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# Sequences of the active-site peptides of three of the high-$M_r$ penicillin-binding proteins of *Escherichia coli* K-12

**(murein/peptidoglycan transpeptidase/serine enzyme)**

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**ABSTRACT** **The amino acid compositions of the radioactive peptides obtained from trypsin digestion of [$^{14}$C]benzylpenicillin-labeled penicillin-binding proteins (PBPs) 1A, 1B, and 3 of *Escherichia coli* have been obtained. Complete digestion of these peptides with a combination of aminopeptidase M and carboxypeptidase Y showed that benzylpenicillin was bound to a serine residue in each of these proteins. Comparison of the compositions of the penicillin-labeled peptides with the complete amino acid sequences of PBPs 1A, 1B, and 3 showed that the acylated serine occurs near the middle of each of the proteins, within the conserved sequence Gly-Ser-Xaa-Xaa-Lys-Pro. The sequence around the acylated serine of these high $M_r$ PBPs shows little similarity to that around the acylated serine of the low-$M_r$ PBPs (D-alanine carboxypeptidases) or of the class A or class C β-lactamases, except that in all of these enzymes which interact with penicillin the acylated serine residue occurs within the sequence Ser-Xaa-Xaa-Lys.**

β-Lactam antibiotics interact with three classes of enzymes. The β-lactamases hydrolyze penicillin and protect bacteria against the lethal effects of the antibiotic (1). The high-$M_r$ penicillin-binding proteins (PBPs) are bifunctional enzymes that catalyze both a penicillin-insensitive peptidoglycan transglycosylase reaction and a penicillin-sensitive peptidoglycan transpeptidase reaction (2–4). These enzymes are required for the synthesis and incorporation of peptidoglycan precursors into the cell wall and are essential for the growth and morphogenesis of the bacterial cell (4, 5). The lethal effects of penicillin are due to the inactivation of the high-$M_r$ PBPs (6). The low-$M_r$ PBPs are D-alanine carboxypeptidases that catalyze the removal of the terminal D-alanine residue from the pentapeptide side chains of peptidoglycan precursors (4, 5). Inhibition of these enzymes is not lethal and their precise role in bacterial growth is unknown (4, 5).

Penicillin has been proposed to be an analog of the terminal acyl-D-alanyl-D-alanine moiety of the pentapeptide sidechain of the precursor in peptidoglycan synthesis (7). It has been suggested that the antibiotic acylates an active-site residue of penicillin-sensitive enzymes to form a stable, inactive, "penicilloyl-enzyme" that is analogous to the transient acyl-enzyme formed with the cell wall peptide substrate during catalysis. In the D-alanine carboxypeptidase reaction, the acyl-enzyme is formed with the release of the terminal D-alanine residue of the substrate, and the acyl moiety is then transferred to $H_2O$ to regenerate the active enzyme (5, 8). For all D-alanine carboxypeptidases so far examined [with the exception of a mechanistically distinct zinc enzyme (9)] penicillin acylates a serine residue (10) that is located close to the $NH_2$ terminus (11–13). Evidence in support of the substrate-analog hypothesis for the mechanism of action of β-lactam antibiotics has been obtained by the demonstration that penicillin and a synthetic substrate (diacetyl-L-lysyl-D-alanyl-D-lactate) acylate the same serine residue of the D-alanine carboxypeptidases of *Bacillus subtilis* and *Bacillus stearothermophilus* (11, 12).

The class A β-lactamases (typified by strain TEM β-lactamase) and the class C β-lactamases (typified by the chromosomal β-lactamase of *Escherichia coli*) have also been shown to utilize an acyl-enzyme mechanism (14, 15) involving acylation of a serine residue close to the $NH_2$ terminus of these proteins (16, 17), and the β-lactamases and D-alanine carboxypeptidases appear to be mechanistically similar.

The transpeptidase reaction catalyzed by the essential high $M_r$ PBPs has been proposed to be similar to that of the D-alanine carboxypeptidases, except that the acyl moiety is transferred from the acyl-enzyme to an amino group of a neighboring peptide side chain to achieve crosslinking of the peptidoglycan (5, 8). However, information about the interaction of penicillin with these physiologically important PBPs is still lacking.

