Fractionation of the mycobacillin-synthesizing enzyme system

Subrata Kumar GHOSH, Nishit Kumar MUKHOPADHYAY, Sekhar MAJUMDER and
Sushil Kumar BOSE

Department of Biochemistry, University College of Science, 35 Ballygunge Circular Road, Calcutta-19, India

(Received 19 April 1983/Accepted 16 August 1983)

The mycobacillin-synthesizing enzyme system was highly purified by fractionation at 30–55% $(\text{NH}_4)_2\text{SO}_4$ saturation. The enzyme concentrate on Sephadex G-200 gel chromatography was resolved into three distinct fragments. Each of the fragments on further purification by DEAE-cellulose ion-exchange chromatography behaved as a single-component system, as clearly indicated by the sharpness of the peaks in the elution diagram. None of the fragments alone nor any two of them in all possible combinations possessed mycobacillin-synthesizing activity, which was restored only when the three fragments were used together in the test system.

Spore-forming bacilli produce a variety of bioactive peptides, detailed studies on the biosynthesis of which have resulted in the discovery of a novel mode of peptide formation mediated by a multi-enzyme complex (Lipmann *et al.*, 1971; Laland & Zimmer, 1973; Kurahashi, 1974; Katz & Demain, 1977; Suzukake *et al.*, 1979).

The biosynthesis of mycobacillin, a cyclic peptide antibiotic produced by *Bacillus subtilis* B₃ (Majumder & Bose, 1958, 1960), did not involve an RNA template, and the enzyme system was found to be present in the soluble supernatant of the producer cell (Sengupta & Bose, 1971). The activation of constituent amino acids occurred as a result of $\text{ATP}-[^{32}\text{P}]\text{P}_i$ exchange and not by $\text{ATP}-[^{32}\text{P}]\text{PP}_i$ exchange, as observed with other peptide antibiotics. These studies indicate that mycobacillin synthesis was initiated with proline (Sengupta & Bose, 1974). An amino acid-deprivation technique was developed for the isolation of possible intermediates (Sengupta & Bose, 1972, 1973). However, these intermediates in the presence of complementary amino acids were not incorporated into mycobacillin (Sengupta & Bose, 1982), which might be taken to mean that the synthesis might not occur as a result of multiple enzymes functioning independently at multiple steps. However, the purification of the enzyme system may prove very useful at this stage. The present paper therefore reports the purification and possible fractionation of mycobacillin-synthesizing enzyme complex.

Materials and methods

Chemicals and radiochemicals

Lysozyme from egg white and ATP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), $\text{U-}^{14}\text{C}$ -labelled *Chlorella*-protein hydrolysate (sp. radioactivity 42 mCi/mg-atom of C) and $\text{Na}_2\text{H}^{32}\text{PO}_4$ (sp. radioactivity 10 mCi/mmol) were purchased from Bhabha Atomic Research Centre (Trombay, India). Other chemicals used in the experiments were obtained from commercial sources.

Organism

Bacillus subtilis B₃ (Majumder & Bose, 1958) was used to study the biosynthesis of mycobacillin.

Preparation of 20 000 g supernatant

The method used to prepare 20 000 g supernatant was based on that described by Sengupta & Bose (1971). The producer organism *B. subtilis* B₃ was grown in a fresh nutrient broth (4.5 litres) supplemented with 1% glucose for 15–16 h at $30 \pm 1^\circ\text{C}$. Then the broth was centrifuged and washed once with 0.9% NaCl and then with 5 mM-MgCl₂. The cells (20 g) were resuspended in 80 ml of 50 mM-Tris/HCl buffer, pH 7.8, containing 2.0 mM-EDTA, 10 mM-MgCl₂ and 1 mM-dithiothreitol (buffer A), to which lysozyme (800 µg/ml) had been added. After incubation for 20 min at room temperature the

cell-free system was quickly chilled at 0°C for 20 min and centrifuged at 20 000 *g* for 30 min.

Ultracentrifugation and streptomycin sulphate/(NH₄)₂SO₄ fractionation

The cell-free extract was centrifuged at 20 000 *g*. A portion of the supernatant was further centrifuged at 105 000 *g* (Beckman ultracentrifuge) for 90 min, and the supernatant from this was retained for biosynthesis studies. To another portion of the 20 000 *g* supernatant 10% (w/v) streptomycin sulphate solution in buffer A was added dropwise to make the final concentration 1%. The precipitate collected by centrifugation at 20 000 *g* for 30 min was discarded. The supernatant was then precipitated with (NH₄)₂SO₄ (30–55% saturation), and the precipitate was collected by centrifugation at 20 000 *g* for 30 min. The precipitate was dissolved in buffer B (50 mM-Tris/HCl buffer, pH 7.8, containing 0.25 mM-EDTA, 10 mM-MgCl₂ and 1 mM-dithiothreitol) and dialysed for 10 h against 2 litres of the same buffer (buffer B).

