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Activation by ATP and an Undefined Substance in the Supernatant of Mycolic Acid Synthesis in an *in vitro* System of *Bacterionema matruchotii*

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ABSTRACT. The present investigation was undertaken to identify the promoting factor in the supernatant that greatly stimulates mycolic acid-biosynthetic activity localized in the cell wall fraction of *B. matruchotii* cells. The data obtained indicated that the factor was composed of at least two components: ATP and an undefined substance. ATP provoked activation in mycolic acid synthesis in the presence of small quantities of the supernatant whereas it had no stimulant effect in the absence of the supernatant. In view of the results of partial characterization of the promoting substance(s), it is assumed that the undefined substance is a cofactor or intermediate different from the those previously expected.

Key words : mycolic acid synthesis — *Bacterionema matruchotii*

Mycolic acids (α-alkyl and β-hydroxy fatty acids) are a major constituent of the cell walls of *Mycobacteria*, *Nocardia*, *Corynebacteria*, and related taxa.

As to the biosynthesis of mycolic acids, several lines of evidence have indicated that two fatty acid molecules directly condense to form one molecule of mycolic acid.[1-8] Since thus, a number of observation was made and many biosynthetic routes for mycolate have been proposed.[5,6,8-10] However, no evidence of the existence of such pathways has been obtained, largely due to difficulty in isolating the enzyme(s) from the cell.

We have found activity for the biosynthesis of mycolic acids occurring in a cell wall fraction prepared from *B. matruchotii* cells,[7,11,12] suggesting that the enzyme(s) is tightly associated with the cell wall membrane. Further, it was found that mycolate synthesis was greatly promoted by adding either a 78,000$\times$g supernatant or glucose.[12]

In the present communication, further investigation of the promoting factor in the supernatant is described. The results showed that both ATP and an undefined substance were necessary for stimulation of mycolate synthesis in the cell wall fraction of *B. matruchotii* cells. Some characteristics of the promoting substance(s) were also clarified.

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MATERIALS AND METHODS

Growth of *Bacterionema matruchotii* (ATCC 14266) and preparations of fractions of the cell wall and the 78,000 ×g supernatant from the same bacterial cells were carried out as described previously.[12]

Brij-58 treatment of the cell wall fraction: Since the biosynthetic activity of mycolic acids is associated with the cell wall membrane,[12] solubilization of the activity with various detergents was attempted, but was unsuccessful. Among detergents examined, Brij-58 (HLB value, 15.7) was promising: 45% of the initial activity remained after 1% of Brij-58 was added to the cell wall fraction and the mixture was stirred for 30 min. However, after centrifugation of the resulting treated solution at 78,000 ×g for 60 min, mycolate-synthetic activity was not recovered in the supernatant but remained in the precipitate. However, as about 20% of the protein was removed from the cell wall fraction by such treatment, we used the precipitate as an enzyme source for mycolic acid synthesis. The method of preparation was as follows: to the cell wall fraction suspended to a protein concentration of about 20 mg per ml in 0.05 M PEMM buffer,[12] which consisted of 0.05 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol, was added a one tenth volume of 10% (w/v) Brij-58 solution to a final concentration of 1%. The mixture was stirred at 4°C for 30 min, after which it was centrifuged at 25,000 ×g for 20 min. The resulting precipitate was suspended in 0.05 M PEMM buffer of a volume equal to that of the original suspension, homogenized well and used for the following experiments (this homogenate is identified as Brij ppt).

Heat treatment of the supernatant: The 78,000 ×g supernatant solution (about 50 mg protein per ml) was heated at 100°C for 20 min, cooled quickly and centrifuged at 25,000 ×g for 20 min. The resulting supernatant was collected and referred to heat-treated supernatant.

