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NOTES

Identification of the Active Site in Penicillin-Binding Protein 3 of *Escherichia coli*

ROBERT A. NICHOLAS,[1] JACK L. STROMINGER,[1]* HIDEHO SUZUKI,[2] AND YUKINORI HIROTA[3]

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138,[1] and Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113[2] and National Institute of Genetics, Mishima, Shizuoka-ken 411,[3] Japan

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We report the sequence of the active site tryptic peptide of penicillin-binding protein 3 from *Escherichia coli*. Purified penicillin-binding protein 3 was labeled with [^{14}C]penicillin G and digested with trypsin, and the resulting radioactive peptides were isolated by a combination of gel filtration and high-pressure liquid chromatography. The major radioactive peak from high-pressure liquid chromatography was sequenced, and the peptide Thr-Ile-Thr-Asp-Val-Phe-Glu-Pro-Gly-Ser-Thr-Val-Lys, which comprises residues 298 to 310 in the amino acid sequence, was identified. This sequence is compared with the active site sequences from other penicillin-binding proteins and β-lactamases.

Penicillin exerts its antibiotic effect by binding to and reacting with several enzymes present in the cytoplasmic membrane that are involved in the final stages of cell wall biosynthesis and cross-linking of the peptidoglycan layer (24, 36). In *Escherichia coli* there are seven proteins that form a covalent bond with penicillin (termed penicillin-binding proteins [PBPs]), numbered by decreasing molecular weight: PBPs 1a, 1b, and 2 to 6 (4, 31). PBPs are detected by incubating cytoplasmic membranes with [^{14}C]penicillin G, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography (31). All of the higher-molecular-weight PBPs (1a, 1b, 2, and 3) have been shown by genetic analysis to be essential to the cell and to be the killing sites for β-lactam antibiotics (30, 32). PBPs 1a and 1b are involved in cell elongation and are probably the major transpeptidases of the cell. Each of these PBPs is also able to compensate for the absence of the other. PBP 2 is important in maintaining the rod shape of the bacterium, and PBP 3 is involved in septation of dividing bacteria. These high-molecular-weight PBPs, with the exception of PBP 2, have also been shown to process both a penicillin-insensitive transglycosylase domain and a penicillin-sensitive transpeptidase domain (12, 13, 25, 35). The lower-molecular-weight PBPs (PBPs 4, 5, and 6) are known to be the major D-alanine carboxypeptidases of the cell in vitro (22, 34). These PBPs may also function in vivo either as D-alanine carboxypeptidases (23) or as secondary transpeptidases (8). Although they account for over 85% of the binding of [^{14}C]penicillin G to cell membranes (30), they are not essential for cell viability (21).

The sequences at the active sites of PBPs 1b (28a), 5 (9), and 5′ (PBP 5 from the *dacA1119*1 mutant of *E. coli*; see reference 28) have been determined recently, and each has been shown to contain a unique serine residue that forms an acyl enzyme intermediate with pencillin G. When these active site sequences are compared with the active sites of class A and C β-lactamases and D-alanine carboxypeptidases from several different species, the sequence -Ser-X-X-Lys- appears in all cases. The amino acid sequence of PBP 3 has been deduced from the sequence of the gene encoding PBP 3 (20, 27), and within its sequence are three regions that meet this requirement. Broome-Smith et al. (6) have recently described the site-directed mutagenesis of a serine residue to a cysteine residue in the putative active site region, which was identified by homology to PBPs 1a and 1b. A stable penicilloyl complex could not be detected with the mutated enzyme. In this paper, we directly identify the active site region within the amino acid sequence by isolating and sequencing the [^{14}C]penicilloyl peptide of PBP 3.

Cells from a multiple PBP mutant of *E. coli* K-12 transformed with pWK7 (18, 35) were used for the isolation of PBP 3. This strain overproduces PBP 3 at about 100 times the wild-type level. The bacteria were grown in L broth and harvested by centrifugation after cooling. Cell membranes were prepared as described previously (34). PBP 3 was purified from differentially solubilized fractions by utilizing ampicillin-Affigel 10 (Bio-Rad Laboratories) and blocking PBP 2 with mecillinam (1, 35). The purity of the preparation of PBP 3 was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The apparent heterogeneity of the preparation, as seen by both Coomassie blue staining and fluorography, results from its extreme sensitivity to degradation during purification and storage. All of the major bands seen in Fig. 1 were reactive immunologically in Ouchterlony diffusion with rabbit antiserum against PBP 3. Thus, the majority of protein (>90%) was PBP 3 (M_r 63,000) and two principal degradation products (M_rs 40,000 and 23,000) derived from PBP 3 during its purification. It is interesting to note that only the M_r 40,000 degradation product contained covalently bound [^{14}C]penicillin G. In an analagous manner, PBP 5′ (M_r 43,000) degrades to fragments of M_r 33,000 and 10,000, of which only the M_r 10,000 fragment contains covalently bound [^{14}C]penicillin G (2, 28). This preparation of PBP 3 was therefore suitable for establishing the active site region of the protein.

