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Functional characterization of constituent enzyme fractions of mycobacillin synthetase

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The enzyme fraction A, a constituent enzyme of the three-fraction enzyme mycobacillin synthetase, independently and sequentially activated five amino acids starting from L-proline, producing the pentapeptide Pro(Asp_1,Glu_1,Tyr_1)Asp. The fractions B and C were unable to function independently. However, the fraction B synthesized the nonapeptide Pro(Asp_3,Glu_1,Tyr_2,Ser_1)Leu, sequentially activating the pentapeptide and next four amino acids, whereas the fraction C synthesized mycobacillin by the sequential activation of the nonapeptide and the remaining four amino acids. The pH optima of the above enzymes are almost identicial (pH 7.8), but their K_m values are a little different.

Biosynthesis of peptide antibiotics elaborated by *Bacillus* species involves multienzyme complexes (Roskoski *et al.*, 1970*a*,*b*; Shimura *et al.*, 1974). Gramicidin S is synthesized by two complementary enzyme fractions (Tomino *et al.*, 1967), and tyrocidine and bacitracin each by three enzyme fractions (Roskoski *et al.*, 1970*a*,*b*; Frøyshov, 1974). Each of the fractions involved in these three syntheses has been characterized (Gevers *et al.*, 1968; Lee *et al.*, 1973). Mycobacillin (Majumder & Bose, 1958), an antifungal cyclic tridecapeptide antibiotic (Majumber & Bose, 1960; Banerjee & Bose, 1963), is elaborated by *Bacillus subtilis* B_3. The biosynthesis occurs through sequential activation, by ATP–P_i exchange reaction, of the mycobacillin amino acids starting from L-proline (Sengupta & Bose, 1972, 1974), and the enzyme complex involved in the synthesis is a three-fraction enzyme (A, B and C) called mycobacillin synthetase (Ghosh *et al.*, 1983). All the fractions are localized in cytosol in the early exponential phase (Sengupta & Bose, 1971), but are subsequently translocated to plasma membrane during the stationary phase (Mukhopadhyay *et al.*, 1985). The present work seeks to characterize the three constituent enzyme fractions A, B and C of the synthetase.

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Materials and methods

Chemicals and radiochemicals

All materials used were of analytical grade where available, and were obtained as follows. Hydrazine hydrate (80%, w/v) and 1-fluoro-2,4-dinitrobenzene were purchased from E. Merck (Darmstadt, Germany). Hydrazine hydrate was distilled to 99% (w/v) as described by Locker (1954). $Na_2H^{32}PO_4$ (sp. radioactivity 10 Ci/mol) was purchased from Bhabha Atomic Research Centre (Trombay, India). Other materials were obtained from commercial sources.

Preparation of enzyme

The producer organism *B. subtilis* B_3 (Majumder & Bose, 1958) was grown in fresh nutrient broth supplemented with 1% (w/v) glucose for 15–16 h at 30 ± 1°C, and the 20000*g* supernatant was prepared from harvested cells. The supernatant was purified by $(NH_4)_2SO_4$ fractionation and column chromatography on Sephadex G-200 and DEAE-cellulose, as described previously by 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 × 6 cm) that had been equilibrated with 0.02 M-phosphate buffer, pH 7.2, containing 0.25 mM-EDTA, 5 mM-$MgCl_2$ and 1 mM-dithiothreitol, and then stepwise elution

was performed with increasing concentrations of the phosphate buffer (0.05M, 0.08M, 0.1M, 0.12M, 0.18M etc.), 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 discontinuous sucrose gradient (10%, 12.5%, 15%, 17.5%, 20% and 22.5%, w/v) and centrifuged at 40000rev./min in a Beckman SW 40 rotor for 8h at 0°C. All the subsequent studies were performed with these active fractions.

Measurement of ATP-[^{32}P]P_i exchange

The exchange was studied by using the method of Stulberg & Novelli (1960) as described by Ghosh *et al.* (1983).

Isolation and characterization of peptides

The incubation mixture for each of the sets contained the following, in a total volume of 2ml: Tris/HCl buffer, pH7.8, 100μM; $MgCl_2$, 10μM; ATP, 10μM; dithiothreitol, 10μM; mycobacillin amino acids, 10μM each (whose composition was varied according to the plan of experiments as indicated in the text); enzyme, 200μg (any one of the three fractions A, B and C of the enzyme complex either alone or in all possible combinations). This mixture was incubated for 20min, and the peptides from the incubation mixture were isolated by using the method described previously by Sengupta & Bose (1972). Each of the new peptides so isolated was then analysed to determine the amino acid composition and the *C*-terminal and *N*-terminal amino acids by the method of Akabori *et al.* (1956) and Sanger (1945) as described by Sengupta & Bose (1972).