Assay for mycolic acid synthesis : The standard reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 5 μM [1-^{14}C] palmitic acid (56.6 mCi/mmole, New England Nuclear), 10 mM ATP, Brij ppt (about 2 mg protein) and either the 78,000 ×g supernatant (0.02 ml) or the heat-treated supernatant (0.02 ml) in a final volume of 1.0 ml, or as indicated. Mixtures not containing ^{14}C-palmitate were preincubated for 20 min at 37°C, and then the reaction was started by the addition of ^{14}C-palmitate and it continued for 80 min at 37°C. The reaction was stopped by the addition of a solution containing 400 mg of KOH, 0.5 ml of distilled water and 2 ml of methanol. Saponification, extraction, TLC and calculation of the activity were carried out as previously described.[12]

Measurement of ATP content: Quantitative analyses of ATP content were carried out as follows: Brij ppt (about 2 mg protein), was incubated for 80 min at 37°C with or without 0.1 M glucose in 0.05 M potassium phosphate buffer (pH 6.5) in a final volume of 1.0 ml, after which ATP was extracted by the boiling buffer (Tris-EDTA) extraction method described by Prioli *et al.*[13] and

a portion of the extract was added to a reaction mixture containing luciferase-luciferin reagent (Sigma). The bioluminescence reaction was monitored with a Packard liquid scintillation counter. ATP content in the 78,000 ×g supernatant was also measured as described above, except that incubation at 37°C was omitted.

RESULTS

Activation by ATP and the 78,000 ×g supernatant: Our previous paper indicated that mycolic acid-synthetic activity in the cell wall fraction of *B. matruchotii* cells was greatly promoted by 78,000 ×g supernatant from the same bacterial cells. Fig. 1 shows again the stimulant effect of the supernatant on the activity. Interestingly, the addition of ATP, along with small quantities

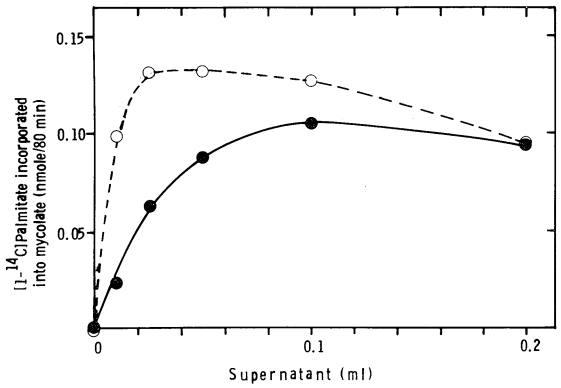

Fig. 1. Activation of mycolic acid synthesis by the supernatant and ATP. Assays were carried out as described in the text, except that indicated amounts of the 78,000 ×g supernatant were added in the presence (○) or absence (●) of 10 mM ATP.

(less than 0.1 ml) of the 78,000 ×g supernatant, caused greater activation in mycolate synthesis than that of the supernatant alone. The stimulant effect of ATP decreased, when the amount of the supernatant added was increased. This seems to be due to the presence of a considerable amount of ATP in the supernatant (see below). The activating activity of ATP and the promoting substance can also be seen in Fig. 2, which shows the time course up to 80 min. Omission of the ATP resulted in a decrease in mycolate-synthetic activity, and removal of the supernatant led to negligible formation of mycolic acids regardless of the existence of ATP as reported previously.[7] The same results were observed using a heat-treated supernatant that was protein-free. These results agree with our previous results; i.e. the promoting factor is heat-stable and trypsin-resistant. The data shown in Fig. 3 indicate that ATP promotes mycolate synthesis in a dose-dependent manner in the presence of a fixed amount of either the supernatant (0.02 ml) or the heat-treated supernatant (0.02 ml). In contrast, there was no ATP-dependent increase in mycolate synthesis when neither the supernatant nor the heat-treated supernatant was added to the reaction mixture.

