Biochem. J. (1989) 260, 601–604 (Printed in Great Britain)

The *Streptomyces* K15 DD-peptidase/penicillin-binding protein

Active site and sequence of the *N*-terminal region

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The *N*-terminal region of the *Streptomyces* K15 DD-peptidase/penicillin-binding protein shows high homology with that of other penicillin-interactive proteins or domains. The active-site serine residue of the conserved tetrad Ser-Xaa-Xaa-Lys occurs at position 35. There is no indication for the presence of a signal peptide or an *N*-terminal hydrophobic sequence, suggesting that the *Streptomyces* K15 enzyme is probably anchored to the membrane by a *C*-terminal peptide segment.

INTRODUCTION

The membrane-bound 26000-M_r DD-peptidase of *Streptomyces* K15 catalyses acyl-transfer reactions with cyclic β-lactam and non-cyclic amide and ester carbonyl donors via formation of an acyl-enzyme that involves an essential serine residue [1,2]. The acyl-enzymes formed by reaction with β-lactam antibiotics have long half-lives [3]. Consequently, the DD-peptidase behaves as a penicillin-binding protein (PBP). The peculiarity of the low-M_r *Streptomyces* K15 DD-peptidase/PBP is that when acting on an amide substrate such as Ac_2-L-Lys-D-Ala-D-Ala it functions almost exclusively as a strict transpeptidase [1]. In water, hydrolysis is negligible because the D-alanine that is released during enzyme acylation is effectively re-utilized in a transfer reaction that maintains the concentration of the amide carbonyl donor at a constant level. In the presence of an exogenous amino compound such as Gly-Gly, the acceptor activity of which overcomes that of water and D-alanine, the substrate is quantitatively converted into the transpeptidated product Ac_2-L-Lys-D-Ala-Gly-Gly. As a basis for the design of nucleotide probes that would help identifying the gene that encodes the *Streptomyces* K15 DD-peptidase, the *N*-terminal region of the purified enzyme was sequenced and the essential serine residue was identified by active-site peptide mapping.

MATERIALS AND METHODS

Sequence of the *N*-terminal region of the DD-peptidase

The enzyme (4.9 nmol in 130 μl), purified in the presence of cetyltrimethylammonium bromide as described previously [4], was supplemented with 5 vol. of cold (−20 °C) acetone and maintained at −20 °C for 30 min, after which time the precipitated enzyme was collected by centrifugation at 20000 *g* for 30 min. The pellet was washed with 4 vol. of cold acetone, dissolved in trifluoroacetic acid and the solution supplemented with an equal volume of water. Automated sequence analysis of 4.9 nmol of protein was carried out on a 470A gas-phase Sequenator. The amino acid phenylthiohydantoin derivatives were analysed off-line the sequencer on a 4.6 mm × 250 mm IBM cyanopropyl column (Biotech Instruments, Hertford, Herts., U.K.) as described in ref. [5]. The h.p.l.c. set-up consisted of an Intelink System connected to a 440 fixed-wavelength detector (Waters, Milford, MA, U.S.A.) set at 254 and 313 nm. Automated sequence analysis of 1.4 nmol of protein was also carried out on a 477A pulsed-liquid Sequenator (Applied Biosystems, Foster City, CA, U.S.A.). The amino acid phenylthiohydantoin derivatives were analysed on-line the sequencer on a 21 mm × 220 mm C_{18} MPLC Cartridge column (Applied Biosystems) as described by the manufacturer.

Preparation of the acyl- ([^{35}S]benzylpenicilloyl-)enzyme

The enzyme (20 nmol) was incubated for 15 min at 37 °C with [^{35}S]benzylpenicillin (230 nmol; 250 nCi/nmol; New England Nuclear) in 1100 μl of 17.5 mM-potassium phosphate buffer, pH 7.5, containing 0.016% cetyltrimethylammonium bromide. Protein acylation was complete. The acyl-enzyme was precipitated with acetone as described above. The pellet was washed with 4.8 ml of cold acetone, dried *in vacuo* and dissolved in 280 μl of 88% (v/v) formic acid. The solution was supplemented with 32 μl of a Triton X-100 solution (3%, w/v) and 2 ml of water, and freeze-dried.

