A PRECURSOR FORM OF THE PENICILLINASE FROM *BACILLUS LICHENIFORMIS*

M. SARVAS, K. P. HIRTH*, E. FUCHS* and K. SIMONS

European Molecular Biology Laboratory, Postfach 10 22 09, 6900 Heidelberg and **Institut für Molekulare Genetik, Universität Heidelberg, 6900 Heidelberg, FRG*

Received 24 August 1978

1. Introduction

All but one of the secretory proteins studied so far are made initially with an amino-terminal extension of 15–30 amino acids. This signal sequence is thought to bind the ribosomal complex containing the nascent chain to the endoplasmic reticulum in eukaryotic cells [1–3] and to the cytoplasmic membrane in prokaryotic cells [4,5]. The vectorial transfer of the peptide chain across the membrane is coupled to translation and as soon as the chain is finished the protein is usually released from the extra-cytoplasmic side of the membrane.

We are studying the secretion of the penicillinase enzyme in *Bacillus licheniformis*. This penicillinase is unique among secretory proteins, since it remains membrane bound after completion of the polypeptide chain [6]. We have shown that the membrane-bound form is attached by an amino-terminal peptide which is hydrophobic [7]. This hydrophobic tail is then removed by a proteolytic cleavage, and the penicillinase is released from the cell [6].

To study the relationship of the amino-terminal extension of the membrane penicillinase to the signal peptides found in other secretory proteins we have synthesized the protein in vitro. DNA containing the penicillinase gene from *B. licheniformis* was transcribed and translated in a cell-free system from *Escherichia coli*. We found that the penicillinase made in vitro was larger than the membrane penicillinase. This extension is located at the amino terminus of the protein and is probably equivalent to the signal sequence present in other secretory proteins.

2. Material and methods

DNA was isolated from a λ vehicle (λ *pen*) kindly provided by Dr W. Brammar, Edinburgh. In this vehicle *Eco*RI restriction fragments E and D have been replaced by a DNA fragment (~3.5 Mdaltons) from *Bacillus licheniformis* 749/C. This fragment contains the structural gene for the penicillinase [8]. λ *pen* was purified [9] and the DNA extracted as in [10]. For some experiments the inserted 3.5 Mdalton fragment DNA in λ *pen* was purified from an *Eco*RI digest of λ *pen* DNA by sucrose gradient centrifugation. Hence it did not contain any λ DNA. The purified 3.5 Mdalton fragment did not hybridize with RNA transcribed from DNA of λ wild type (K. P. Hirth, unpublished results). DNA, either from λ wild type or from $\lambda_{imm\ 434}$ phage was used for controls.

Proteins were synthesized in vitro using the cell-free coupled transcription–translation assay in [10]. When the products were labelled with [^{3}H]leucine or [^{35}S]-methionine these aminoacids were decreased to 20 μM; the other 19 amino acids were kept at 100 μM.

Immunoprecipitations were performed in 0.1% sodium dodecyl sulphate (SDS) 0.15 M Tris–HCl (pH 8.2). Antibody–antigen complexes were then bound to protein A–Sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala) (5 mg/μl rabbit antiserum), washed 4 times with the above buffer, and eluted with the sample buffer used for gel electrophoresis [11].

Penicillinase activity was determined using chromogenic cephalosporin (Nitrocephin, Glaxo) [12]. One unit of penicillinase hydrolyses 1 μmol penicillin G in 1 h at 30°C. The specific activity of purified

membrane penicillinase is 350 units/μg protein [13].

Cleavage of the protein with (2-(2-nitrophenyl-sulphenyl)-3-methyl-3′-bromoindolenine, BNPS-skatol, was performed as in [7,14]. SDS polyacrylamide gels were fluorographed as in [15].

3. Results and discussion

The DNA of both λ *pen* and the 3.5 Mdalton fragment of bacillar DNA inserted in λ *pen*, programmed the synthesis of enzymatically active penicillinase in the cell-free coupled system (table 1). No penicillinase activity was found if DNA of λ wild-type or λ_{434} was used.

Products programmed by DNA of λ_{434} and λ *pen* were further analysed with SDS–acrylamide gel electrophoresis (fig.1). Only one significant difference was seen. At the position of the O protein of λ (lane A) there was a much heavier band in the case of λ *pen* (lane B).