Identification of mycobacillin formed by the enzyme fractions

The incubation mixture and the method of isolation of radioactive mycobacillin were the same as described by Ghosh *et al.* (1983), and that the radioactive material was mycobacillin was further confirmed by silica-gel t.l.c (Kieselgel F 1500; Schleicher und Schüll, Dassel, Germany) with the solvent system propanol/conc. NH_3 (2:1, v/v), the radioactivity in the scraped material being found in the range of the R_F value of authentic mycobacillin.

Determination of protein concentration

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

Table 1. *ATP-[^{32}P]P_i exchange in the activation of mycobacillin amino acids, added in a sequential manner, catalysed by the three enzyme fractions*

The assays were carried out by incubating 50μg each of enzyme fractions A, B and/or C in a medium with the following composition: 0.1M-Tris/HCl buffer, pH8.0, 3mM-ATP, 5mM-$MgCl_2$, 10mM-KF, 2mM-dithiothreitol, 5mM of each amino acid and 2mM-$Na_2H^{32}PO_4$ (10μCi; sp. radioactivity 10Ci/mol). The incubation was for 30min at 30°C in a final volume of 300μl. Under these conditions the time course for enzyme activity is linear. Incorporation into ATP was determined as previously described (Ghosh *et al.*, 1983). Results are expressed as means ± S.E.M. for four different experiments. Mycobacillin is cyclo-(L-Pro-D-Asp-D-Glu-L-Tyr-L-Asp-L-Tyr-L-Ser-D-Asp-L-Leu-D-Glu-D-Asp-L-Ala-D-Asp-).

Mycobacillin amino acids added	10^{-3} × Amount of radioactivity in ATP (c.p.m.)						
	Fraction A	Fraction B	Fraction C	Fractions A+B	Fractions B+C	Fractions C+A	Fractions A+B+C
Mycobacillin amino acids except L-proline	0.77 ± 0.01	0.60 ± 0.04	0.96 ± 0.02	0.60 ± 0.03	0.83 ± 0.04	0.80 ± 0.04	0.70 ± 0.04
L-Pro	8.47 ± 0.02	0.85 ± 0.03	0.86 ± 0.03	7.93 ± 0.42	0.97 ± 0.04	5.96 ± 0.35	9.32 ± 0.34
L-Pro + D-Asp	9.55 ± 0.06	0.69 ± 0.08	0.53 ± 0.04	10.50 ± 0.46	0.69 ± 0.04	7.63 ± 0.49	11.65 ± 0.26
L-Pro + D-Asp + D-Glu	11.06 ± 0.08	0.81 ± 0.04	0.61 ± 0.04	12.71 ± 0.45	0.38 ± 0.05	11.03 ± 0.42	15.58 ± 0.15
L-Pro + D-Asp + D-Glu + L-Tyr	12.44 ± 0.03	0.72 ± 0.04	0.46 ± 0.05	15.74 ± 0.72	0.71 ± 0.03	15.20 ± 0.23	16.85 ± 0.17
L-Pro + DL-Asp + D-Glu + L-Tyr	13.45 ± 0.02	0.50 ± 0.05	0.72 ± 0.03	19.01 ± 0.32	0.66 ± 0.04	16.41 ± 0.25	22.42 ± 0.24
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser	13.12 ± 0.03	0.79 ± 0.03	0.89 ± 0.05	20.62 ± 0.76	0.89 ± 0.03	16.36 ± 0.35	23.06 ± 0.33
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + L-Leu	12.87 ± 0.11	0.85 ± 0.01	0.54 ± 0.03	22.48 ± 0.38	0.92 ± 0.03	16.89 ± 0.16	25.95 ± 0.56
L-Pro + DL-Asp + D-Glu + L-Tyr + L-Ser + L-Leu + L-Ala	13.37 ± 0.25	0.69 ± 0.04	0.92 ± 0.02	22.10 ± 0.31	0.87 ± 0.04	16.86 ± 0.30	29.01 ± 0.27

Results

Functional characterization of enzyme fractions by ATP–[^{32}P]P_i exchange reaction

The ATP–[^{32}P]P_i exchange reactions involved in the activation of mycobacillin amino acids by the three enzyme fractions either alone or in different combinations were studied. Table 1 shows that no exchange reaction occurred in the absence of L-proline with any of the three enzyme fractions either alone or in combination. The enzyme fraction A effected the exchange reaction starting from L-proline (the initiating amino acid) to L-tyrosine (the fifth amino acid in the sequence). However, neither enzyme fraction B nor fraction C alone activated any amino acid. From studies on the exchange reaction by combinations of any two fractions out of the three it appears that fractions A plus B effected the exchange reaction up to L-leucine (the ninth amino acid in the sequence), whereas fractions B plus C did not activate any amino acid, and the activation by fractions A plus C was similar to that by fraction A alone. The combination of all fractions effected the activation of all the mycobacillin amino acids sequentially.