Pronase digestion of the acyl-([^{35}S]benzylpenicilloyl-)enzyme

Trypsin, subtilisin, thermolysin, *Staphylococcus aureus* proteinase V8 and Pronase were tested as proteolytic agents. Pronase, previously used for similar purposes [6,7], caused the rapid disappearance of the acyl-([^{35}S]benzylpenicilloyl-)enzyme (as monitored by SDS/polyacrylamide-gel electrophoresis) and the appearance of four major radioactive peptide fragments (as monitored by paper electrophoresis at pH 6.5, 60 V/cm for 1 h). One of these fragments (I) migrated 2 cm towards the cathode, another (II) remained at the origin of the electrophoretograms and the two others migrated 9 cm

Abbreviation used: PBP, penicillin-binding protein.

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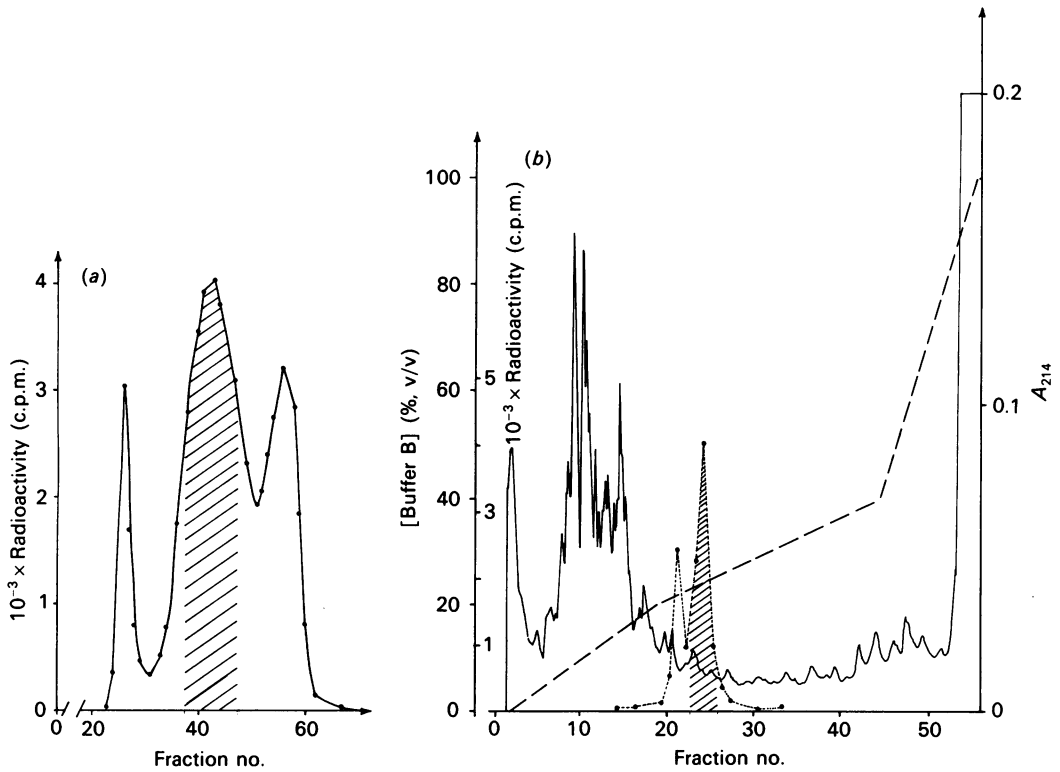

Fig. 1. Purification of the ^{35}S-labelled active-site-containing fragment (III) of the *Streptomyces* K15 DD-peptidase/PBP by chromatography on Sephadex LH20 (*a*) and by f.p.l.c. on a PepRPC column (*b*)

For details see the text. (*a*) —·—, Radioactivity. (*b*) -------, Radioactivity; ——, A_{214}; ——, buffer B gradient. Hatched areas indicate the fragment-(III)-containing fractions.

(III) and 28 cm (IV) towards the anode. Compound (IV) was [^{35}S]benzylpenicilloate. Maximal yield of fragment (III) (31% of total radioactivity) was obtained by incubating 20 nmol of acyl- ([^{35}S]benzylpenicilloyl-)enzyme for 2 h at 37 °C with 58 μg of Pronase in 1.6 ml of 0.4% NH_4HCO_3. The solution was freeze-dried.

Isolation of the ^{35}S-labelled active-site fragment (III)

Step 1. The Pronase digest was dissolved in 100 μl of 88% (v/v) formic acid. After addition of 250 μl of 95% (v/v) ethanol, the solution was loaded on a 35 ml column of Sephadex LH20 equilibrated against an 88% (v/v) formic acid/95% (v/v) ethanol (1:4, v/v) mixture [8]. Elution with the same mixture yielded three major fractions (Fig. 1*a*). Fractions 25–30 contained fragment (II), fractions 38–47 contained fragments (I) and (III) and fractions 53–60 contained [^{35}S]benzylpenicilloate (IV).