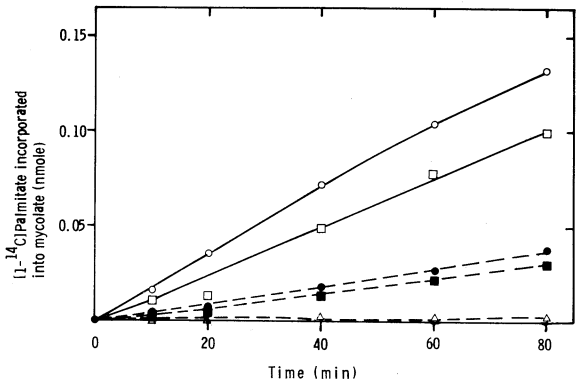

Fig. 2. Time course of mycolic acid synthesis. The standard reaction mixtures were modified as follows: △, minus supernatant (excluding heat-treated supernatant) plus 10 mM ATP; ■, plus supernatant minus ATP; ●, plus heat-treated supernatant minus ATP; □, plus supernatant plus 10 mM ATP; ○, plus heat-treated supernatant plus 10 mM ATP.

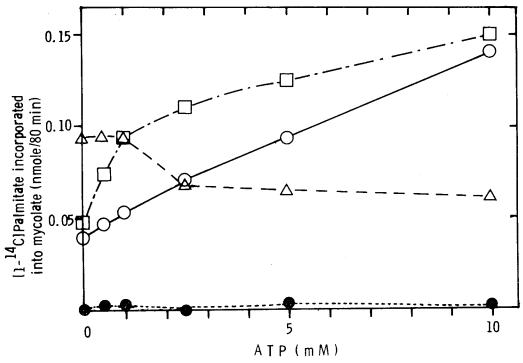

Fig. 3. Dose response of mycolate-synthetic activity to ATP. Assays were carried out as described in the text, except that the amount of ATP added was changed as indicated and the reaction mixtures were as follows: ●, without supernatant (excluding heat-treated supernatant); ○, plus supernatant (0.02 ml); □, plus heat-treated supernatant (0.02 ml); △, without supernatant (excluding heat-treated supernatant) plus 10 mM glucose.

When the effect of ATP on the glucose-induced activity for mycolate synthesis was examined, it was found that ATP, far from promoting further activation, inhibited more than 30% of the activity induced by 10 mM glucose at concentrations above 2.5 mM (Fig. 3). The activity induced by 5 mM and 50 mM glucose (incorporation of 0.05 and 0.27 nmole palmitate, respectively, into mycolate per 80 min using the same amount of Brij ppt as in Fig. 3) was also inhibited by the addition of more than 2.5 mM of ATP to a degree similar to the case of 10 mM glucose.

In general, it is well known that the catabolism of glucose takes place via the glycolytic and oxidative pathways, thereby inducing energy production. Therefore, the amount of ATP synthesized during the incubation of the cell wall fraction with glucose was measured. After incubation of the cell wall fraction alone or together with 0.1 M glucose for 80 min, the ATP content

was 1.58×10^{-10} mole and 2.73×10^{-10} mole, respectively, per 3.35 mg of protein of the cell wall fraction (data not shown). The addition of glucose caused a 1.73-fold increase in ATP content. On the other hand, the freshly prepared 78,000 ×g supernatant contained 7.38×10^{-9} mole of ATP per 12.95 mg of protein (data not shown), but this value varied considerably from preparation to preparation.

When nucleotide phosphate specificity was examined, ATP most effectively stimulated mycolate synthesis, with ADP being second. AMP, GTP, UTP and CTP were inert.

Some characteristics of the promoting substance: Since the heat-treated supernatant contained a fair amount of ATP and the promoting substance was close to ATP in molecular weight (Fig. 4), the heat-treated supernatant was pretreated with luciferase-luciferin reagent to consume its ATP content prior to gel filtration on Bio-Gel P-2. As shown in Fig. 4, the promoting substance was eluted into two peaks at No 56 and 61. These two positions corresponded to about 600 and 500 daltons, respectively. The substance was completely separated from coenzyme A, trehalose and glucose, and absolutely required ATP for the occurrence of promoting activity. Removal of ATP resulted in almost complete loss of activity (Fig. 4). The appearance of a small peak (No 60-62) after removal of ATP seems to have been caused by the presence of fractionally remaining ATP. The structural relationship between the larger substance and the smaller one is undefined.

