Biochem. J. (1986) 240, 265–268 (Printed in Great Britain)

Role of ATP and enzyme-bound nascent peptides in the control of elongation for mycobacillin synthesis

Subrata Kumar GHOSH, Sekhar MAJUMDER, Nishit Kumar MUKHOPADHYAY and Sushil Kumar BOSE*
Department of Biochemistry, University College of Science, 35 Ballygunge Circular Road, Calcutta-700019, India

The enzyme fraction A, a constituent of the three-fraction (A, B and C) enzyme complex mycobacillin synthetase, elongated tri- and tetra-peptides, under enzyme-bound conditions, to tetra- and penta-peptides respectively in the presence of the 'next' amino acid (in the mycobacillin sequence). The enzyme fraction B synthesized hexapeptide from free pentapeptide and the next amino acid, but synthesized heptapeptide from hexapeptide only under enzyme-bound conditions in the presence of the next amino acid. Similarly, the enzyme fraction C synthesized decapeptide from free nonapeptide in the presence of the next amino acid, but undecapeptide only from enzyme-bound decapeptide in the presence of the next amino acid during the elongation process. The K_m values for the initiating reactions for each of the three enzyme fractions were 6–7-fold lower than those for the succeeding reactions catalysed by each of the enzyme fractions. The specificity of the initiation and elongation is discussed in the light of these findings.

INTRODUCTION

In most non-ribosomal syntheses of peptides such as gramicidin S, tyrocidin and bacitracin the elongation of a specific peptide chain is achieved by the transfer of thiol-bound peptides to thiol-bound amino acids (Roskoski *et al.*, 1970*a*,*b*; Kleinkauf *et al.*, 1971; Lee *et al.*, 1973; Kurahashi, 1980). This transfer occurs via the 4′-phosphopantetheine arm to which the peptide is linked by a thiol bond. The peptide is then transferred by the arm to the next amino acid covalently bound on the enzyme surface also by a thiol bond. All the thiol groups have to be accessible to the cofactor pantetheine, which plays a key role in elongation. Intermediate peptides remain bound to the enzyme, and only completed molecules are released.

The synthesis of mycobacillin (Majumder & Bose, 1958), a cyclotridecapeptide antibiotic (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) (Majumder & Bose, 1960), is catalysed by the three-fraction (A, B and C) enzyme complex mycobacillin synthetase (Ghosh *et al.*, 1983), which was purified to homogeneity (Ghosh *et al.*, 1986). The synthesis occurs by three-step process, the enzyme fraction A synthesizing pentapeptide in the first step, fraction B the nonapeptide in the second step, and fraction C completing the molecules in the third step (Ghosh *et al.*, 1985). None of the fractions contains the pantothenic acid arm (Mukhopadhyay *et al.*, 1986), but instead a sequential activation of an amino-acid-dependent ATP$\rightleftharpoons$[^{32}P]P_i exchange reaction (Sengupta & Bose, 1972, 1974) is carried out instead of individual activation, as is the case for other bioactive peptides (Itoh *et al.*, 1968; Gevers *et al.*, 1968).

The present study therefore sought to examine (i) the elongation of the peptide chain of mycobacillin by the above three-fraction enzyme in the absence of the 4′-phosphopantetheine arm and (ii) specific binding sites for amino acids via a thiol bond.

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.

Organism

Bacillus subtilis B_3 (Majumder & Bose, 1958) was used to study the biosynthesis of mycobacillin.

Preparation of enzyme

The producer organism *B. subtilis* B_3 was grown in a fresh nutrient broth supplemented with 1% glucose for 15–16 h at 30±1 °C and a 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 (Ghosh *et al.*, 1983), and subsequently the final purification was done by hydroxyapatite chromatography and sucrose-density-gradient centrifugation (Ghosh *et al.*, 1986).

Measurement of ATP$\rightleftharpoons$[^{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 2 ml: Tris/HCl buffer, pH 7.8, 100 μM; $MgCl_2$, 10 μM; ATP, 10 μM; dithiothre-

* To whom correspondence and reprint requests should be addressed.