Functional characterization of enzyme fractions by peptide synthesis

Table 2 shows the peptide formation by the three enzyme fractions individually, in all possible combinations of two and finally in the combination of all three. The enzyme fraction A alone synthesized the pentapeptide Pro(Asp$_1$,Glu$_1$,Tyr$_1$)Asp beginning with *N*-terminal proline and ending with *C*-terminal aspartic acid; however, neither enzyme fraction B nor fraction C alone synthesized any peptide. From studies of peptide synthesis by combinations of any two fractions out of the three it appears that enzyme fractions A plus B synthesized the nonapeptide Pro(Asp$_3$,Glu$_1$,Tyr$_2$,Ser$_1$)Leu beginning with *N*-terminal proline and ending with *C*-terminal leucin, whereas fractions B plus C did not synthesize any peptide, and fractions A plus C, the pentapeptide that enzyme fraction A alone could produce. The combination of all three fractions synthesized mycobacillin as before.

Characterization of enzyme fractions B and C by ATP–[^{32}P]P_i exchange reaction and peptide synthesis

The characterization of the enzyme fraction B was done in the presence of pentapeptide (the product of enzyme fraction A action; Table 2) and the next four amino acids in the mycobacillin sequence both by ATP–[^{32}P]P_i exchange and peptide synthesis. Studies on the exchange reaction showed (Table 3) that enzyme fraction B activated the pentapeptide alone and also the pentapeptide plus the next four amino acids in the sequence, and those on peptide synthesis by fraction B indicate that it synthesized the nonapeptide (Table 2) in the presence of pentapeptide and the next four amino acids in the sequence. Similar studies were carried out with fraction C. Fraction C effected the exchange reaction in the presence of nonapeptide (the product of fraction B action) and the remaining four mycobacillin amino acids, and the peptide product formed under this condition by fraction C was mycobacillin.

Physical characteristics of the three enzyme fractions

pH optima by ATP–[^{32}P]P_i exchange reaction. The ATP–[^{32}P]P_i exchange reaction effected by enzyme fraction A in the presence of five constituent amino acids at different pH values showed that the maximum exchange occurred at pH 7.8. The ATP–[^{32}P]P_i exchange effected by enzyme fraction B in the presence of pentapeptide and the remaining four constituent amino acid residues of the nonapeptide was similarly studied, and in this case maximum exchange occurred at pH 7.6. The same procedure was followed in determining the optimum pH for the exchange reaction effected by enzyme fraction C, and this was found to be at pH 7.8 (Fig. 1).

Table 2. *Formation of peptides by the three enzyme fractions in possible combinations*
For experimental details see the text.

Added enzyme fractions	R_F of peptide in solvent: Butan-1-ol/acetic acid/water (4:1:1, by vol.)	R_F of peptide in solvent: Ethanol (70%, v/v)	*C*-Terminal amino acid	*N*-Terminal amino acid	Amino acid composition
A	0.61	0.72	Asp	Pro	Pro$_1$,Asp$_2$,Glu$_1$,Tyr$_1$
B	—	—	—	—	—
C	—	—	—	—	—
A+B	0.56	0.79	Leu	Pro	Pro$_1$,Asp$_3$,Glu$_1$,Tyr$_2$,Ser$_1$,Leu$_1$
B+C	—	—	—	—	—
C+A	0.61	0.72	Asp	Pro	Pro$_1$,Asp$_2$,Glu$_1$,Tyr$_1$

Table 3. *Peptides synthesis and amino acid activation tested by using ATP-[^{32}P]P_i exchange reaction*
Incorporations were measured by the procedures given in Table 1. Results are expressed as means ± S.E.M. for four different experiments. Mycobacillin is cyclo-(L-Pro-D-Asp-D-Glu-L-Tyr-L-Asp-L-Tyr-L-Ser-D-Asp-L-Leu-D-Glu-D-Asp-L-Ala-D-Asp-).