Sephadex G-200 gel filtration

The dialysed 30–55%-satn.-(NH₄)₂SO₄ fraction (8 ml) was applied to a Sephadex G-200 column (2.5 cm × 70 cm) equilibrated with buffer B and eluted with the same buffer in 5 ml fractions (flow rate 15 ml/h). The protein content and ATP-[³²P]-P_i-exchange activity dependent on mycobacillin constituent amino acids of each of the fractions were measured. The peak fractions as indicated by ATP-[³²P]P_i exchange capacity were separately pooled together and precipitated with (NH₄)₂SO₄, and the precipitate was dissolved in a small volume of buffer B and dialysed for 5 h against 2 litres of buffer B.

DEAE-cellulose column chromatography

The three dialysed peak fractions from the Sephadex G-200 gel column were applied separately to a DEAE-cellulose column (1.5 cm × 16 cm) equilibrated with buffer B and eluted stepwise with the same buffer containing increasing concentrations of KCl in 4 ml fractions (flow rate 24 ml/h), and the activity of each fraction was determined on the basis of ATP-[³²P]P_i-exchange capacity. The peak fractions as indicated by the exchange activity were pooled together and further concentrated separately by freeze-drying.

Determination of mycobacillin biosynthesis

The incubation mixture contained the following, in a total volume of 2 ml: Tris/HCl buffer, pH 7.8, 100 μM; MgCl₂, 10 μM; ATP, 5 μM; phosphoenolpyruvate, 5 μM; pyruvate kinase, 20 μg; dithiothreitol, 10 μM; U-¹⁴C-labelled *Chlorella*-protein hydrolysate (sp. radioactivity 42 mCi/mg-atom of

C), 2.5 μCi; L-leucine, L-alanine, L-proline, L-serine, L-aspartic acid, 1 μM-each; D-aspartic acid, 4 μM; D-glutamic acid, 2 μM; L-tyrosine, 2 μM; enzyme to be assayed, 5 mg. The reaction was terminated by adding 4 vol. of butan-1-ol, and 2 mg of unlabelled mycobacillin was added as carrier. Mycobacillin was isolated from the butanol extract as described by Majumder & Bose (1960) and finally dissolved in 2 ml of ethanol, and the ethanolic solution was used directly for counting of radioactivity.

Measurement of ATP-[³²P]P_i exchange dependent on mixture of constituent amino acids of mycobacillin

The exchange was studied as described by Stulberg & Novelli (1960). The reaction mixture contained, in a final volume of 300 μl, 0.1 M-Tris/HCl buffer, pH 8.0, 3 mM-ATP, 5 mM-MgCl₂, 10 mM-KF, 2 mM-dithiothreitol, a mixture of constituent amino acids of mycobacillin (each 5 mM), 2 mM-Na₂H³²PO₄ (10 μCi; sp. radioactivity 10 mCi/mmol) and 200 μl of Sephadex G-200 eluate as a source of the enzyme. The mixture was incubated for 30 min at 30°C, and the reaction was terminated by mixing with 0.5 ml of a solution of 2.5% of charcoal (acid-washed Norit A) in 0.02 M-Na₂HPO₄ and 10% (w/v) trichloroacetic acid. After 30 min in the cold the suspension was filtered on a Whatman GF/C disc (2.5 cm diam.), washed with 5 × 5 ml of water, and the filter was dried and radioactivity of the adsorbed materials measured in a liquid-scintillation counter.

Determination of protein concentration

The protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results

Purification of mycobacillin-synthesizing enzyme by ultracentrifugation

The attempt to purify the mycobacillin-synthesizing enzyme system by centrifugation shows that the protein content was 8.0 mg/ml for the 20 000 *g* supernatant, as against 6.2 mg/ml for the 105 000 *g* supernatant, and that the incorporating ability of the mycobacillin-synthesizing enzyme per mg of protein was also slightly greater for the 20 000 *g* supernatant than for the 105 000 *g* supernatant, which retained approx. 80% of the activity.