To show that this band contained penicillinase, the proteins synthesized in vitro were precipitated with antiserum against penicillinase (fig.2). Only one band was seen in the immunoprecipitation in SDS–gel electrophoresis, at the position of the O protein, when the assay was programmed either with DNA from λ *pen* (lane C) or with DNA from 3.5 Mdalton fragment (lane B). SDS–gel electrophoresis of the supernatant after immunoprecipitation showed that in both cases most of the material at the position of O protein was removed, whereas all other bands were unchanged. No bands were seen in immunoprecipitate of products made with DNA of λ wild-type or λ_{434} or if antiserum against bovine serum albumin was used.

Table 1
Synthesis of enzymatically active penicillinase in vitro

Source of DNA	Amount of DNA (μg/0.1 ml)[a]	Penicillinase (units/0.1 ml)[a]
λ *pen*	4	7.1
3.5 Mdalton fragment	~0.5	5.3
λ wild-type	4	< 0.3
None	–	< 0.3

[a] Volume of in vitro assay

The penicillinase protein synthesized in vitro (fig.1,2) had the same apparent molecular weight as O protein (~34.5×10^3 [16], fig.1) and migrated in SDS–gels more slowly than the membrane penicil-

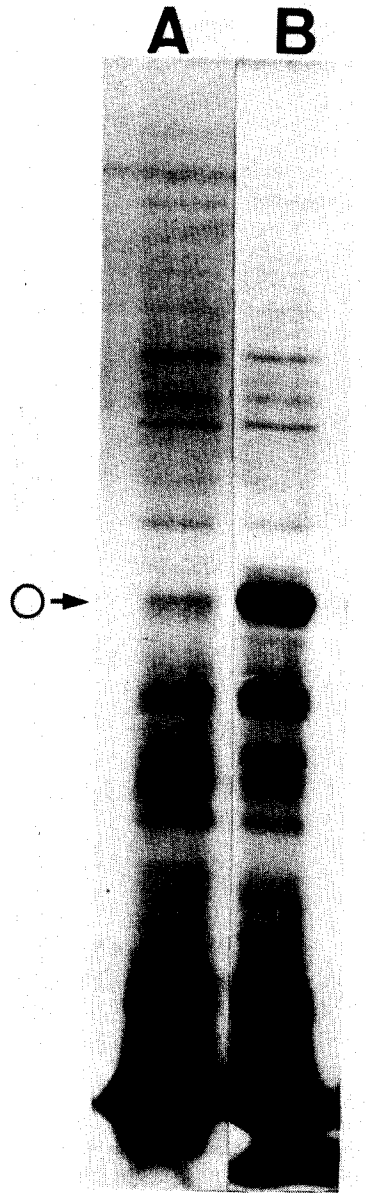

Fig.1. Fluorogram of SDS–polyacrylamide gel electrophoresis of [^{3}H]leucine labelled products synthesized in vitro using: (A) DNA of λ_{434}; (B) DNA of λ *pen* as template. (o) Shows the position of the λ O protein.

linase (fig.2, lane A, apparent molecular weight in SDS gels 31 X 10^3 [7]).

To determine the location of the extension of the 34.5 X 10^3 dalton precursor we have studied the peptides obtained by partial cleavage at tryptophan residues with BNPS-skatol [14]. We know from [7] that the membrane penicillinase has an amino-terminal extension when compared to the exopenicillinase. There are only three tryptophan residues in the exopenicillinase, all located in the carboxy-terminal region [17]. Cleavage with BNPS-skatol produces two sets of peptides from either exopenicillinase or membrane penicillinase (fig.3, lanes 1 and 2): one set derived from the carboxy terminus (2–8 X 10^3 daltons) indicated by C in fig.3; and another set of larger peptides from the amino-terminus (> 21.5 X 10^3 daltons, indicated by N in fig.3).