Table 1. Role of substrate-bound enzyme fraction in peptide-chain elongation

The incubation mixture for each of the sets contained the following, in a total volume of 2 ml: Tris/HCl buffer, pH 7.8, 100 μM; $MgCl_2$, 10 μM; ATP, 10 μM; dithiothreitol, 10 μM; amino acid (according to the plan of the experiment), 10 μM; enzyme, 200 μg; and peptide (free or enzyme-bound). The mixture was incubated for 20 min and the peptide was isolated by the method described in the Materials and methods section. Myobacillin 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	Peptides used	Amino acid added next in sequence	R_F value of peptide: Butan-1-ol/acetic acid/water (4:1:1, by vol.)	R_F value of peptide: Ethanol (70%, v/v)	C-Terminal amino acid	Amino acid composition
A	$Pro(Asp_1)Glu$	L-Tyr	—	—	—	—
	$Pro(Asp_1)Glu$ bound to the enzyme surface	L-Tyr	0.59	0.75	Tyr	Pro_1,Asp_1,Glu_1,Tyr_1
	$Pro(Asp_1,Glu_1)Tyr$	L-Asp	—	—	—	—
	$Pro(Asp_1,Glu_1)Tyr$ bound to the enzyme surface	L-Asp	0.61	0.72	Asp	Pro_1,Asp_2,Glu_1,Tyr_1
B	$Pro(Asp_1,Glu_1,Tyr_1)Asp$	L-Tyr	0.57	0.80	Tyr	Pro_1,Asp_2,Glu_1,Tyr_2
	$Pro(Asp_2,Glu_1,Tyr_1)Tyr$	L-Ser	—	—	—	—
	$Pro(Asp_2,Glu_1,Tyr_1)Tyr$ bound to the enzyme surface	L-Ser	0.55	0.81	Ser	$Pro_1,Asp_2,Blu_1,Tyr_2,Ser_1$
C	$Pro(Asp_3,Glu_1,Tyr_2,Ser_1)Leu$	D-Glu	0.50	0.76	Glu	$Pro_1,Asp_3,Glu_2,Tyr_2,Ser_1,Leu_1$
	$Pro(Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)Glu$	D-Asp	—	—	—	—
	$Pro(Asp_3,Glu_1,Tyr_2,Ser_1,Leu_1)Glu$ bound to enzyme surface	D-Asp	0.41	0.82	Asp	$Pro_1,Asp_4,Glu_2,Tyr_2,Ser_1,Leu_1$

itol, 10 μM; mycobacillin amino acids, 10 μM each [the number of amino acids added corresponded to the peptide required (tri-, penta-, nona- etc.)]; enzyme, 200 μg. This mixture was incubated for 20 min, and the peptides from the incubation mixture were isolated as described previously (Sengupta & Bose, 1972). Each of the new peptides so isolated was then analysed to determine its amino acid composition and *C*-terminal and *N*-terminal amino acids by the methods of Akabori *et al.* (1956) and Sanger (1945), as described by Sengupta & Bose (1972).

Preparation of nascent peptide chains bound to the enzyme fractions

The incubation mixture for the preparation of nascent peptides was same as that described above, but the method of preparation of enzyme-bound nascent peptides was different. After 20 min the incubation mixture was immediately chilled on ice and diluted with buffer B (10 mM-Tris/HCl buffer, pH 7.8, containing 0.25 mM-EDTA, 10 mM-$MgCl_2$ and 1 mM-dithiothreitol) and then the mixture was poured into a Millipore filter (0.45 μm pore size). After rapid washing with an excess volume of cold buffer B, the filter paper containing adsorbed enzyme-bound nascent peptide was used for the extension of the peptide chain after reincubation with the next amino acid(s) in the mycobacillin sequence.

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

Elongation by the enzyme fraction A

Stepwise elongation of tri- and tetra-peptide was studied by incubating unbound peptide and enzyme fraction A or enzyme-bound peptides in the presence of the next amino acid in the sequence (Table 1). It appears from Table 1 that the enzyme-bound tripeptide $Pro(Asp_1)Glu$ is elongated to tetrapeptide $Pro(Asp_1,Glu_1)Tyr$ in presence of the next amino acid, L-tyrosine (as confirmed by the determination of amino acid composition and *C*-terminal amino acid), whereas the tripeptide when free and not bound to the enzyme surface was not utilized by the enzyme fraction A for the synthesis of tetrapeptide. In the same way the enzyme-bound tetrapeptide is converted into the pentapeptide $Pro(Asp_1,Glu_1,Tyr_1)Asp$ in the presence of the next amino acid, L-aspartic acid, as confirmed by amino acid composition and *C*-terminal amino acid of the peptide, whereas free tetrapeptide is not converted into pentapeptide under identical conditions.