Purification by streptomycin sulphate/(NH₄)₂SO₄ fractionation

Table 1 indicates that streptomycin sulphate did not increase the purification (fold) of the enzymes. However, the step was retained, since it removed much of the non-protein macromolecular constituents. Attempts at further purification with

Table 1. *Streptomycin/(NH₄)₂SO₄ fractionation of the mycobacillin-synthesizing system*

The data below refer to the enzyme protein obtained from 4.5 litres of culture broth of *B. subtilis* B₃.

Sample	Total protein (mg)	Radioactivity incorporated into mycobacillin (c.p.m./mg of protein)	Purification (fold)
Crude extract	688	1513	—
Streptomycin sulphate fraction	668	1560	—
30–55%-satn.-(NH ₄) ₂ SO ₄ fraction	144	5368	3.5

(NH₄)₂SO₄ shows that most of the active enzyme protein was precipitated at 30–55% (NH₄)₂SO₄ saturation, which resulted in 3.5-fold purification with respect to the crude-cell-free extract.

Purification of mycobacillin-synthesizing enzyme on the Sephadex G-200 gel column

The protein content and ATP-[³²P]P_i-exchange-activity profiles of the different fractions from a Sephadex G-200 column are shown in Fig. 1, which clearly indicates that the mycobacillin-synthesizing enzyme system was resolved into three peak fractions, on the basis of both protein content and exchange activity in the presence of mycobacillin constituent amino acids. It is noteworthy that the three peaks of protein content almost coincided with those of exchange activity.

Mycobacillin-synthesizing activity of three peak fractions either alone or in different combinations

The fractions constituting the three different peaks obtained from Sephadex G-200 gel filtration on the basis of ATP-[³²P]P_i-exchange activity were separately pooled together, and mycobacillin-synthesizing activity was examined in each fraction alone, or in all possible combinations of two fractions, and also in a combination of the three. The results are shown in Table 2, which indicates that the mycobacillin-synthesizing enzyme system could be separated into three fractions and that neither any one nor any two of them in any combination was active, whereas all the three peak fractions taken together could bring about the synthesis.

DEAE-cellulose ion-exchange-chromatographic behaviour of peak fractions obtained from Sephadex G-200 gel column

All the three peak fractions from the Sephadex column on the basis of ATP-[³²P]P_i-exchange activity were separately pooled together and passed separately through a DEAE-cellulose column; the results are shown in Figs. 2(a), 2(b) and 2(c). It

Fig. 1. *Sephadex G-200 gel filtration and ATP-[³²P]P_i exchange studies on the mycobacillin-synthesizing enzyme system*

The 30–55%-satn.-(NH₄)₂SO₄ fraction obtained from 20 g wet wt. of cells (4.5 litres of culture) was loaded on a Sephadex G-200 column (2.5 cm × 70 cm) equilibrated with buffer B at 5°C. The column was then eluted with buffer B (15 ml/h). The A₂₈₀ was measured in each fraction (5 ml). ATP-[³²P]P_i-exchange activity dependent on a mixture of mycobacillin constituent amino acids was measured in a 200 μl portion of each fraction. The total amount of radioactivity in each incubation mixture was 10 μCi. The radioactivity measured in control tubes (without amino acid mixture) was subtracted. ●, A₂₈₀; ○, ATP-[³²P]P_i exchange dependent on the amino acid mixture.

Table 2. *Role of three fractions of the mycobacillin-synthesizing system obtained from a Sephadex G-200 gel column in mycobacillin biosynthesis*

The three Sephadex G-200 fractions were designated as peaks A, B and C (see Fig. 1). To each of the fractions (19–27 for A, 30–38 for B and 50–59 for C) was added solid (NH₄)₂SO₄ to 80% saturation, and the precipitate was dissolved in 4 ml of buffer B and dialysed against 1 litre of the same buffer for 2 h. Mycobacillin synthesis was measured as described in the Materials and methods section. The radioactivity in the control (0 h incubation) was subtracted from that in the experiments.

Protein fraction used	Radioactivity incorporated into mycobacillin (c.p.m.)
A + B + C	3806
A + B	360
B + C	360
A + C	231
A	327
B	199
C	17

appears that the peaks in the elution diagram on the basis of either protein concentration or exchange activity were much sharper and showed greater coincidence.

Fig. 2. DEAE-cellulose column chromatography of components A (a), B (b) and C (c) of the mycobacillin-synthesizing enzyme system

In each case the enzyme protein was eluted as described in the Materials and methods section (concns. of KCl in buffer are indicated). A 200 μ l sample of each fraction was assayed for ATP-[32 P]P_i-exchange activity dependent on the amino acid mixture. ●, A_{280} ; ○, exchange activity.

