Characterization of three-fraction mycobacillin synthetase

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

Department of Biochemistry, University College of Science, 35 Ballygunge Circular Road, Calcutta 700 019, India

Mycobacillin synthetase lacks aspartic acid racemase, alanine racemase and glutamic acid racemase activities. The enzyme also does not respond to ATP- $[^{32}\text{P}]\text{P}_i$ exchange, nor does it catalyse the antibiotic synthesis in presence of amino acids of configuration opposite to that present in the molecule. Preincubation with optical isomers of opposite configuration inhibited the ATP- $[^{32}\text{P}]\text{P}_i$ exchange reaction to the extent of 60–90%. None of the three fractions of mycobacillin synthetase contained a pantothenic acid arm. Two molecules of ATP are required to synthesize one peptide bond of mycobacillin. Intermediate peptides of mycobacillin are not covalently linked to the three-fraction mycobacillin synthetase.

INTRODUCTION

Mycobacillin synthetase, a multienzyme complex consisting of three distinct enzyme fractions, A, B and C (Ghosh *et al.*, 1983), catalyses the non-ribosomal biosynthesis of mycobacillin (Majumdar & Bose, 1958; Sengupta & Bose, 1971), an antifungal cyclotridecapeptide (Banerjee & Bose, 1963; Sengupta & Bose, 1973). The enzyme complex initially present in the cytosol during early exponential phase was found to be translocated from soluble cytosol to membrane fraction as the culture ages (Mukhopadhyay *et al.*, 1985). The enzyme catalyses L-proline-dependent ATP- $[^{32}\text{P}]\text{P}_i$ exchange, which was sequentially stimulated by mycobacillin into amino acids, starting with L-proline (Sengupta and Bose, 1972, 1974). The present paper deals with the different characteristic properties of mycobacillin synthetase together with the energy requirement for peptide-bond synthesis.

MATERIALS AND METHODS

Chemicals and radiochemicals

D-Amino acid oxidase (from pig kidney), L-glutamic acid decarboxylase (from *Clostridium Welchii*), catalase, alkaline phosphatase, ATP, FAD and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L- $[^{14}\text{C}]$ Proline, L- $[^{14}\text{C}]$ alanine, DL- $[^{14}\text{C}]$ glutamic acid, L- $[^{14}\text{C}]$ aspartic acid and $\text{Na}_2\text{H}^{32}\text{PO}_4$ were purchased from Bhabha Atomic Research Centre (Trombay, India). D- $[^{14}\text{C}]$ Glutamic acid was prepared from DL- $[^{14}\text{C}]$ glutamic acid by destruction of the L isomer with L-glutamic acid decarboxylase. The residual D-glutamic acid and γ -aminobutyric acid (decarboxylated product of L-glutamic acid) were desalted by the use of Dowex 50 (H^+ form) resin and separated by paper chromatography. Other chemicals used were from commercial sources.

Strain, media and growth conditions

Bacillus subtilis B₃ was grown in nutrient broth supplemented with 1% glucose with shaking at pH 7.2 and at 30 °C as reported previously (Mukhopadhyay *et al.*, 1984).

Purification of mycobacillin synthetase

The three-fraction mycobacillin synthetase was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, Sephadex G-200 gel filtration and DEAE-cellulose ion-exchange chromatography (Ghosh *et al.*, 1983). All the enzyme fractions (A, B and C) from the DEAE-cellulose column were concentrated, dialysed and finally passed, separately, through a hydroxyapatite column (2 cm $\times$ 6 cm) which had been equilibrated with 0.02 M-phosphate buffer, pH 7.2, containing 0.25 mM-EDTA, 5 mM- MgCl_2 , 1 mM-dithiothreitol and then stepwise elution was performed with the increasing concentration of the phosphate buffer (0.05 M, 0.08 M, 0.1 M, 0.12 M, 0.14 M, 0.18 M) and the fractions, as monitored by A_{280} and confirmed by enzyme assay, were collected. Finally each of the active fractions was concentrated and separately layered over a discontinuous sucrose gradient (10%, 12.5%, 15%, 17.5%, 20%, 22.5%, w/v) and centrifuged for 8 h at 40000 rev./min in Beckman SW 40 rotor at 0 °C. All the subsequent studies were performed with these active fractions (Ghosh *et al.*, 1985).