Step 2. Fractions 38–47 were pooled and freeze-dried and the pellet was dissolved in 1 ml of 0.1% (v/v) trifluoroacetic acid. Purification of fragment (III) was achieved on a f.p.l.c. apparatus (Pharmacia, Uppsala, Sweden) equipped with a 0.5 cm × 5 cm PepRPC column, with various mixtures of buffer A (0.1% trifluoroacetic acid in water) and buffer B [0.1% trifluoroacetic acid in water/acetonitrile (3:7, v/v)]. The gradient started with 1 ml of buffer A, and then went on from 0% to 20% buffer B over 8 ml, from 20% to 40% buffer B over 12 ml and from 40% to 100% buffer B over 5 ml (flow rate 0.5 ml/min; volume of the collected fractions 460 μl). Fractions 24–26 (Fig. 1*b*) were pooled and freeze-dried. The final preparation contained 0.72 nmol of purified fragment (III). Paper electrophoresis at pH 5.6 revealed the presence of a single radioactive compound. As shown below, it was, however, contaminated by free glycine.

RESULTS AND DISCUSSION

The sequence of the *N*-terminal region of the protein is shown in Fig. 2. Ambiguity was observed at some cycles following cycle 30 with the gas-phase Sequenator and following cycle 41 with the pulsed-liquid Sequenator. The enzyme preparation was very pure, valine being the only *N*-terminal residue detected.

Although the first cycle of the Edman degradation was made difficult because of the presence of large amounts of glycine, analysis of the active-site peptide fragment (III) yielded the sequence (Ser)-Thr-Gly-Ser-Thr-Xaa-(Lys), thus demonstrating that the active-site serine residue is at position 35 in the native protein. (The amount of glycine phenylthiohydantoin derivative at cycle 1 was so large that, on the basis of the radioactivity of the peptide, it was obviously a contamination. As a result of the wash-out effect, the amount of the glycine phenylthiohydantoin derivative gradually decreased during the subsequent cycles except at cycle 3.)

It has been proposed that all the penicillin-interactive active-site-serine proteins and domains, i.e. the β-lactamases of classes A, C and D, the low-M_r PBPs and the penicillin-binding domains of the high-M_r PBPs,

Fig. 2. Amino acid alignments of the *N*-terminal regions of the *Streptomyces* K15 DD-peptidase/PBP and other penicillin-interactive proteins and domains

Identities (*) and similarities (°) with the *Streptomyces* enzyme are indicated; parentheses indicate an ambiguous residue. VD⊥LD (insertion) reads VDEGKLD. The active-site serine residue is at position 35.
EGK

form a super-family of evolutionarily related proteins [20]. One of their common features is the occurrence of the conserved tetrad Ser*-Xaa-Xaa-Lys, where Ser* is the active-site serine residue, close to the *N*-terminus of the proteins or domains. The alignments of Fig. 2 reveal an especially high similarity between the 20-amino-acid-residue stretch around the active-site serine residue of the *Streptomyces* K15 DD-peptidase/PBP (from Thr-21 to Met-40) and the corresponding regions of the penicillin-binding domain of the high-M_r PBP1B of *Escherichia coli* (40% identity), the low-M_r PBP5 of *E. coli* (45% identity) and the low-M_r PBP of *Bacillus subtilis* (50% identity). The amino acid sequence failed to reveal the presence of a signal peptide or an *N*-terminal hydrophobic sequence, thus suggesting that the *Streptomyces* K15 enzyme, like the other known low-M_r PBPs, is probably anchored to the membrane by a *C*-terminal segment [21,22].

The work in Liège was supported in part by the Fonds de la Recherche Scientifique Médicale (Contract no. 3.4507.83), an Action Concertée with the Belgian Government (Convention 86/91-90), the Fonds de Recherche de la Faculté de Médecine U.Lg. and a contract with the E.E.C. (BAP-0197-B). The work in Gent was supported by the Belgian National Incentive Program of Fundamental Research in Life Sciences, initiated by the Belgian State–Prime Minister's Office–Science Policy Programming Department.

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Received 16 January 1989/14 March 1989; accepted 28 March 1989