Fragmentation of mycobacillin-synthesizing activity

The three peak fractions from the DEAE-cellulose column were tested again for mycobacillin-synthesizing activity either alone, or in groups of two in all possible combinations, or finally in a combination of all three peak fractions. Table 3 shows that mycobacillin synthesis occurred only when all the three peak fractions were taken together.

Table 3. Synthesis of mycobacillin by the enzyme purified through DEAE cellulose column

Active fractions (A, B and C; see Fig. 1) obtained from ion-exchange chromatography were concentrated by means of freeze-drying and used in the assay of mycobacillin synthesis as described in the Materials and methods section.

Enzyme fraction used	Mycobacillin synthesis (c.p.m.)
A	110
B	194
C	328
A + B	250
B + C	132
C + A	85
A + B + C	3168

Discussion

Although exhaustive studies on the biosynthesis of oligopeptides indicate the presence of two enzyme components in gramicidin-S synthetase (Gevers *et al.*, 1968) and of three in tyrocidine synthetase (Roskoski *et al.*, 1970), the present work was undertaken to purify and if possible to fractionate the mycobacillin-synthesizing enzyme to test whether the synthesizing system is a multi-site single enzyme or a multiple-component enzyme complex. Different techniques of protein purification were tried. Ultracentrifugation at 105 000 *g* did not purify the enzyme, although it directly proved that the enzyme was present in a soluble form in the cytosol. $(\text{NH}_4)_2\text{SO}_4$ fractionation considerably purified the enzyme; a 3.5-fold purification was achieved by fractionation at 30–55% saturation. The active enzyme concentrate so obtained on further purification on Sephadex G-200 was resolved into three components on the basis of both their protein content and also their ATP-[32 P]P_i-exchange activity. The three components on the basis of their exchange activity, when tested for mycobacillin-synthesizing activity either alone or in all possible combinations, gave results clearly indicating that the mycobacillin-synthesizing system consists of three enzyme components. DEAE-cellulose column chromatography further purified each of the components, and also confirmed the three-component nature of the mycobacillin-synthesizing enzyme complex, which resembles tyrocidine and bacitracin (cyclic oligopeptide with a side chain) synthetase (Frøyskov, 1974) but not gramicidin-S synthetase.

The fold purification, which was 3.5 after streptomycin/ $(\text{NH}_4)_2\text{SO}_4$ fractionation, could not be calculated for the components obtained after gel or ion-exchange chromatography. However, on the basis of protein requirement for equal synthesis in the presence of equal but not equivalent amounts of these components, the values were 22-fold and

36-fold for gel and ion-exchange chromatography respectively.

Thus the purification of mycobacillin-synthesizing enzyme complex and its fractionation into three components have been achieved.

We thank the Science and Engineering Research Council, Department of Science and Technology, Government of India, for their financial support.

References

- Frøyshov, Ø. (1974) *FEBS Lett.* **44**, 75–78
- Gevers, W., Kleinkauf, H. & Lipmann, F. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 269–276
- Katz, E. & Demain, A. L. (1977) *Bacteriol. Rev.* **41**, 449–474
- Kurahashi, K. (1974) *Annu. Rev. Biochem.* **43**, 445–459
- Laland, S. G. & Zimmer, T. L. (1973) *Essays Biochem.* **9**, 31–57
- Lipmann, F., Gevers, W., Kleinkauf, H. & Roskoski, R., Jr. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol.* **35**, 1–34
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Majumder, S. K. & Bose, S. K. (1958) *Nature (London)* **181**, 134–135
- Majumder, S. K. & Bose, S. K. (1960) *Biochem. J.* **74**, 596–599
- Roskoski, R., Jr., Gevers, W., Kleinkauf, H. & Lipmann, F. (1970) *Biochemistry* **9**, 4839–4845
- Sengupta, S. & Bose, S. K. (1971) *Biochim. Biophys. Acta* **237**, 120–122
- Sengupta, S. & Bose, S. K. (1972) *Biochem. J.* **128**, 47–52
- Sengupta, S. & Bose, S. K. (1973) *Biochem. J.* **131**, 623–624
- Sengupta, S. & Bose, S. K. (1974) *Indian J. Biochem. Biophys.* **11**, 335–336
- Sengupta, S. & Bose, S. K. (1982) *Pept. Antibiot. Biosynth. Funct. Pap. Meet.* 109–113
- Stulberg, M. P. & Novelli, G. D. (1960) *Methods Enzymol.* **5**, 703–707
- Suzukake, K., Fujigama, T., Hayashi, H. & Hori, M. (1979) *J. Antibiot.* **32**, 523–530