Assay of mycobacillin synthetase activity

The incubation mixture and the assay procedure was the same as described by Mukhopadhyay *et al.* (1985).

Determination of ATP- $[^{32}\text{P}]\text{P}_i$ exchange

The ATP- $[^{32}\text{P}]\text{P}_i$ exchange was studied by using the method of Stulberg & Novelli (1960) as described by Ghosh *et al.* (1983).

Assay of alanine racemase and aspartic acid racemase activity

The assay is based on the conversion of L-alanine and L-aspartic acid into their D-isomers by specific racemases, followed by their oxidation with D-amino acid oxidase to corresponding oxo acids. The oxo acids were then allowed to react with 2,4-dinitrophenylhydrazine and hydrazone derivatives were extracted with ethyl acetate.

The incubation mixture for determining racemase activity contained 0.1 M-sodium phosphate buffer, pH 7, 10 μM - MgCl_2 , 10 μM -mercaptoethanol, 0.5 mg of purified

* To whom correspondence and requests for reprints should be sent.

Table 1. Assay of aspartic acid racemase, glutamic acid racemase and alanine racemase activities

The method of assay and the composition of incubation mixture are detailed in the text. In each case, 0.5 mg of the purified whole enzyme (ABC) was used.

¹⁴ C-labelled amino acid added (10 μ M each)	10 ⁻³ $\times$ Radioactivity (c.p.m.) of hydrazone of oxo acid obtained by D-amino acid oxidase treatment*		10 ⁻³ $\times$ Radioactivity (c.p.m.) of γ -aminobutyric acid obtained by L-glutamic acid decarboxylase treatment*	
	+ ATP	- ATP	+ ATP	- ATP
L-Alanine	0.100 $\pm$ 0.002	0.110 $\pm$ 0.003	0.095 $\pm$ 0.004	0.100 $\pm$ 0.003
L-Aspartic acid	0.090 $\pm$ 0.002	0.092 $\pm$ 0.006	0.089 $\pm$ 0.005	0.090 $\pm$ 0.002
D-Glutamic acid	0.085 $\pm$ 0.003	0.080 $\pm$ 0.006	0.080 $\pm$ 0.005	0.082 $\pm$ 0.004

* Results are expressed as means $\pm$ S.E.M. for four different experiments.

enzyme and 5 μ M of ¹⁴C-labelled L-amino acid with or without 10 μ M-ATP, the total volume being 1 ml. The reaction was allowed to proceed for 30 min and then terminated by heating in a boiling-water bath and centrifuged. The supernatant was then applied to a Dowex 50 (H⁺ form) column and eluted with ammoniacal ethanol. The ethanol extract was then dried and dissolved in 0.5 ml of 0.02 M-sodium pyrophosphate buffer, pH 8.3, and incubated with D-amino acid oxidase. The incubation mixture contained 0.5 ml of substrate in 0.02 M-pyrophosphate buffer, pH 8.3, 10 μ M-D-alanine or D-aspartic acid in 0.2 ml of buffer, 1.0 ml of 0.02 M-sodium pyrophosphate buffer containing 0.02 ml of catalase and 0.5 ml of D-amino acid oxidase (5 mg/ml) in the same buffer. The reaction was conducted in a Warburg apparatus at 37 °C for 90 min. Thereafter 0.1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M-HCl was added to the incubation mixture, which was left for 5 min at 37 °C and diluted to 4 ml with 10% (v/v) metaphosphoric acid. The aqueous phase was then shaken with 4 ml of ethyl acetate and 0.5 ml of the clear ethyl acetate layer taken out, dried and counted for radioactivity in a liquid-scintillation counter.

