3':5'-Cyclic Nucleotide Phosphodiesterase of *Mycobacterium smegmatis*

By C. H. LEE*

Department of Clinical Biochemistry, University of Otago,
Medical School, Dunedin, New Zealand

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INTRODUCTION

The intracellular concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) at any given instant depends on the relative activities of adenylate cyclase and phosphodiesterase, the latter enzyme being known to break down cyclic AMP to AMP in mammalian and bacterial systems (Robison, Butcher & Sutherland, 1971). The elucidation of the properties of the phosphodiesterase in *M. smegmatis* provides a sound basis for further investigations on the role of cyclic AMP in the regulation of mycobacterial cell function.

METHODS

Culture and harvest of micro-organisms. *Mycobacterium smegmatis* NCTC 523 has been maintained in our laboratory since 1966. Cultures were maintained without shaking at 37 °C in 25 ml synthetic medium in 100 ml conical flasks and subcultured every 4 d. The bacteria grew as a thick creamy surface pellicle. The synthetic growth medium (Clark, 1971) contained (per litre distilled water): L-asparagine.H₂O, 4 g; citric acid.H₂O, 2 g; glycerol, 30 g; MgSO₄.7H₂O, 0.5 g; thiamin, 10 mg; KH₂PO₄, 0.5 g; ammonium ferric citrate, 50 mg; inorganic salt solution, 1 ml; DL-lactic acid solution (about 85 %, w/w; boiled and neutralized), 10 g. The pH was adjusted to 7.2 with solid NaOH. The inorganic salt solution contained (per litre): ammonium molybdate.4H₂O, 64 mg; ZnSO₄.7H₂O, 2 g; and MnCl₂.4H₂O, 45 mg.

For large scale growth, bacteria were grown for 3.5 d in Roux flasks each containing 200 ml growth medium and harvested by decanting the medium and filtering through three layers of muslin. The harvested bacteria were washed three times with distilled water, packed by centrifuging and stored at -80 °C.

Preparation of extracts. Frozen bacteria were thawed, suspended in 60 mM-Tris/HCl buffer (pH 8.0 or 9.0) and sonicated in a 100 W ultrasonic disintegrator (MSE) for 20 min at 0 °C. The disrupted cells were centrifuged at 39100 g for 40 min at 4 °C. The pellet was discarded and the supernatant was kept at -80 °C. The enzyme was stable for at least 1 month at this temperature. Enzyme dilution was carried out with 0.1 % (w/v) albumin. Protein was determined by the method of Lowry *et al.* (1951).

Assay of phosphodiesterase (3':5'-cyclic-AMP 5'-nucleotidohydrolase; EC 3.1.4.17). This assay was based on the hydrolysis of cyclic AMP to AMP by phosphodiesterase and subsequent conversion of the AMP to adenosine by the nucleotidase of *Crotalus atrox* venom (Sigma) as described by Loten & Sneyd (1970). The assay mixture (total volume 50 µl) contained: 2.5 µmol 0.05 M-Tris/HCl buffer, pH 8.0 (or 9.0), 0.5 µmol MgSO₄, cyclic [³H]AMP (about 30000 c.p.m.), cyclic AMP (various concentrations) and enzyme preparation. The mixture was incubated at 37 °C for 10 min. Snake venom (20 µl, 7.2 mg ml⁻¹ in 60 mM-Tris/HCl buffer, pH 8.0) was then added and the mixture was incubated for a further 10 min. The reaction was stopped by adding 50 µl 5 mM-adenosine in 50 mM-EDTA (pH 4.6) and the tube was immediately transferred to an ice bath. Samples could be kept in an ice bath for at least 3 h without any change in the amount of [³H]adenosine present. EDTA was inhibitory to the enzyme (see Results). The [³H]adenosine was separated from unchanged cyclic [³H]AMP using a column (25 × 5 mm) of anion exchange resin (Bio-Rad AG1-X2, 200 to 400 mesh). Samples were washed through with 3 ml water which eluted adenosine. Recovery of adenosine from columns (calculated from the absorbance at 260 nm) was usually better than 96 %. Radioactive adenosine was determined by counting a sample (1.0 ml) in 10 ml dioxan-based scintillation fluid.

