
Li ⁺	0.33	106

Liquid scintillation counting. Radioactivity was determined as described by Lee (1978).

Materials. All the organic compounds tested for their effects on adenylate cyclase activity were obtained from Sigma.

RESULTS

Stability of enzyme. The adenylate cyclase of *M. smegmatis* was reasonably stable and could be left at 3 °C for 5 h or stored at -80 °C for at least 4 d without any loss of enzyme activity.

Determination of K_m . The K_m value for ATP at pH 7.3 and 9.0 was from 1.0 to 1.3 mM.

Effect of pH. Maximum enzyme activity occurred at pH 7.5, with only 45 % of the maximum activity at pH 9.0.

Effect of cations. At 3.0 mM-Mg²⁺, adenylate cyclase activity was stimulated by 175 % and increasing the Mg²⁺ concentration up to 13.3 mM did not decrease enzyme activity. Amongst the metal ions tested (Table 1), Mn²⁺, Co²⁺ and Fe²⁺ were the most stimulatory. Enzyme activity was maximum with 8.5 mM-Mn²⁺ and remained unchanged up to 26.6 mM. Mn²⁺ was about 20 times as effective as Mg²⁺ if both were tested at their optimum concentrations. At relatively low concentrations (0.6 mM), Cu²⁺ was inhibitory, while Zn²⁺ was inhibitory only at 9.0 mM. In the presence of Fe³⁺ (1.33 mM) a slight stimulation of enzyme activity was observed but the enzyme was inhibited at concentrations greater than 2.3 mM. Al³⁺ at all concentrations inhibited enzyme activity. EDTA (6.3 mM) abolished enzyme activity in the absence of added cations. No marked effect on the adenylate cyclase was observed with any monovalent cation tested; NH₄⁺, Cs⁺ and K⁺ had no effect on the enzyme.

Effect of fluoride and hormones. Fluoride (1 to 5 mM) inhibited enzyme activity by 72 % but adrenaline (0.3 to 0.85 $\mu\text{g ml}^{-1}$) and glucagon (0.3 to 0.75 $\mu\text{g ml}^{-1}$) had no effect on the activity.

Effect of metabolic intermediates and related compounds. All compounds were tested at 1 mM, and percentage inhibitions are shown in parentheses. The following compounds inhibited the adenylate cyclase of *M. smegmatis*: phosphoenolpyruvate (74 %), citrate (71 %), L-malate (70 %), oxaloacetate (32 %), succinate (31 %), L-lactate (20 %), glucose 6-phosphate (18 %), 2-oxoglutarate (16 %) and pyruvate (14 %). This inhibition was not caused by depletion of Mn²⁺ which was present in excess in the reaction mixture.

In view of the importance of glycerol metabolism in this bacterium (Clark, 1971), the following compounds were tested and all were shown to inhibit activity: NADP⁺ (76 %), NAD⁺ (65 %), NADH (26 %), dihydroxyacetone (19 %), α -glycerophosphate (17 %), glycerol (8 %) and NADPH (7 %).

Thiamin pyrophosphate (90 %), aneurine (64 %) and pyridoxine (56 %) inhibited adenylate cyclase activity. Lysine plus pyridoxine inhibited enzyme activity by 81 % whereas lysine relieved inhibition in *Escherichia coli* (Tao & Huberman, 1970).

Since extracts of *M. smegmatis* contain carboxylating enzymes (Clark, 1971), the effects of Na₂CO₃ and NaHCO₃ on enzyme activity were tested; they stimulated activity by 30 and 41 %, respectively.

Asparagine and ammonium molybdate, which are constituents of the growth medium, inhibited cyclic AMP synthesis by 35 and 85 %, respectively. Coenzyme A (85 %), ribose 5-phosphate (18 %), pyrophosphate (17 %) and glucose 1-phosphate (5 %) all inhibited adenylate cyclase activity.

DISCUSSION

Adenylate cyclase of *M. smegmatis* was associated only with the particulate fraction and activity was detected in the absence of added pyruvate. Membrane-bound adenylate cyclases have also been reported in *Neurospora crassa* (Flawia & Torres, 1972), *Tetrahymena pyriformis* (Kassis & Kindler, 1975), *Saccharomyces cerevisiae* (Wheeler *et al.*, 1974), *Escherichia coli* (Ide, 1969; Tao & Huberman, 1970) and other organisms (Ide, 1971). This localization contrasts with that in *Brevibacterium liquefaciens* (Hirata & Hayaishi, 1967), *Streptococcus salivarius* (Khandelwal & Hamilton, 1971) and *Bordetella pertussis* (Hewlett & Wolff, 1976) where the adenylate cyclase is soluble. The enzyme in *M. smegmatis* has a similar K_m to the enzymes of *E. coli* (Ide, 1969), *B. liquefaciens* (Hirata & Hayaishi, 1967) and *S. salivarius* (Khandelwal & Hamilton, 1971) but its pH optimum (pH 7.5) is lower than that found for *E. coli* (pH 9 to 10) or *B. liquefaciens* (Tao & Lipmann, 1969; Hirata & Hayaishi, 1967). The effects of metal ions on the *M. smegmatis* enzyme (Table 1) resemble those for the *E. coli* enzyme (Tao & Lipmann, 1969) although NH₄⁺ and Na⁺ were slightly stimulatory in *Nocardia erythropolis* (Ide, 1971). A greater stimulatory effect of Mn²⁺ compared with Mg²⁺ was also found in *E. coli*, *B. liquefaciens* and *Nocardia erythropolis* (Ide, 1971). The membrane-bound adenylate cyclase of *N. crassa* specifically required Mn²⁺ (Flawia & Torres, 1972). The enzyme from *M. smegmatis*, like those from *E. coli* (Tao & Lipmann, 1969), *Bordetella pertussis* (Hewlett & Wolff, 1976), *S. cerevisiae* (Wheeler *et al.*, 1974) and *T. pyriformis* (Kassis & Kindler, 1975), was inhibited by fluoride. It resembled other bacterial adenylate cyclases in its unresponsiveness to hormones (Flawia & Torres, 1972; Wheeler *et al.*, 1974; Hewlett & Wolff, 1976). Pyruvate inhibited adenylate cyclase activity in *M. smegmatis*, as in *E. coli* (Tao & Lipmann, 1969) but not *B. liquefaciens* (Takai *et al.*, 1974; Lynch *et al.*, 1975). NADP⁺ inhibited much more severely than NADPH. It is possible that metabolic intermediates and related compounds are important in controlling the rate of cyclic AMP formation in this bacterium. The inhibition found with pyrophosphate, one of the products of the adenylate cyclase reaction, resembled that reported for the adenylate cyclase of *S. cerevisiae* (Wheeler *et al.*, 1974), *Bordetella pertussis* (Hewlett & Wolff, 1976) and some mammalian tissues (Birnbaumer *et al.*, 1971; Wolff & Jones, 1971).

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