PBP 3 was labeled with [^{14}C]penicillin G and digested with

* Corresponding author.

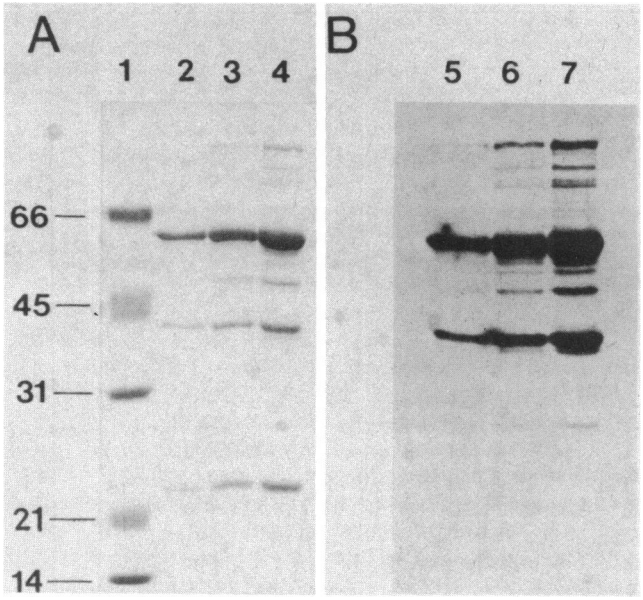

FIG. 1. Analysis of purified PBP 3 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified PBP 3 labeled with [^{14}C]penicillin G was submitted to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel with the gel system of Laemmli (19). Fluorography was carried out by treating the gel with En^{3}Hance (New England Nuclear Corp.) before exposing it to Kodak XAR-5 film at −70°C. (A) Coomassie brilliant blue staining; (B) fluorography. Lanes: 1, molecular weight standards (numbers are in kilodaltons); 2 and 5, 2.5 μg of protein; 3 and 6, 5 μg of protein; and 4 and 7, 10 μg of protein.

trypsin at 37°C for 1 h. After this incubation, the digest was lyophilized, dissolved in 700 μl of formic acid-ethanol (1:4, vol/vol), and chromatographed on a column (1.5 by 85 cm) of Sephadex LH-20 in the same solvent (16). The distribution of radioactivity on Sephadex LH-20 (Fig. 2) revealed a major radioactive peak eluting very close to, but not in, the void volume of the column. The elution position of this peak indicated that the active site tryptic peptide was larger than the corresponding active site peptides from PBPs 5′ (28) and 1b (28a). Pool I (PI) from Fig. 2 was lyophilized, dissolved in 0.1% trifluoroacetic acid, and further purified by high-pressure liquid chromatography (HPLC). The profiles of radioactivity and A_{214} are shown in Fig. 3. Surprisingly, at least seven different peaks of radioactivity were observed. The major radioactive peak, peptide PI-D (Fig. 3), was chosen for further analysis and submitted to sequence analysis (Table 1). The sequence of PI-D was determined to be Thr-Ile-Thr-Asp-Val-Phe-Glu-Pro-Gly-Ser-Thr-Val-Lys, which comprises residues 298 to 310 in the amino acid sequence of PBP 3. This is also the same peptide that was predicted to be the active site peptide by Maruyama et al. (20) and Broome-Smith et al. (6) on the basis of homology to other PBPs, β-lactamases, and D-alanine carboxypeptidase. It is assumed that serine-307 is the nucleophile that forms the acyl enzyme intermediate; although we cannot exclude the possibility that one of the three threonine residues is the site of acylaction, all other PBPs examined were acylated at a serine residue. Additionally, it is assumed that the site of penicilloyl acylation is identical to the site of substrate acylation (compare references 37 and 38), and therefore the peptide truly represents the active site of the transpeptidase domain. The size of tryptic peptide (13 residues) was larger than the corresponding peptides from PBPs 5′ (8 residues) and 1b (7 residues), as was deduced from its elution position on Sephadex LH-20. The origin of the other peaks on HPLC was unclear, although two other major radioactive peptides, PI-A and PI-B (Fig. 3), both yielded the same sequence as PI-D (data not shown). These three peptides accounted for 55% of the total radioactivity, leaving no doubt that the sequence obtained is that of PBP 3. The heterogeneity of a penicilloyl peptide has also been observed for both PBP 5′ and 1b, although not to this extent. It is likely that the minor peaks of radioactivity (PI-C, PI-E, PI-F, and PI-G) were either heterogeneous peptides (due to degradation or rearrangement of the penicilloyl moiety), incompletely digested peptides, or peptides from the minor penicillin-binding components of the PBP 3 preparation, and their identification was not pursued.