A modified version of this assay was used in some experiments. Phosphodiesterase activity was stopped by

* Present address: Department of Biochemistry and Microbiology, University of Agriculture Malaysia, Serdang, Selangor, Malaysia.

adding 50 μ l 50 mM-EDTA and heating at 100 °C for 2 min. The mixture was brought to 37 °C, 20 μ l snake venom (7.2 mg ml⁻¹ in 60 mM-Tris/HCl buffer, pH 8.0) was added and the mixture was incubated for 10 min. The action of snake venom was stopped by heating in a boiling water bath for 2 min after adding 50 μ l 5 mM-adenosine solution. The tube was cooled and 1 ml of a 50% (w/v) suspension of anion exchange resin (AG1-X2, 200 to 400 mesh) was added; the mixture was shaken and allowed to equilibrate for 10 min before centrifuging to sediment the resin. Radioactive adenosine remained in the supernatant. A sample of the supernatant (usually 400 μ l) was added to 10 ml dioxan-based scintillation fluid for radioactivity determinations. Less than 9% of the substrate was utilized in both assays. Under these conditions, formation of [³H]adenosine was linear with time and enzyme concentration.

For experiments involving the effect of cations (used as chlorides) on phosphodiesterase activity, the enzyme preparation was first passed through a Sephadex G-25 column (25 $\times$ 110 mm) equilibrated with 0.25 M-Tris/HCl buffer (pH 9.0) to remove low molecular weight compounds. The various cations did not affect the second stage of the two-step assay.

Inorganic phosphate and pyrophosphate interfered with the two-stage assay method. When these compounds were present, AMP formed in the reaction was separated from cyclic AMP and other products by paper chromatography. The assay mixture (total volume 40 μ l) contained: 2.5 μ mol 0.06 M-Tris/HCl buffer (pH 9.0), Mg²⁺ (various concentrations), 0.005 μ mol cyclic AMP, cyclic [³H]AMP (about 40000 to 50000 c.p.m.), inorganic phosphate or pyrophosphate and enzyme preparation. The mixture was incubated at 37 °C for 35 min. Formation of AMP was linear with time and enzyme concentration. No 5'-mononucleotidase activity was detectable under these assay conditions. The reaction was stopped by adding 5 μ l 50% (w/v) trichloroacetic acid. After centrifuging, 35 μ l of the supernatant was spotted on Whatman no. 1 paper and developed with 2-propanol/14.8 M ammonia/water (7:1:2, by vol.). The AMP spots (clearly separated from cyclic AMP) were detected under ultraviolet light (about 254 nm), cut out and counted in 10 ml toluene scintillation fluid.

To show that AMP was the only product formed in the reaction, a sample of the reaction mixture was spotted on cellulose thin-layer plates. Thin-layer chromatograms developed in solvent systems (i) isobutyric acid/0.5 M-ammonium hydroxide (5:3, by vol.), (ii) 2-propanol/ammonia/water (7:1:2, by vol.), (iii) 1-butanol/acetone/acetic acid/14.8 M-ammonia/water (90:30:20:1:60, by vol.), (iv) 1-butanol/acetic acid/water (5:2:3, by vol.) and (v) *tert*-butyl alcohol/11 M-HCl/water (70:13:2:16:8, by vol.) showed that AMP was the only product.