Assay of glutamic acid racemase activity

The method is based on the conversion of D-glutamic acid into L-isomer by racemase, followed by decarboxylation of the L-isomer into γ -aminobutyric acid with L-glutamic acid decarboxylase.

The incubation mixture for racemase activity and method of isolation of residual amino acids were the same as for alanine or aspartic acid. The dried residue obtained from a Dowex 50 (H⁺ form) column was then dissolved in 0.5 ml of 0.2 M-sodium acetate buffer, pH 4.5. The incubation mixture for decarboxylation contained 1.3 ml of 0.2 M-sodium acetate buffer, pH 4.5, 0.5 ml of L-glutamic acid decarboxylase suspension (24 mg/ml), 0.5 ml of substrate and 0.2 ml of 10 μ M-L-glutamic acid in acetate buffer. The reaction was conducted in a Warburg apparatus at 37 °C and terminated after 4 h with 4 vol. of ethanol and the mixture centrifuged. The clear supernatant was then applied to the Dowex 50 (H⁺ form) column and eluted with ammoniacal ethanol. The elute was then dried and chromatographed in two dimensions by using (a) butan-1-ol/acetic acid/water (4:1:1, v/v) and (b) ethyl acetate/pyridine/acetic acid/water (60:20:6:11, by vol.). The spot corresponding

to γ -aminobutyric acid was then eluted, dried, and counted for radioactivity in a liquid-scintillation counter.

Estimation of ATP

A 200 μ l portion of the mycobacillin synthetase sucrose-density-gradient eluate was incubated in presence of dithiothreitol, mycobacillin amino acids and 0.5–10 μ mol of ATP in a final volume of 1.5 ml. A sample (0.5 ml) was taken and an equal amount of methanol was added and the mixture centrifuged and the clear supernatant freeze-dried. ATP was determined by the method of Jaworek *et al.* (1970). Concomitantly, mycobacillin synthesis was also measured by L-[¹⁴C]proline incorporation.

Assay of pantothenic acid

The three different enzyme fractions of mycobacillin synthetase in 100 μ M-Tris/HCl buffer, pH 8, were incubated with 0.2 M-NaOH at 37 °C for 2 h in 1 ml of reaction mixture. The mixtures were then adjusted to pH 9.5 with 1 M-HCl and incubated with 1 mg of alkaline phosphatase at 37° for 1 h. The reactions were then stopped by boiling the mixtures in a water bath for 10 min and the precipitated protein was separated by centrifugation. The presence of pantothenic acid in the supernatant solution from each of the fractions was assayed microbiologically against *Lactobacillus plantarum* A.T.C.C. 8014.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Absence of aspartic acid racemase, alanine racemase and glutamic acid racemase activities in mycobacillin synthetase

It appears from Table 1 that no detectable radioactivity was present in the hydrazone derivative of the oxo acid obtained by treatment with D-amino acid oxidase, a mixture of non-labelled D-alanine (added as carrier) and labelled L-alanine previously treated with mycobacillin synthetase, a result that indicated the absence of alanine racemase. Similarly, no detectable radioactivity was obtained in the hydrazone derivative obtained from

Table 2. Amino-acid-dependent ATP-[³²P]P_i exchange catalysed by mycobacillin synthetase

A portion (60 µg each) of the enzyme fractions A, B and C was incubated with 0.1 M-Tris/HCl buffer, pH 7.8, 3 mM-ATP, 5 mM-MgCl₂, 10 mM-KF, 2 mM-dithiothreitol, 5 mM of each amino acid and 2 mM-Na₂H³²PO₄ (10 µCi, sp. radioactivity 10 mCi/mmol). The incubation was carried out for 30 min at 30 °C in a final volume of 300 µl. Incorporation into ATP was determined as described in the text.