The electrophoretic pattern of peptides produced

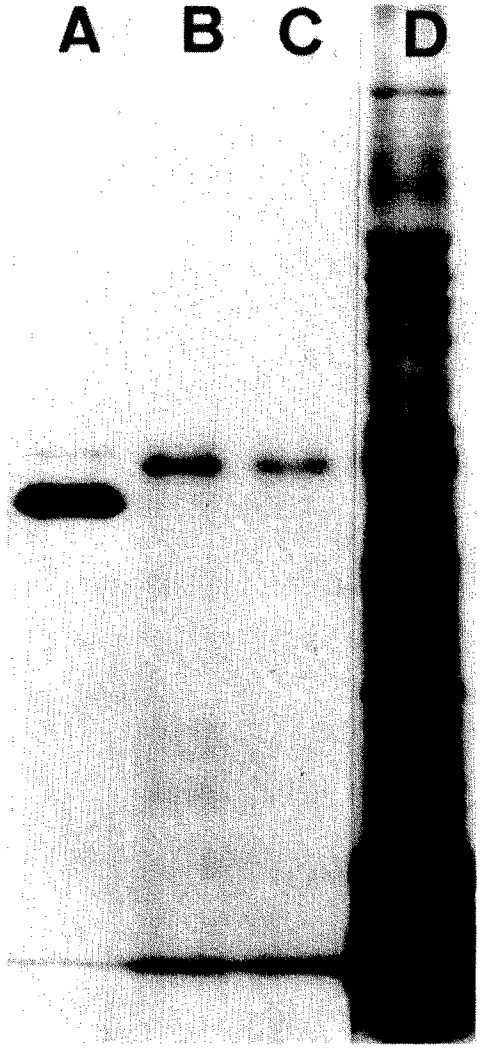

Fig.2. Gel electrophoresis and fluorography of [^{3}H]leucine labelled immunoprecipitates. Products of an in vitro assay of 0.1 ml, supplemented with 100 μCi [^{3}H]leucine, were precipitated with antiserum to penicillinase and electrophoresed. (A) purified membrane penicillinase labelled in vivo with [^{14}C]-leucine; (B) immunoprecipitate of products from 3.5 Mdalton fragment DNA; (C) immunoprecipitate of products from λ *pen* DNA; (D) products from λ *pen* DNA before immunoprecipitation.

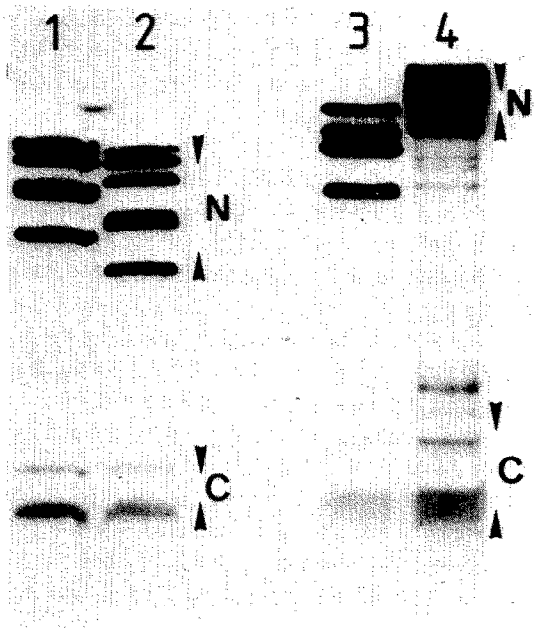

Fig.3. Peptide patterns obtained by cleavage with BNPS-skatol. [^{35}S]Methionine labelled products of in vitro assays programmed with DNA from 3.5 Mdalton fragment were electrophoresed in SDS–polyacrylamide gels. The band at the position of the precursor of the penicillinase was cut out, eluted in 0.1% SDS, subjected to cleavage by BNPS-skatol, and electrophoresed again in SDS–acrylamide gels with an acrylamide gradient of 15–22% [2]. Samples of [^{14}C]leucine-labelled exo- and membrane penicillinase were treated similarly: (1) membrane penicillinase; (2, 3) exopenicillinase; (4) precursor of penicillinase. 1 and 2 are from a different run than 3 and 4. N and C stand for amino and carboxy-terminal peptides (see text).

by BNPS-skatol cleavage from exopenicillinase and the precursor made in vitro are shown in fig.3, lanes 3 and 4. The precursor was made from DNA of the 3.5 Mdalton fragment to eliminate the possibility of O protein contamination. The peptides derived from the carboxyterminal region are identical in their mobility (fig.3, compare lanes 3 and 4). Among the cleavage products there is a peptide migrating slightly slower than the carboxy terminal peptides. The origin of this peptide is unclear. It was not consistently seen in cleavage patterns of precursor preparations made either from DNA of the 3.5 Mdalton fragment or from λ *pen* DNA.