Elongation by the enzyme fraction B

The stepwise elongation process for the synthesis of longer peptides (hexa and hepta) from one-amino-acid-shorter ones (penta and hexa) was studied by incubating the unbound shorter peptides and enzyme fraction B or the enzyme-fraction-bound shorter peptides in the presence of the next amino acid in the sequence. It appears from Table 1 that the free pentapeptide $Pro(Asp_1,Glu_1,Tyr_1)Asp$ in the presence of the next amino acid, L-tyrosine, is converted into the hexapeptide $Pro(Asp_2,Glu_1,Tyr_1)Tyr$ by the enzyme fraction B. The enzyme-bound hexapeptide is elongated to the heptapeptide $Pro(Asp_2,Glu_1,Tyr_2)Ser$ in the presence of the next amino acid, L-serine, whereas free hexapeptide in the presence of the next amino acid is not converted into the heptapeptide.

Elongation by the enzyme fraction C

An analogous experiment was carried out with the enzyme fraction C for stepwise elongation of nonapeptide to decapeptide in the presence of the next amino acid,

D-glutamic acid, and for further elongation of decapeptide to undecapeptide in presence of the next amino acid, D-aspartic acid. The substrate peptides were added to the incubation system either in the free condition or under enzyme-bound conditions. It appears from Table 1 that free nonapeptide Pro(Asp_2,Glu_1,Tyr_2,Ser_1)-Leu in the presence of the next amino acid is converted into decapeptide Pro(Asp_2,Glu_1,Tyr_2,Ser_1,Leu_1)-Glu by the enzyme fraction C. The enzyme-bound decapeptide is elongated to the undecapeptide Pro(Asp_3,Glu_2,Tyr_2,Ser_1,Leu_1)Asp in the presence of the next amino acid, D-aspartic acid, whereas free decapeptide is not converted into undecapeptide in the presence of the next amino acid.

K_m values of the enzyme fractions, A, B and C for the respective initiating and succeeding substrates

It appears from Table 2 that the K_m values of the enzyme fraction A for the initiating substrate L-proline was 0.084 mM, whereas those for succeeding amino acids during the elongation process up to the formation of pentapeptide were about 5–6 times higher than that for L-proline. The same trend was observed for fractions B and C. The K_m values for the initiating substrates pentapeptide and nonapeptide for the enzyme fraction B and C respectively were usually 7–8 times lower than those for the succeeding substrates in the case of both fractions B and C.

Table 2. K_m values of the enzyme fractions of mycobacillin synthetase for initiation and elongation reactions

The K_m values of the enzyme fractions for initiation and elongation reactions were derived on the basis of ATP$\rightleftharpoons$[^{32}P]Pi exchange experiments from Lineweaver–Burk plots. For initiation the exchange was studied with the free enzyme fraction in the presence of various concentrations of initiating amino acid, proline or penta- or nona-peptides. For elongation the exchange was studied with the peptide-bound enzyme in the presence of various concentrations of the next amino acid in the sequence

Enzyme	Substrate	K_m (mM)
Fraction A	L-Proline	0.084
	D-Aspartic acid	0.43
	D-Glutamic acid	0.51
	L-Tyrosine	0.39
	D-Aspartic acid	0.36
Fraction B	Pentapeptide	0.026
	L-Tyrosine	0.14
	L-Serine	0.23
	D-Aspartic acid	0.091
	L-Leucine	0.18
Fraction C	Nonapeptide	0.018
	D-Glutamic acid	0.13
	D-Aspartic acid	0.095
	L-Alanine	0.17
	D-Aspartic acid	0.098

DISCUSSION

We here report a continuation of our previous work on the functional characterization of constituent enzymes of the three-fraction enzyme mycobacillin synthetase, which showed that fraction A formed the first initiating complex with L-proline in the presence of ATP, that fraction B formed the second initiation complex with pentapeptide in the presence of ATP and that fraction C formed the third or last initiation complex with nonapeptide in the presence of ATP (Ghosh *et al.*, 1985). The present work reports elongation studies demonstrating that fraction A converted tripeptide in the presence of ATP and the next amino acid in the sequence into tetrapeptide and, again, converted tetrapeptide into pentapeptide (also in the presence of ATP and the next amino acid in the sequence), thus lengthening the chain by one amino acid in the presence of ATP for the two successive steps. This may be taken to mean that the formation of tripeptide *in situ* on the surface of the enzyme fraction A, followed by ATP binding, might have altered its recognition capability in such a way as to accommodate the specific binding of the next amino acid, L-tyrosine, in the sequence for tetrapeptide synthesis. The synthesis of pentapeptide follows exactly the same mechanism as in the case of tetrapeptide. Interestingly, no such specific binding sites pre-existed (before nascent peptide formation followed by ATP binding) on the surface of the native enzyme. However, in the case of gramicidin S and tyrocidin synthesis, a specific amino-acid-binding site pre-existed on the enzyme in the presence of ATP but in the absence of nascent peptide (Kurahashi, 1980; Frøyshov *et al.*, 1978; Kleinkauf & Koischwitz, 1980).