The comparison of the active site sequence of PBP 3 to the sequences at the active sites of other PBPs, penicillin-sensitive D-alanine carboxypeptidases (CPases), and β-lactamases is shown in Table 2. As mentioned previously, the sequence -Ser-X-X-Lys- is conserved among different species as well as among different proteins that interact with β-lactam antibiotics. The serine residue is the nucleophile

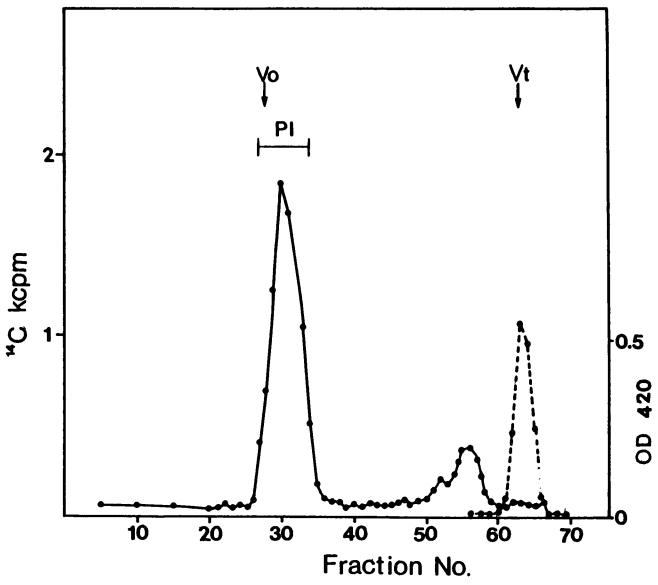

FIG. 2. Gel filtration of a tryptic digest of [^{14}C]penicilloyl PBP 3. PBP 3 (1.9 mg; 30.3 nmol) was mixed with [^{14}C]penicillin G (10 μCi; 45 μg/ml) and incubated at 37°C for 30 min, after which the labeled protein was precipitated with cold (−20°C) acetone. The precipitated protein was collected by centrifugation, washed with acetone, and dried under a stream of N_2. [^{14}C]penicilloyl PBP 3 (2×10^6 cpm) was suspended in 0.4% NH_4HCO_3 containing 0.05% Triton X-100 and incubated with trypsin (1/10 weight ratio to PBP 3) at 37°C for 1 h. After this incubation, the digest was lyophilized and dissolved in 200 μl of 88% formic acid, and then 500 μl of 95% ethanol and 3 μl of 2-mercaptoethanol were added. The solution was loaded onto a column of Sephadex LH-20 (1.5 by 85 cm) equilibrated in formic acid-ethanol (1:4, vol/vol), and the tryptic peptides were eluted with the same solvent at 8 ml/h. Fractions of 1.7 ml were collected, and 10 μl was analyzed for radioactivity. The indicated fractions (PI) were pooled for further analysis. Samples (5 μl) from fractions near the total volume of the column were mixed with 1 mM 5,5′-dithiobis(2-nitrobenzoate) in 50 mM Tris hydrochloride (pH 7.8), and the A_{420} was determined (broken line). The peak fraction represents the total volume of the column. V_0, Void volume; V_t, total volume; OD, optical density.