Liquid scintillation counting. Radioactivity of all samples was determined in a Packard model 3375 scintillation spectrometer. Materials for liquid scintillation counting were obtained from Packard Instrument Co. (Downers Grove, Illinois, U.S.A.). Dioxan-based scintillation fluid contained: naphthalene, 62.5 g; 2,5-diphenyloxazole (PPO), 3.75 g; 1,4-di-(2-methylstyryl)benzene, 0.19 g; 1,4-dioxan, 500 ml. Toluene scintillation fluid contained: PPO, 4 g; 1,4-di-2-(5-phenyloxazoly)benzene, 0.05 g; sulphur-free toluene to make 1 litre. Cyclic [³H]AMP (6.5 or 14.2 Ci mmol⁻¹) was supplied by The Radiochemical Centre, Amersham, and was purified before use by passage through a column of ion exchange resin (AG1-X2, 200 to 400 mesh, chloride form).

RESULTS

Determination of K_m . The range of values obtained for the K_m of the phosphodiesterase was 57 to 66 μ M.

Effect of temperature on enzyme activity. Enzyme activity was measured at various temperatures between 20 and 60 °C. The temperature for maximum phosphodiesterase activity was 55 °C. The activity was 45% of the maximum at 37 °C and the enzyme was completely inactive at 20 °C. The enzyme was reasonably heat-stable. After 10 min at 55 °C, approximately 80% of its original activity remained. Less than 10% of its original activity remained after 10 min at 70 °C. The enzyme was stable at 37 °C or at room temperature for at least 24 h.

Effect of pH. The phosphodiesterase of *M. smegmatis* exhibited its optimum activity in the alkaline range, with highest activity at pH 9.0. At pH 7.0, only 37% of its maximum activity was observed.

Effect of cations. The optimum concentrations of various metal ions producing maximum phosphodiesterase activity in *M. smegmatis* are shown in Table 1. The most stimulatory metal ion was Mg²⁺. All the metal ions tested had their maximum effect on phosphodiesterase activity at concentrations below about 0.05 mM with the exception of Mg²⁺, Ba²⁺ and Co²⁺. In general, monovalent cations caused only a small stimulation in phospho-

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

Activity was assayed by the modified procedure described in Methods. Phosphodiesterase activity in the absence of added cation (taken as 100%) was 95.9 pmol adenosine present in 400 μ l supernatant (see Methods).

Cation	Optimum concn (mM)	Relative activity
Mg ²⁺	2.5	202
Ni ²⁺	0.05	182
Co ²⁺	1.0	176
Ca ²⁺	0.025	173
Al ³⁺	0.025	159
Fe ³⁺	0.25	157
Ba ²⁺	5.0	148
Mn ²⁺	0.063	141
Cd ²⁺	0.025	140
Fe ²⁺	0.25	140
Cs ⁺	0.025	136
K ⁺	0.25	133
Na ⁺	0.05	132
Cu ²⁺	0.05	125
NH ₄ ⁺	0.05	119

diesterase activity. Of the four ions tested, Cs⁺ was the most stimulatory and NH₄⁺ the least.

All divalent metal ions tested stimulated phosphodiesterase activity. However, unlike other divalent cations, Cd²⁺ was inhibitory at concentrations greater than 0.2 mM while Cu²⁺ was only slightly active at its optimum concentration and inhibitory at concentrations above 0.27 mM.

Only two trivalent metal ions were tested. Al³⁺ was slightly more potent in stimulating phosphodiesterase activity than Fe³⁺. Neither ion was inhibitory at concentrations up to 1.0 mM.

Effect of compounds known to affect mammalian phosphodiesterase. Caffeine and theophylline at concentrations up to 30 mM had no effect on the *M. smegmatis* enzyme. Pyrophosphate and inorganic phosphate (2.0 mM) stimulated the enzyme by 51% and 25%, respectively, while EDTA (25 mM) inhibited the enzyme by 98%.