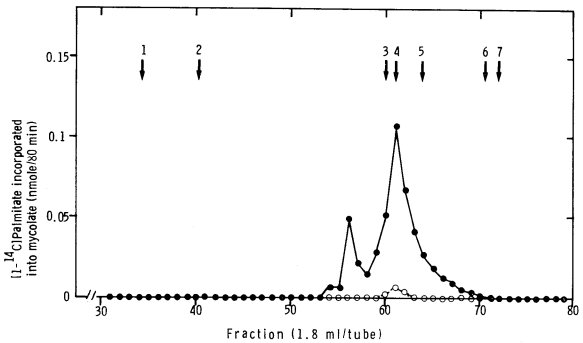

Fig. 4. Gel filtration of heat-treated supernatant on Bio-Gel P-2 column. To the solution containing 30 mg of lyophilized heat-treated supernatant in 1 ml of distilled water was added 10 mg of luciferase-luciferin reagent (Sigma). The mixture was incubated for 30 min at 37°C, after which it was applied to a Bio-Gel P-2 column (2×46 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0), eluted with the same buffer and collected in 1.7 ml fractions. Aliquots of 0.1 ml were supplied to assays of mycolate synthesis, which were conducted in the presence (●) or absence (○) of 10 mM ATP. Standard markers eluted in the same conditions peaked at the positions indicated: 1, cytochrome c; 2, coenzyme A; 3, ATP; 4, raffinose; 5, trehalose; 6, glucose; 7, ribose.

On the other hand, by methylation of the heat-treated supernatant with diazomethane or trimethylsilylation with N,O-bis (trimethylsilyl) trifluoroacetamide, its promoting activity was almost completely inactivated in both cases of the presence and absence of ATP (data not shown).

DISCUSSION

The mechanism whereby mycolic acid and its derivatives are synthesized from two fatty acid molecules is our primary concern. However, there is a paucity of information concerning the reaction mechanism.

As described above, we found that ATP provoked activation of mycolic acid synthesis in the presence of an undefined substance in the supernatant. But ATP did not promote mycolate synthesis in the absence of the supernatant. On the other hand, ATP did not further stimulate glucose-induced activity for mycolate synthesis. This result may be explained by the fact that the ATP content in Brij ppt increased after incubation with glucose (see text). Accordingly, the stimulant effect of ATP was not observable. In this case, Brij ppt must contain the smallest amount of the supernatant containing the promoting substance. However, the degree of increase of ATP was too slight to explain the great activation of mycolate synthesis by glucose,[12] even if the ATP synthesized decomposed during incubation. Therefore, the other possibility that should also be considered is that the mechanism of activation of mycolate synthesis by glucose differs from that by ATP and the supernatant. This subject is under investigation.

Concerning the reaction products, mycolic acids synthesized from palmitate in the reaction mixture containing ATP and the supernatant were not free form but were bound to trehalose and an unknown compound (monosaccharide?, data not shown), as observed in previous results obtained using glucose.[12] In addition, acylated sugar was also formed. However, it could not be demonstrated that it was an intermediate or substrate for the mycolate-synthesizing system.

It is unclear whether ATP acts on any of the steps in the overall pathway for mycolate biosynthesis.[10] To clarify the role of ATP, the promoting substance was partially characterized, and the data obtained are as follows: (i) the substance is heat-stable and water-soluble; (ii) the data from inactivation by methylation or trimethylsilylation show that it contains one or more hydroxyl group; (iii) on Bio-Gel P-2 gel filtration (Fig. 4), it was eluted into two peaks, whose positions corresponded to about 600 and 500 daltons, and it was completely separated from coenzyme A, trehalose, glucose and, of course, acyl carrier protein, which are thought to be likely candidates for cofactors involved in mycolate synthesis.[10–12]

Judging from these and previous[12] data, the promoting substance described herein seems to be a kind of cofactor or intermediate different from those previously expected or a modulator such as inositol phosphate glycan that regulates the activities of cyclic GMP phosphodiesterase, pyruvate dehydrogenase and adenylate cyclase.[14] Clarification of the chemical structure of the promoting substance(s) should result in much progress in elucidation of the complicated reaction sequence for mycolate synthesis.

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