Amino acid(s) added	Enzyme fraction(s) added	10 ⁻³ × Radioactivity in ATP (c.p.m.)*
L-Pro	A	10.094 ± 0.055
D-Pro	A	0.600 ± 0.017
L-Pro + D-Asp	A	12.900 ± 0.163
L-Pro + L-Asp	A	10.005 ± 0.081
L-Pro + D-Asp + D-Glu	A	14.890 ± 0.072
L-Pro + D-Asp + L-Glu	A	12.010 ± 0.046
L-Pro + D-Asp + D-Glu + L-Tyr	A	15.668 ± 0.112
L-Pro + D-Asp + D-Glu + D-Tyr	A	14.682 ± 0.071
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser	AB	21.540 ± 0.246
L-Pro + DL-Asp + D-Glu + L-Tyr + D-Ser	AB	16.600 ± 0.116
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + L-Leu	AB	23.320 ± 0.098
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + D-Leu	AB	21.410 ± 0.070
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + D-Leu + L-Ala	ABC	26.615 ± 0.069
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + L-Leu + D-Ala	ABC	23.525 ± 0.063
L-Pro + mycobacillin amino acids added non-sequentially	ABC	11.120 ± 0.092

* Results are expressed as means ± S.E.M. for four different experiments.

D-aspartic acid under similar conditions, indicating the absence of aspartic acid racemase. In an analogous manner, the treatment, with L-glutamic acid decarboxylase, of a mixture of non-labelled L-glutamic acid (added as carrier) and labelled D-glutamic acid previously treated

with mycobacillin synthetase did not lead to the formation of labelled γ-aminobutyric acid, a result indicating the absence of glutamic acid racemase. Also, no racemization either of glutamic acid or of alanine or aspartic acid took place in the presence of ATP.

Table 3. Incorporation of D- and L-amino acids into mycobacillin by purified mycobacillin synthetase

Enzyme activity was measured under the standard assay conditions described in the text. The purified enzyme used was at 50 µg in a total volume of 1 ml. Different radioactive amino acids were used as a requirement, but each was diluted with unlabelled carrier to attain a concentration whereby 5 µM-amino acid = 0.1 mCi.

Incubation mixture	10 ⁻³ × Radioactivity (c.p.m.) in mycobacillin*
Complete	3.800 ± 0.048
—L-Pro	0.200 ± 0.004
—L-Pro + D-Pro	0.350 ± 0.001
—D-Asp	0.425 ± 0.008
—L-Asp	0.420 ± 0.066
—D-Glu	0.330 ± 0.011
—D-Glu + L-Glu	0.440 ± 0.008
—L-Tyr	0.240 ± 0.006
—L-Tyr + D-Tyr	0.350 ± 0.002
—L-Ser	0.200 ± 0.001
—L-Ser + D-Ser	0.275 ± 0.005
—L-Leu	0.240 ± 0.002
—L-Leu + D-Leu	0.300 ± 0.005
—L-Ala	0.250 ± 0.007
—L-Ala + D-Ala	0.325 ± 0.008

* Results are expressed as means ± S.E.M. for four different experiments.

Effect of L or D isomers of constituent amino acids on ATP-[³²P]P_i exchange

It is obvious from Table 2 that the mycobacillin amino acids, if added sequentially from L-proline, enhance the ATP-[³²P]P_i exchange activity. Replacement of L- by D-amino acids and vice versa anywhere in the polypeptide chain completely inhibited the sequential enhancement in exchange activity.

Role of different L or D isomers of constituent amino acids on ¹⁴C-labelled-amino-acid incorporation into the antibiotic

Studies undertaken to discover whether mycobacillin was synthesized by alteration of D- or L-amino acids in their respective sequential position in the molecule indicated that complete inhibition of antibiotic synthesis occurred when such alterations were made (Table 3).