That elongation more or less followed the same pattern in the case of enzyme fractions B and C was revealed by the formation of a one-amino-acid-higher homologue of a peptide from a lower one (bound as the nascent peptide) by the enzyme fraction B or C in the presence of ATP and the next amino acid in the sequence. Thus ATP binding at each of the elongation steps brought about a continuous alteration of the peptide-bound enzyme complex, generating one after another active site for the specific binding of the next amino acid until the synthesis was complete. Obviously ATP has a two-fold function in the synthesis: one to supply energy and the other to bring about the necessary change in the enzyme conformation. It is noteworthy that mycobacillin, a tridecapeptide, required, per mol of the antibiotic, 26 mol of ATP (Mukhopadhyay *et al.*, 1986), whereas tyrocidin, a decapeptide, required only 10 (Roskoski *et al.*, 1970*a*).

Again, each of the enzyme fractions of the complex has five reactive sites for binding: in the case of enzyme fraction A, five specific amino acids; in the case of enzyme fraction B, one specific pentapeptide and four specific amino acids; and in the case of enzyme fraction C, the specific nonapeptide and four specific amino acids. The K_m values for the three enzyme fractions and for each of these five respective reactive sites were separately determined. We found that the K_m values for the initiating reactions of each of three enzyme fractions were 6–7-fold lower than those for the four succeeding reactions catalysed by each of the enzyme fractions. This might be due to a long evolutionary process in the interest of the economy of synthesis of the antibiotic. Thus ATP binding at each step prepares the peptide-bound enzyme complex, by means of a conformational alteration, for its multi-site catalytic activities.

REFERENCES

Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Isugita, A., Sugas, K. & Matsushima, T. (1956) Bull. Chem. Soc. Jpn. **29**, 507

Frøyshov, Ø., Zimmer, T. L. & Laland, S. G. (1978) Int. Rev. Biochem. (Amino Acid Protein Biosynth. II) **18**, 49–78

Gevers, W., Kleinkauf, H. & Lipmann, F. (1968) Proc. Natl. Acad. Sci. U.S.A. **60**, 269–276

Ghosh, S.K., Mukhopadhyay, N. K., Majumder, S. & Bose, S. K. (1983) Biochem. J. **215**, 539–543

Ghosh, S. K., Majumder, S., Mukhopadhyay, N. K. & Bose, S. K. (1985) Biochem. J. **230**, 785–789

Ghosh, S. K., Mukhopadhyay, N. K., Majumder, S. & Bose, S. K. (1986) Biochem. J. **235**, 81–85

Itoh, H., Yamada, M., Tomino, S. & Kurahashi, K. (1968) J. Biochem. (Tokyo) **64**, 259–261

Kleinkauf, H. & Koischwitz, H. (1980) in Multifunctional Proteins (Bisswanger, H. & Schminckeott, E., eds.), p. 217, Wiley–Interscience Publications, New York

Kleinkauf, H., Roskoski, R. & Lipmann, F. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 2069–2072

Kurahashi, K. (1980) in Antibiotic Biosynthesis (Concoran, J. W., ed.), vol. 4, pp. 325–352, Springer-Verlag, Berlin, Heidelberg and New York

Lee, S. G., Roskoski, R., Jr., Bauer, K. & Lipmann, F. (1973) Biochemistry **12**, 398–405

Locker, R. H. (1954) Biochim. Biophys. Acta **14**, 533–542

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**, 569–599

Mukhopadhyay, N. K., Majumder, S., Ghosh, S. K. & Bose, S. K. (1986) Biochem. J. **235**, 638–643

Roskoski, R., Jr., Kleinkauf, H., Gevers, W. & Lipmann, F. (1970*a*) Biochemistry **9**, 4839–4845

Roskoski, R., Jr., Kleinkauf, H., Gevers, W. & Lipmann, F. (1970*b*) Biochemistry **9**, 4846–4851

Sanger, F. (1945) Biochem. J. **39**, 507–515

Sengupta, S. & Bose, S. K. (1972) Biochem. J. **128**, 47–52

Sengupta, S. & Bose, S. K. (1974) Indian J. Biochem. Biophys. **11**, 335–336

Stulberg, M. P. & Novelli, G. D. (1960) Methods Enzymol. **5**, 703–707

Received 7 July 1986/1 September 1986; accepted 8 September 1986