Enzyme fraction used	Intermediate peptide and amino acids added in a sequential manner	10^{-3} × Amount of radioactivity in ATP (c.p.m.)
B	(Pro_1,Asp_2,Glu_1,Tyr_1)	2.69 ± 0.09
	(Pro_1,Asp_2,Glu_1,Tyr_1)+Tyr	3.51 ± 0.11
	(Pro_1,Asp_2,Glu_1,Tyr_1)+Tyr+Ser	4.96 ± 0.12
	(Pro_1,Asp_2,Glu_1,Tyr_1)+Tyr+Ser+D-Asp	6.50 ± 0.28
	(Pro_1,Asp_2,Glu_1,Tyr_1)+Tyr+Ser+D-Asp+Leu	7.21 ± 0.16
C	(Pro_1,Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)	3.21 ± 0.28
	(Pro_1,Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)+D-Glu	5.16 ± 0.10
	(Pro_1,Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)+D-Glu+D-Asp	6.21 ± 0.17
	(Pro_1,Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)+D-Glu+D-Asp+Ala	7.61 ± 0.19

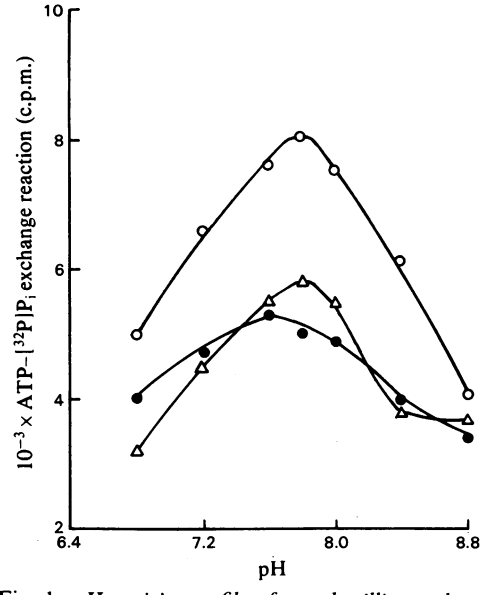

Fig. 1. *pH–activity profile of mycobacillin synthetase*
ATP-[^{32}P]P_i exchange measurements over 30min were carried out (as a measure of the velocity of the reaction) with enzyme fraction A (50μg) in the presence of the first five amino acids (Table 1) in the mycobacillin sequence, with enzyme fraction B (100μg) in the presence of pentapeptide (Table 2) plus the next four amino acids in the mycobacillin sequence, and with enzyme fraction C (100μg) in the presence of nonapeptide (Table 2) plus the remaining four amino acid residues (in the mycobacillin sequence, in 0.1M-Tris/HCl buffer, pH6.8–8.8. ○, Activity of fraction A; ●, activity of fraction B; △, activity of fraction C.

K_m values of the enzyme fractions. The amount of ATP-[^{32}P]P_i exchange by enzyme fraction A in the presence of different concentrations of L-proline was determined, and the Lineweaver–Burk plot of these data shows that the K_m of fraction A for L-proline is 0.084mM. By the same method the K_m of fraction B for the pentapeptide and that of fraction C for the nonapeptide are 0.026mM and 0.018mM respectively.

Discussion

In continuation of our previous findings (Ghosh *et al.*, 1983) that mycobacillin synthetase, a three-fraction enzyme complex, produces mycobacillin only when the three fractions A, B and C are all present, we observed that fraction A alone activated sequentially, but not individually, by ATP-P_i exchange, the five mycobacillin amino acids starting with L-proline, producing the pentapeptide Pro(Asp_1,Glu_1,Tyr_1)Asp. With other peptide antibiotics, such as gramicidin S, tyrocidine, bacitracin etc. (Gevers *et al.*, 1969; Roskoski *et al.*, 1970*a*,*b*; Frøyshov, 1975), intermediate peptide synthesis occurs sequentially, although the constituent amino acids are activated individually by ATP-PP_i exchange producing amino acid adenylates.

The functional independence of fraction B is restored in the presence of the pentapeptide and the next four amino acids in the mycobacillin sequence, the nonapeptide Pro(Asp_3,Glu_1,Tyr_2,-Ser_1)Leu being produced through sequential activation. Similarly, the functional independence of fraction C is restored in the presence of the nonapeptide and the remaining four amino acids in the mycobacillin sequence, with the formation of the final product mycobacillin by sequential activation. Thus mycobacillin synthesis appears to be a three-step process, carried out by three independent enzymes, the product of the first enzyme fraction A being accepted as the substrate for the

second enzyme fraction B, leading to the addition of the next four amino acids in the sequence, and the product of fraction B being the substrate for the third enzyme fraction C, giving the addition of the remaining four amino acids in the sequence. With other peptide antibiotics, neither the synthesis of gramicidin S appears to be a two-step independent process nor that of tyrocidine or bacitracin to be a three-step independent process but rather to be a stepwise interdependent process in each case. The intermediate peptides, although formed sequentially, remained bound to the enzyme through a thioester bond, and are not released until the completion of the molecule. In fact, the intermediate peptides formed by the preceding enzyme are not accepted as substrate by the following one unless the peptides are presented to it in the covalently bound form.

The pH optima for all these reactions, occurring in the same microenvironment of the cytosol, are almost identical, being about 7.8. Interestingly, the K_m values of the three enzyme fractions towards the incoming first substrate are in slightly decreasing order.

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