DISCUSSION

In *M. smegmatis*, repeated experiments showed that only one form of phosphodiesterase able to hydrolyse cyclic AMP was present. The presence of a low K_m enzyme (K_m less than 10 μ M) could not be demonstrated in cell-free extracts. The K_m of the enzyme was similar to that of *Chlamydomonas reinhardtii* (Fischer & Amrhein, 1974), but lower than those of other micro-organisms including *Saccharomyces carlsbergensis* (Speziali & van Wijk, 1971), *Saccharomyces cerevisiae* (Fujimoto, Ichikawa & Tomita, 1974), *Serratia marcescens* (Okabayashi & Ide, 1970a), *Brevibacterium liquefaciens* and *Escherichia coli* (Okabayashi & Ide, 1970b). The enzyme was more heat-stable than those of mammalian origin (Song & Cheung, 1971).

The enzyme from *M. smegmatis* exhibited maximum activity at pH 9.0. Maximum phosphodiesterase activity in animal tissues (Nair, 1966) and unicellular organisms (Speziali & van Wijk, 1971; Chang, 1968; Okabayashi & Ide, 1970a; Fujimoto *et al.*, 1974; David, 1977) generally occurs in the alkaline range. Cheung (1971) has pointed out that the optimum pH for phosphodiesterase activity can vary with the exogenous ions added. The values mentioned above were determined in the presence of Mg²⁺.

The effect of various metal ions on phosphodiesterase activity has been studied in animal

tissues (Song & Cheung, 1971) and micro-organisms but, in most cases, only divalent metal ions were investigated and at only one or a few concentrations (Fischer & Amrhein, 1974; Fujimoto *et al.*, 1974). In this investigation, a range of metal ion concentrations was used. Although Cu^{2+} (Okabayashi & Ide, 1970*a*) and Ca^{2+} (Song & Cheung, 1971) inhibit phosphodiesterase activity in most tissues, both metal ions stimulated phosphodiesterase activity in *M. smegmatis* when tested at the appropriate concentration. All the ions tested produced appreciable stimulation of enzyme activity at appropriate concentrations.

The stimulatory effect of NH_4^+ has been reported in dog heart (Nair, 1966). Drummond & Perrott-Yee (1961) found that the brain enzyme required Mg^{2+} but that partial activity was obtained in the presence of Co^{2+} and Mn^{2+} . The enzymes from *C. reinhardtii* (Fischer & Amrhein, 1974) and *S. carlsbergensis* (Speziali & van Wijk, 1971) were stimulated by Mn^{2+} at the appropriate concentration. Stimulatory effects of Co^{2+} (Drummond & Perrott-Yee, 1961; Cheung 1971), Ba^{2+} (Okabayashi & Ide, 1970*a*) and Ca^{2+} (Okabayashi & Ide, 1970*a*; Fischer & Amrhein, 1974) have also been reported in several tissues.

Pyrophosphate and inorganic phosphate were slightly stimulatory to the *M. smegmatis* enzyme. The enzymes from *S. carlsbergensis* (Speziali & van Wijk, 1971) and *C. reinhardtii* (Fischer & Amrhein, 1974) were inhibited by pyrophosphate, but in *Serratia marcescens* (Okabayashi & Ide, 1970*a*) and *S. cerevisiae* (Fujimoto *et al.*, 1974) pyrophosphate (10 mM) did not affect the enzymes.

The methylxanthines are inhibitors of mammalian phosphodiesterase (Butcher & Sutherland, 1962) and have been of considerable use in studying the role of cyclic AMP in hormone action. Inhibition by methylxanthines varies among micro-organisms. The enzymes from *Dictyostelium discoideum* (Chang, 1968) and *E. coli* (Nielsen, Monard & Rickenberg, 1973) were apparently not affected by these compounds although Aboud & Burger (1971) have reported that the *E. coli* enzyme was inhibited by methylxanthines. The enzymes from *Serratia marcescens* (Okabayashi & Ide, 1970*a*), *S. cerevisiae* (Fujimoto *et al.*, 1974) and *C. reinhardtii* (Fischer & Amrhein, 1974) were inhibited by theophylline.