In *E. coli* the high $M_r$ PBPs 1A, 1B, 2, and 3 have been identified as the essential PBPs that are the targets of the killing action of β-lactam antibiotics (4, 6). We show here that penicillin binds to a serine residue in PBPs 1A, 1B, and 3 of *E. coli* and that the acylated serine residue is located close to the middle of these proteins, within the conserved sequence Gly-Ser-Xaa-Xaa-Lys-Pro.

## MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** PBP 1A was purified from *E. coli* KN126 *trp tyr ilv sup-126* carrying the runaway-replication plasmid pWK12-4, a derivative of pOU71 (18) containing the *ponA* gene (unpublished data). PBP 1B was purified from *E. coli* JE5615 *ponA(ts) ponB dap lys metB proA* carrying the runaway-replication plasmid pHK231, a derivative of pBEU17 (19) containing the *ponB* gene (kindly provided by H. Kraut and Y. Hirota). PBP 3 was purified from *E. coli* JE7627 *ponB dacB dap lys metB proA ilvH leuA* carrying pWK7, a derivative of pBEU17 containing the *pbpB* gene (unpublished data).

**Purification of PBPs 1A, 1B, and 3.** *E. coli* KN126(pWK12-4) was grown at 30°C to mid-logarithmic phase and then transferred to 42°C for 3 hr to allow runaway replication of the plasmid encoding PBP 1A. Approximately 50-fold overproduction of PBP 1A was obtained under these conditions; the protein was purified by chromatography on heparin-Sepharose CL-6B followed by 6-aminopenicillanic acid-substituted CH-Sepharose 4B. *E. coli* JE5615(pHK231) was grown at 30°C and was shifted to 42°C (as above) to achieve 150-fold overproduction of PBP 1B. The protein was purified by affinity chromatography on ampicillin-substituted CH-Sepharose 4B as described (20). *E. coli* JE7627(pWK7) was

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Abbreviation: PBP, penicillin-binding protein.

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grown as above to achieve 150-fold overproduction of PBP 3. The protein was purified by affinity chromatography on cephalexin-substituted CH-Sepharose 4B as described (20).

**Isolation and Analysis of [$^{14}$C]Benzylpenicillin-Labeled Tryptic Peptides.** Purified PBPs (17–99 nmol) were labeled with a 3- to 6-fold molar excess of [$^{14}$C]benzylpenicillin (54 Ci/mol, Amersham International; 1 Ci = 37 GBq) for 15 min at 37°C. Under these conditions, PBPs 1A, 1B, and 3 were 32%, 25%, and 2% saturated with penicillin, respectively. The cause of the low level of binding to purified PBP 3 is unclear, but such binding was obtained in several independent experiments. The penicilloylated PBPs were denatured and digested with trypsin as described (13). The tryptic digests were dissolved in 200 μl of $H_2O$, and 180–190 μl was used for HPLC fractionation on a Hypersil ODS column (250 × 4.6 mm; 3-μm particles, Bischoff, F.R.G.) as described (13). The column was eluted with a linear 0–100% gradient of buffer B in buffer A over 3 hr at a column temperature of 30°C and a flow rate of 0.7 ml/min. [Buffer A was 50 mM phosphate buffer, pH 4.0, containing 0.17 mM sodium azide; buffer B was 70% (vol/vol) methanol in 50 mM phosphate buffer, pH 4.0.] Fractions (210 μl) were collected at 18-sec intervals and the radioactivity of 2-μl aliquots was measured. The radioactive fractions were pooled and lyophilized. The radiolabeled fragments for PBP 1A and PBP 1B were further purified by HPLC with a linear 30–100% gradient of buffer D in buffer C over 70 min. [Buffer C was 50 mM phosphate buffer, pH 5.5, containing 0.06 mM sodium azide; buffer D was 70% (vol/vol) methanol in 50 mM phosphate buffer, pH 5.5.] The radiolabeled fragments of PBP 3 were analyzed without further purification. The purified [$^{14}$C]benzylpenicillin-labeled tryptic peptides were hydrolyzed in 4 M HCl for 14 hr at 104°C under nitrogen. The hydrolysates were dansylated essentially as described (21) and analyzed by HPLC, using a linear 35–80% gradient of methanol in 35 mM phosphate buffer, pH 6.5, with a column temperature of 40°C and a flow rate of 0.8 ml/min. Fluorescence was monitored with a Perkin-Elmer 3000 micro flow cell (excitation at 330 nm, emission at 545 nm). For calibration, standards with 50 pmol of each amino acid and of acid-hydrolyzed benzylpenicillin were run; the detection limit was 1 pmol for a pure standard. Blank values were used for background correction.