Effect of preincubation with optical isomers of opposite configuration on ATP-[³²P]P_i exchange

The effect of preincubation of mycobacillin synthetase with amino acids of opposite configuration on the sequential-amino-acid-dependent ATP-[³²P]P_i exchange was studied. It was observed that preincubation with D-proline inhibited L-proline-dependent ATP-[³²P]P_i exchange to the extent of 90%, whereas other amino acids of opposite configuration inhibited the sequential ATP-[³²P]P_i exchange to the extent of 60–80%.

Fig. 1. Requirement of ATP for mycobacillin synthesis by purified mycobacillin synthetase

The details of the method are given in the text. Key to symbols: ○, mycobacillin synthesis; ●, ATP consumed.

Absence of pantothenic acid from different enzyme fractions of mycobacillin synthetase

Pantothenic acid, which actively takes part in oligopeptide biosynthesis, was absent from any of the fractions of the three-fraction mycobacillin synthetase.

Total ATP requirement for mycobacillin synthesis

It is obvious from Fig. 1 that mycobacillin synthesis did not occur when the amount of ATP added was below 0.5 μmol. At or above this level, a linear relationship was observed between mycobacillin synthesis and ATP utilization. The computation of the data for the several pairs of points over the linear range (Fig. 1) shows that, on average, 7.25 nmol or 7250 pmol of ATP are consumed for the synthesis of 271 pmol of mycobacillin. In other words, 25.63 mol i.e., 26 mol of ATP, are consumed for the synthesis of 1 mol of mycobacillin.

DISCUSSION

In an attempt to show whether the D-amino acids of mycobacillin originate from L isomers by specific racemases, we assayed the racemase activity of different enzyme fractions of mycobacillin synthetase; the results indicated that the enzyme system did not contain any ATP-dependent or -independent specific racemases for aspartic acid, glutamic or alanine, unlike the situation for gramicidin S or tyrocidin, whose enzyme system contained ATP-dependent racemase activity (Gevers *et al.*, 1969).

Like those pertaining to gramicidin S and tyrocidin (Barry & Ichihara, 1958; Mach & Tatum, 1964), the whole-cell fermentation experiments revealed that the constituent D-amino acids of mycobacillin did not inhibit

its biosynthesis (Banerjee & Bose, 1967, 1968). But unlike the situation with gramicidin S and tyrocidin, whose cell-free enzyme system can incorporate both D- and L-amino acids with almost equal facility into the peptide chain, highly purified mycobacillin synthetase was unable to incorporate optical isomers of opposite configuration into the polypeptide chain. A study of the effect of preincubation with amino acids of opposite configuration in equimolar amounts on ATP-[³²P]P_i exchange showed that they were competitive inhibitors of each other.

Interestingly, none of the three enzyme fractions of mycobacillin synthetase contained pantothenic acid, which is a unique requirement for peptide antibiotic synthesis, highlighting the fact that the elongation of the peptide chain of the mycobacillin to completion does not require the participation of the pantothenic-acid-arm system that is observed in the synthesis of gramicidin S, tyrocidin etc. (Gilhuus-Moe *et al.*, 1970; Kleinkauf *et al.*, 1970; Lipmann, 1971).

Experiments on the energy requirement for mycobacillin synthesis indicated that only two molecules of ATP were needed for the synthesis of one peptide bond of mycobacillin, whereas in protein synthesis about 4 molecules of ATP are required per peptide bond. This shows that the former synthesis was less expensive in energy terms than the latter, and did not require its elaborate machinery, a finding that agreed with the present-day concept of antibiotic peptide synthesis.

It is noteworthy that isolation of intermediate peptides of mycobacillin (extraction with butanol and without the use of performic acid and alkaline treatment needed for the release of thioesterified peptide) from the enzyme-peptide complex was easy, indicating that the peptides were non-covalently bound to the enzyme, a finding further supported by the instability of the complex to trichloroacetic acid treatment (results not shown).

Thus mycobacillin synthetase, which appears to form non-covalent bonds with intermediate peptides, lacks both racemase activity and a pantothenic acid arm.

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