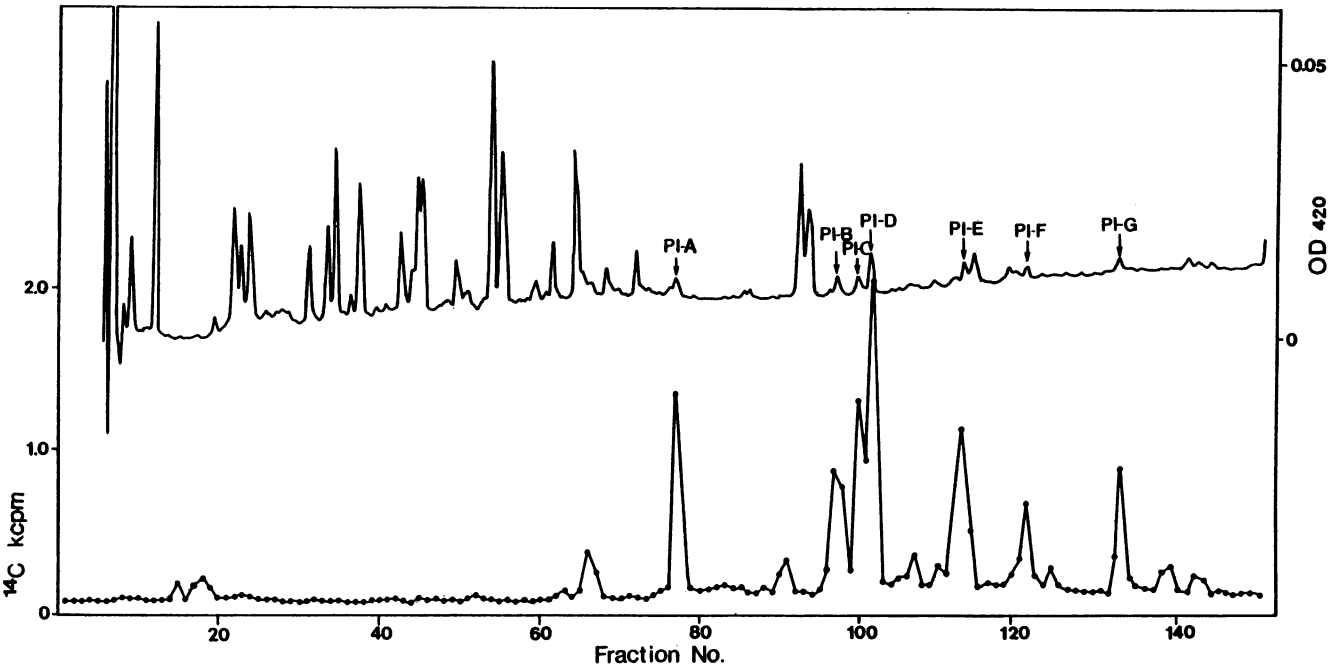

FIG. 3. Purification of [^{14}C]penicilloyl peptides by HPLC. PI from the column described in the legend to Fig. 2 was dissolved in 500 μl of 0.1% trifluoroacetic acid and a portion was injected onto a Vydac 5-μm C_{18} column equilibrated with CH_3CN-H_2O (2:8, vol/vol) containing 0.1% trifluoroacetic acid. The peptides were eluted over 75 min with a linear gradient from CH_3CN-H_2O (2-8, vol/vol) containing 0.1% trifluoroacetic acid to CH_3CN-H_2O (4:6, vol/vol) containing 0.1% trifluoroacetic acid. Fractions of 0.5 ml were collected, and 15 μl of each fraction was analyzed for radioactivity. The radioactive peaks described in the text are identified. OD, Optical density.

that forms a covalent bond with peptide substrates and β-lactam antibiotics, and it is possible that the conserved lysine interacts with the carboxyl group of peptide substrates and β-lactam antibiotics. Other similarities are also evident. The phenylalanine residue four amino acids to the amino-terminal side of the active site serine is identical throughout class A and class C β-lactamases. It is also interesting to note that phenylalanine does not appear at this position in all of the other PBPs and CPases (arginine occupies this position in PBP 1b [5]). There is also a strong similarity between PBP 3 and class C β-lactamases in the active site region; five of eight residues are identical.