**Proteolytic Digestion of Purified Tryptic Peptides.** Partial aminopeptidase M digestion of the [$^{14}$C]benzylpenicillin-labeled tryptic peptide II of PBP 1B was carried out for 90 min at 25°C in 40 mM Tris Cl buffer, pH 6.7/8 mM $MgSO_4$ with 0.2 unit of enzyme (4 units/mg; Boehringer Mannheim) and 450 pmol of peptide in a total volume of 250 μl. The reaction was terminated by the addition of ice-cold glacial acetic acid to a final concentration of 10%, and the sample was lyophilized and then dissolved in 200 μl of water. The sample (190 μl) was fractionated by HPLC; elution was with

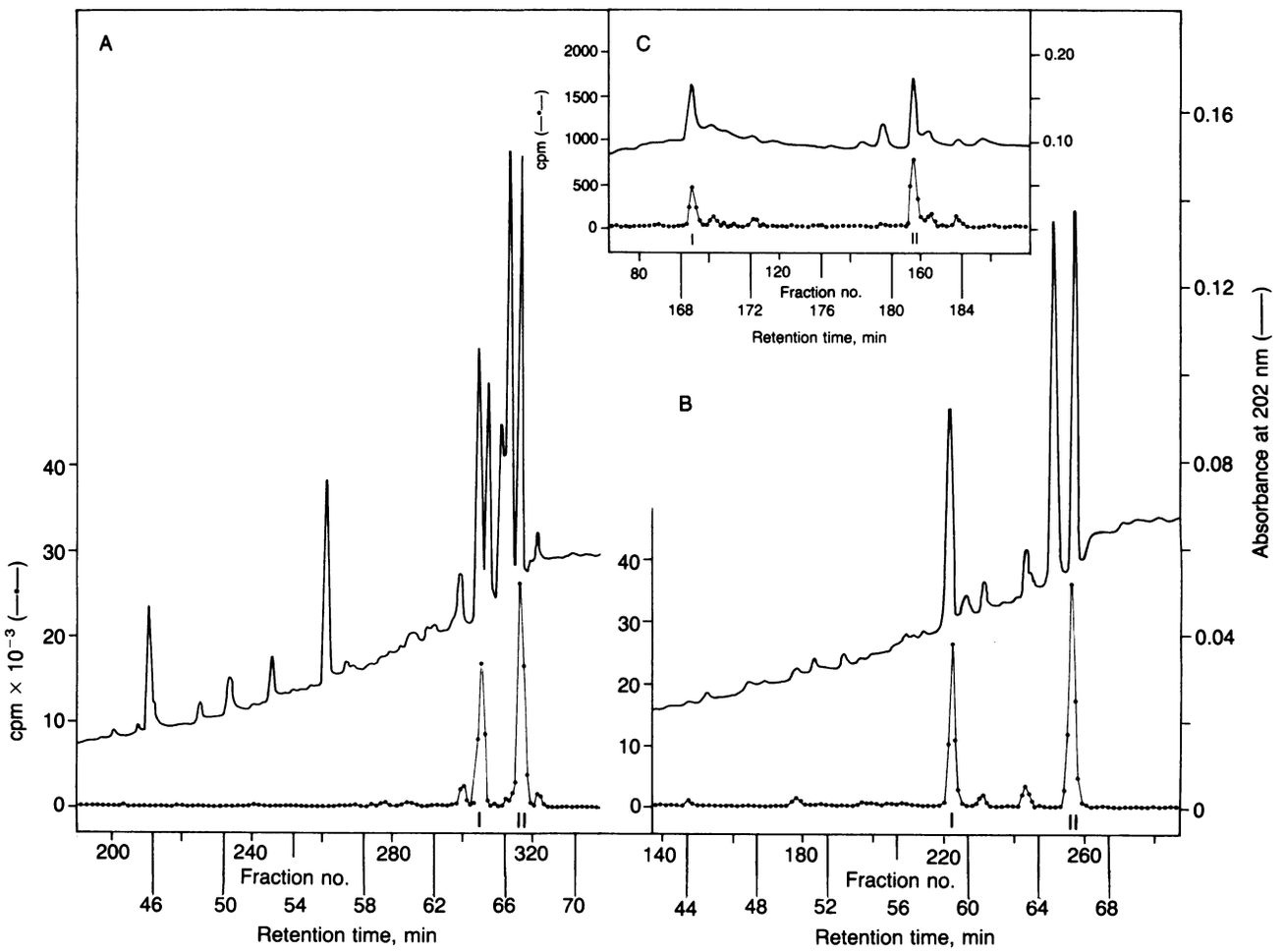


FIG. 1. Purification of [$^{14}$C]benzylpenicillin-labeled tryptic peptides of PBPs 1A, 1B, and 3. Purified PBPs 1A, 1B, and 3 (17, 33, and 99 nmol, respectively) were labeled with [$^{14}$C]benzylpenicillin, digested with trypsin, and the peptides were fractionated by HPLC. The radiolabeled peptides obtained from PBP 1A (*A*) and PBP 1B (*B*) were purified further by HPLC, and the relevant parts of the elution profiles of the peptides from the latter column are shown. The radiolabeled peptides from PBP 3 were not purified further, and the relevant part of the elution profile from the initial HPLC column is shown (*C*).

Table 1. Amino acid analysis of [$^{14}$C]benzylpenicillin-labeled peptides

| Fragment | Amount analyzed, pmol | Molar ratios of components of the peptides | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Asx | Pen* | Glx | Ser | Gly | Thr | Ala | Pro | Val | Ile | Leu | Phe | Lys |