The peptides derived from the amino terminal region of exopenicillinase, membrane penicillinase and the precursor made from DNA of 3.5 Mdalton fragment are shown in fig.4. As shown in [7], the set of amino-terminal peptides cleaved from the membrane penicillinase migrated slower than those deriving from the exopenicillinase (fig.4, lanes B and A). A corresponding decrease in mobility was seen when the peptides deriving from the precursor were compared to those from membrane penicillinase (fig.4, lanes C and B). These results suggest that there is an extension in the precursor located in the amino-terminal region of the protein. Small differences in the carboxy-termini cannot be excluded [18], although the carboxy-terminal peptides (fig.3) migrated with identical mobilities.

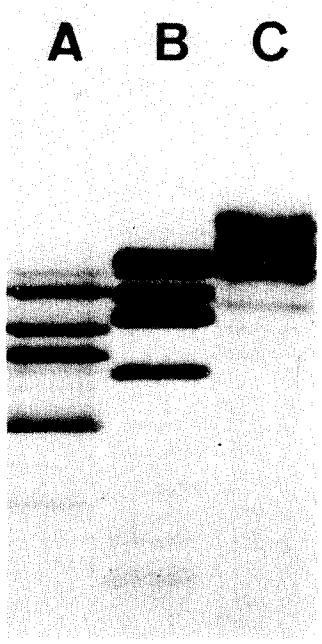

Fig.4. Amino-terminal peptides obtained by cleavage with BNPS-skatol from: (A) Exopenicillinase; (B) membrane penicillinase; (C) ^{3}H-labelled penicillinase precursor made from 3.5 Mdalton fragment DNA. Experimental conditions as in fig.3 except that the 15–22% gradient gels were run for 16 h instead of 11 h.

Our results show that the penicillinase protein is made as a precursor in vitro. Whether this precursor is also made in the bacterial cell remains to be proven. The amino-terminal extension in the precursor is likely to be analogous to the signal peptides found in most secretory proteins [1–5]. Secretion of penicillinase thus seems to involve at least two proteolytic cleavages. First the signal peptide is cleaved, but the protein remains membrane bound. The second cleavage removes the amino-terminal hydrophobic peptide anchoring the enzyme to the cytoplasmic membrane. This releases the water-soluble exoenzyme from the cell. Further studies on the mechanisms of this secretion process are in progress.

Acknowledgement

M.S. has received an EMBO long term fellowship. The work has been partially supported by a grant from the Deutsche Forschunggemeinschaft no. Fu 70/4 to E.F. and from Sigrid Juselius Foundation, Helsinki to M.S.

References

[1] Milstein, C., Brownlee, G. G., Harrison, T. M. and Matthews, M. D. (1972) Nature New Biol. 239, 117–120.
[2] Blobel, G. and Dobberstein, B. (1975) J. Cell. Biol. 67, 835–851.
[3] Campbell, N. and Blobel, G. (1976) FEBS Lett. 72, 215–226.
[4] Inouye, H. and Beckwith, J. (1977) Proc. Natl. Acad. Sci. USA 74, 1440–1444.
[5] Randall, L. L., Hardy, S. J. S. and Josefsson, L.-G. (1978) Proc. Natl. Acad. Sci. USA 75, 1209–1212.

[6] Lampen, J. O. (1978) in: Relations between structure and function in the prokaryotic cell (Stanier, R. Y. et al.) pp. 231–247, Cambridge University Press, Cambridge.
[7] Simons, K., Sarvas, M., Garoff, H. and Helenius, A. (1978) submitted.
[8] Brammar, W. J. (1977) Biochem. Soc. Trans. 5, 1633–1652.
[9] Thomas, M. and Davies, R. W. (1975) J. Mol. Biol. 91, 315–328.
[10] Fuchs, E. (1976) Eur. J. Biochem. 63, 15–22.
[11] Laemmli, U. K. (1970) Nature 227, 680–685.
[12] O'Callaghan, C. H., Morris, A., Kirby, S. and Shingler, A. H. (1972) Antimicrob. Ag. Chemother. 1, 283–288.
[13] Yamamoto, S. and Lampen, J. O. (1976) J. Biol. Chem. 251, 4095–4101.
[14] Fontana, A. (1972) Methods Enzymol. 25, 419–423.
[15] Laskey, R. A. and Mills, A. D. (1975) Eur. J. Biochem. 56, 335–341.
[16] Raab, C., Klein, A., Kluding, H., Hirth, P. and Fuchs, E. (1977) FEBS Lett. 80, 275–278.
[17] Meadway, R. J. (1969) Biochem. J. 115, 12.
[18] Kelly, L. E. and Brammar, W. J. (1973) J. Mol. Biol. 80, 135–147.