Now that the active site region of the transpeptidase domain of PBP 3 has been conclusively identified, it is interesting to speculate on the topography of PBP 3. PBP 3 is able to catalyze both glycan polymerization and transpeptidation (12). Since these two activities are so different, it seems likely that they are catalyzed by separate domains located at different regions in the primary structure. In fact, there is convincing evidence that the penicillin-binding domain of PBP 3 is located within the carboxy-terminal 349 amino acids (residues 240 to 588 [10]). This is consistent with the placement of the active site nucleophile at residue 307, which is well within the carboxy-terminal portion defined by Hedge and Spratt (10). There is also evidence that this is true for PBP 1b, on the basis of deletions within the *ponB* gene (15, 26). In both of these studies, it was concluded that the transglycosylase activity resides within the amino-terminal half of PBP 1b and that the transpeptidase activity was on the carboxy-terminal side of the transglycosylase domain. Thus, even though there is little homology between the higher-molecular-weight PBPs that have been sequenced, they appear to have similar topographies with respect to their catalytic functions.

Analysis of the protein sequence also suggests the presence of several possible membrane-spanning peptides that are located within a region making up residues 413 to 558 (20). These regions are seen as two stretches of hydrophobic amino acids that appear to flank a lysine or arginine residue. Although the presence of a hydrophilic residue within the interior of a transmembrane sequence seems unlikely, there have been several sequences determined recently that ap-

TABLE 1. Yields and identification of phenylthiohydantoin-amino acids from PI-D by automated degradation[a]

Cycle no.	Residue No.[b]	PTH-amino acid	Amt (pmol)
1	298	Thr	NQ[c]
2	299	Ile	855
3	300	Thr	NQ
4	301	Asp	622
5	302	Val	722
6	303	Phe	665
7	304	Glu	545
8	305	Pro	467
9	306	Gly	502
10	307	Ser	NQ
11	308	Thr	NQ
12	309	Val	515
13	310	Lys	422

[a] The penicilloyl peptide PI-D (1.1 nmol [Fig. 3]), purified by HPLC from PI (Fig. 2), was submitted to automated degradation on a Beckman 890M amino acid sequencer. Phenylthiohydantoin (PTH)-amino acids were identified and quantitated by HPLC.

[b] The residue number corresponds to the gene sequence of Maruyama et al. (20).

[c] NQ, Not quantitated; the fragmentation of the serine and threonine derivatives into their characteristic by-products during conversion prevented quantitation.

TABLE 2. Active site sequences of PBPs, CPases, and β-lactamases[a]

Enzyme	Microorganism	Active site sequence	Reference
CPase	*Bacillus subtilis*	-Leu-Pro-Ile-Ala-Ser*-Met-Thr-Lys-	37
CPase	*Bacillus stearothermophilus*	-Leu-Gly-Ile-Ala-Ser*-Met-Thr-Lys-	38
PBP 5, 5′	*Escherichia coli*	-Arg-Asp-Pro-Ala-Ser*-Leu-Thr-Lys-	9, 28
PBP 1b	*Escherichia coli*	-Ser-Ile-Gly-Ser*-Leu-Ala-Lys-	28a
PBP 3	*Escherichia coli*	-Phe-Glu-Pro-Gly-Ser*-Thr-Val-Lys-	This study
Class A β-lactamase	*Staphylococcus aureus*	-Phe-Ala-Tyr-Ala-Ser*-Thr-Ser-Lys-	3
	Bacillus cereus	-Phe-Ala-Phe-Ala-Ser*-Thr-Tyr-Lys-	3
	Bacillus licheniformis	-Phe-Ala-Phe-Ala-Ser*-Thr-Ile-Lys-	3
	Escherichia coli	-Phe-Pro-Met-Met-Ser*-Thr-Phe-Lys-	3
Class C β-lactamase	*Escherichia coli*	-Phe-Glu-Leu-Gly-Ser*-Val-Ser-Lys-	14
	Pseudomonas aeruginosa	-Phe-Glu-Ile-Gly-Ser*-Val-Ser-Lys-	17

[a] The active site serine residues are denoted by an asterisk (*).

pear to have transmembrane sequences that contain a lysine or arginine residue (7, 11, 29). The possibility that these hydrophilic residues are involved in complex formation with other cytoplasmic membrane proteins is intriguing. The idea that PBP 3 may be involved in a complex has been raised (6, 33).

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ADDENDUM IN PROOF

The same result has recently been deduced from the amino acid composition of the [^{14}C]penicilloyl tryptic peptide of PBP 3 (W. Keck, B. Glauner, U. Schwarz, J. K. Broome-Smith, and B. G. Spratt, Proc. Natl. Acad. Sci. USA **82**:1999–2003, 1985).

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