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REFERENCES

- ABOUD, M. & BURGER, M. (1971). Cyclic 3':5'-adenosine monophosphate-phosphodiesterase and the release of catabolite repression of β -galactosidase by exogenous cyclic 3':5'-adenosine monophosphate in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **43**, 174-182.
- BUTCHER, R. W. & SUTHERLAND, E. W. (1962). Adenosine-3':5'-phosphate in biological materials. *Journal of Biological Chemistry* **237**, 1244-1250.
- CHANG, Y. Y. (1968). Cyclic 3':5'-adenosine monophosphate phosphodiesterase produced by the slime mold *Dictyostelium discoideum*. *Science* **161**, 57-59.
- CHEUNG, W. Y. (1971). Cyclic 3':5'-nucleotide phosphodiesterase. Effect of divalent cations. *Biochimica et biophysica acta* **242**, 395-409.
- CLARK, D. G. S. (1971). *Intermediary metabolism in Mycobacterium smegmatis*. Ph.D. thesis, University of Otago, New Zealand.
- DAVID, H. L. (1977). Alkaline phosphatases from *Mycobacterium smegmatis*. *Journal of General Microbiology* **101**, 99-102.
- DRUMMOND, G. I. & PERROTT-YEE, S. (1961). Enzymatic hydrolysis of adenosine-3':5'-phosphoric acid. *Journal of Biological Chemistry* **236**, 1126-1129.
- FISCHER, U. & AMRHEIN, N. (1974). Cyclic nucleotide phosphodiesterase of *Chlamydomonas reinhardtii*. *Biochimica et biophysica acta* **341**, 412-420.
- FUJIMOTO, M., ICHIKAWA, A. & TOMITA, K. (1974). Purification and properties of adenosine-3':5'-monophosphate phosphodiesterase from baker's yeast. *Archives of Biochemistry and Biophysics* **161**, 54-63.
- LOTEN, E. G. & SNEYD, J. G. T. (1970). An effect of insulin on adipose-tissue adenosine-3':5'-cyclic monophosphate phosphodiesterase. *Biochemical Journal* **120**, 187-193.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- NAIR, K. G. (1966). Purification and properties of 3':5'-cyclic nucleotide phosphodiesterase from dog heart. *Biochemistry* **5**, 150-157.

- NIELSEN, L. D., MONARD, D. & RICKENBERG, H. V. (1973). Cyclic 3':5'-adenosine monophosphate phosphodiesterase of *Escherichia coli*. *Journal of Bacteriology* **116**, 857-866.
- OKABAYASHI, T. & IDE, M. (1970*a*). Cyclic 3':5'-nucleotide phosphodiesterase of *Serratia marcescens*. *Biochimica et biophysica acta* **220**, 116-123.
- OKABAYASHI, T. & IDE, M. (1970*b*). Effect of dipicolinic acid on bacterial cyclic 3':5'-nucleotide phosphodiesterase. *Biochimica et biophysica acta* **220**, 124-126.
- ROBISON, G. A., BUTCHER, R. W. & SUTHERLAND, E. W. (1971). Formation and metabolism of cyclic AMP. In *Cyclic AMP*, pp. 84-90. Edited by G. A. Robison, R. W. Butcher & E. W. Sutherland. New York: Academic Press.
- SONG, S. Y. & CHEUNG, W. Y. (1971). Cyclic 3':5'-nucleotide phosphodiesterase. Properties of the enzyme of human blood platelets. *Biochimica et biophysica acta* **242**, 593-605.
- SPEZIALI, G. A. G. & VAN WIJK, R. (1971). Cyclic 3':5'-AMP phosphodiesterase of *Saccharomyces carlsbergensis*. Inhibition by adenosine 5'-triphosphate, inorganic pyrophosphate and inorganic polyphosphate. *Biochimica et biophysica acta* **235**, 466-472.