| PBP 1A fragment II | 71 | 1.1 | 0.8 | 1.0 | 0.9 | 0.9 | 0 | 0 | 1.3 | 1.2 | 0.9 | 0 | 1.1 | 0.8 |
| PBP 1A fragment II after aminopeptidase and carboxypeptidase digestion | 62 | 0 | 1.2 | 0.1 | 1.0 | 0.1 | 0 | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 |
| PBP 1B fragment II | 131 | 0 | 0.7 | 0 | 2.0 | 1.0 | 0 | 1.2 | 0 | 0 | 0.9 | 1.0 | 0 | 1.0 |
| PBP 1B fragment II after partial aminopeptidase digestion | 100 | 0 | 1.1† | | 1.0 | 0 | 0 | 1.2 | 0 | 0 | 0 | 1.1 | 0 | 0.9 |
| PBP 1B fragment II after aminopeptidase and carboxypeptidase digestion | 35 | 0 | 1.1 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PBP 3 fragment I | 24 | 0.9 | 2.0† | | 0.8 | 1.0 | 3.2 | −0.1 | 2.2 | 2.1 | 1.1 | 0 | 0.8 | 1.1 |
| PBP 3 fragment I after aminopeptidase and carboxypeptidase digestion | 14 | 0 | 1.3 | 0 | 1.0 | 0.1 | 0.1 | 0 | −0.1 | 0 | 0 | 0 | 0 | 0 |

The purified [$^{14}$C]benzylpenicillin-labeled peptides were hydrolyzed and, after dansylation, the hydrolysates were analyzed by HPLC. Complete digestion of radiolabeled peptides with aminopeptidase M and carboxypeptidase Y and partial digestion with aminopeptidase M were carried out as described in *Materials and Methods*. Blank values were used for background correction; the molar proportions of the components of the peptides are given.

*Pen, degradation product of benzylpenicillin.

†Under the standard conditions used the penicillin degradation product (Pen) and Glx (i.e., Glu) were eluted together. Where individual values of Pen and Glx are given, the two components were quantitated separately by HPLC of the dansylated hydrolysates at pH 3.3 (13).

a linear 20–100% gradient of buffer F in buffer E over 144 min at a column temperature of 20°C and a flow rate of 0.7 ml/min. [Buffer E was 50 mM phosphate buffer, pH 4.0, containing 0.15 mM sodium azide; buffer F was 70% (vol/vol) methanol in 50 mM phosphate buffer, pH 4.0]. Radioactively labeled peptides were lyophilized, hydrolyzed, and analyzed as described above. The [$^{14}$C]benzylpenicillin-labeled tryptic peptides were completely digested with a mixture of aminopeptidase M and carboxypeptidase Y and the reaction products were separated by HPLC as described (13). The pooled fractions from the radioactive peak were lyophilized, hydrolyzed, and analyzed.

## RESULTS

**Isolation of the [$^{14}$C]Benzylpenicillin-Labeled Tryptic Peptides of PBPs 1A, 1B, and 3.** Strains that carry the PBP 1A, PBP 1B, and PBP 3 gene on runaway replication plasmids were used to provide substantial amounts of these proteins for purification. Purified PBPs 1A, 1B, and 3 (17, 33, and 99 nmol, respectively) were labeled with [$^{14}$C]benzylpenicillin, denatured, and digested with trypsin. The tryptic peptides were separated by HPLC as described (13). Fractionation of the peptides from both PBP 1A and PBP 1B resulted in only one major radioactive peak; this material was further purified by HPLC under different elution conditions. With both PBP 1A and PBP 1B, two major radioactive peptides were resolved by the second HPLC separation (Fig. 1 *A* and *B*), and their amino acid compositions were determined. Fractionation of the tryptic digest of PBP 3 resulted in two radioactive peptides (Fig. 1*C*) which were analyzed without further purification. The radioactivity in the purified tryptic peptides from PBPs 1A, 1B, and 3 accounted for 26–35% of the radioactivity initially applied to the HPLC columns.

**Amino Acid Compositions of [$^{14}$C]Benzylpenicillin-Labeled Tryptic Peptides of PBPs 1A, 1B, and 3.** The two radioactive peptides obtained from each of the PBPs were hydrolyzed and their compositions were determined (Table 1). Fragments I and II of PBP 1A (Fig. 1*A*) were identical in amino acid composition, as were fragments I and II of PBP 1B (Fig. 1*B*). The differences in the retention times of the two fragments obtained from PBP 1A and from PBP 1B were found to be due to an unknown modification of the benzylpenicillin moiety. In both cases, fragment I yielded a benzylpenicillin degradation product that could not be dansylated, whereas fragment II yielded a product that could be dansylated and that was identical with that obtained by acid hydrolysis of native benzylpenicillin. Fragment I from PBP 3 had the amino acid composition shown in Table 1 and, in addition, contained an unidentified compound. Fragment II from PBP 3 had the same amino acid composition as fragment I but contained an extra valine as well as the unidentified compound. The latter components may have arisen from fragmentation of the benzylpenicillin and were only found in the analysis of PBP 3.

**Benzylpenicillin Is Bound to a Serine Residue of PBPs 1A, 1B, and 3.** Complete digestion of the radiolabeled fragments from PBP 1A, 1B, and 3 with a combination of aminopepti-

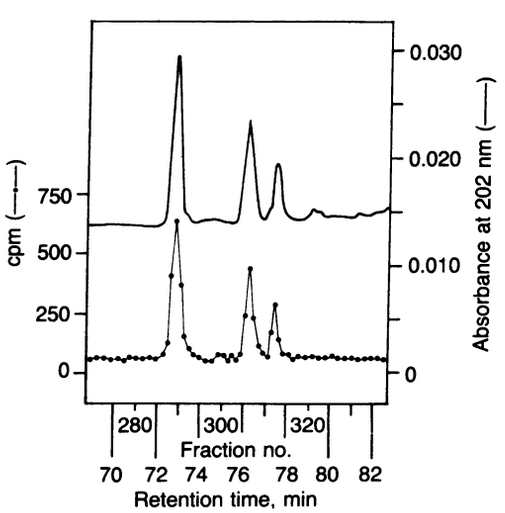


FIG. 2. Purification of [$^{14}$C]benzylpenicillin-labeled fragments produced by partial aminopeptidase M digestion of tryptic fragment II of PBP 1B. The radiolabeled tryptic fragment II (450 pmol; see Fig. 1*B*) was digested partially with aminopeptidase M, and the resulting peptides were separated by HPLC. Fractions (210 μl) were collected and those containing the three major radioactive peptides were pooled (pool 1, fractions 288–290; pool 2, fractions 305–307; pool 3, fractions 311–313), lyophilized, and hydrolyzed for amino acid analysis.

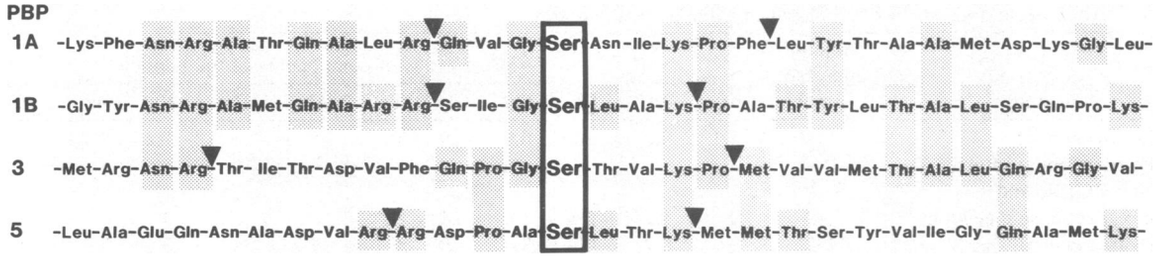


FIG. 3. Comparison of the penicillin-binding sites of PBP 1A, PBP 1B, PBP 3, and PBP 5 of *E. coli*. The amino acid sequences of PBPs 1A, 1B, and 3 were examined for regions with the amino acid composition obtained from the analysis of the benzylpenicillin-labeled peptides. The peptide compositions corresponded to residues 462–470 of PBP 1A, residues 507–513 of PBP 1B, and residues 298–311 of PBP 3; the sequences of these regions are shown. Arrowheads indicate the trypsin cleavage sites. The sequence around the active-site serine residue of the low $M_r$ PBP 5 is shown for comparison (13, 23). The amino acid sequences of PBP 1A and PBP 1B are derived from the nucleotide sequences of the *ponA* and *ponB* genes (unpublished data), and the sequences of PBP 3 (22) and PBP 5 (23) have been reported. The cleavage sites on the COOH-terminal side of the penicilloylated serine residues are atypical. Contamination of trypsin with chymotrypsin would explain the cleavage site observed with PBP 1A but not the cleavage between lysine and proline that occurs in PBP 1B. Proline at this position normally prevents trypsin cleavage (24), and the presence of the penicilloylated serine may explain the unusual cleavage site. An influence of the amino acid composition on the $NH_2$-terminal side of potential trypsin cleavage sites has been described (24).

dase M and carboxypeptidase Y yielded radioactive products that were shown to contain penicillin and serine in equimolar proportions (Table 1). Benzylpenicillin therefore binds to a serine residue in each of these high $M_r$ PBPs. The radiolabeled peptide from PBP 1B contained two serine residues, either of which could have been the residue acylated by penicillin (Table 1). Amino acids were removed from the $NH_2$ terminus of fragment II of PBP 1B by partial digestion with aminopeptidase M and the products were separated by HPLC (Fig. 2). Two of the three radiolabeled products were obtained in sufficient quantities for amino acid analysis. The major product (retention time, 73 min) contained serine, leucine, alanine, lysine, and penicillin in equimolar amounts (Table 1). The second product (retention time, 76.3 min) had an identical composition except that it lacked lysine and presumably resulted from carboxypeptidase contamination of the aminopeptidase preparation. The removal of one of the serine residues of fragment II by aminopeptidase digestion allowed the unambiguous identification of the serine residue of PBP 1B that was acylated by penicillin (see below).

**Sequence of the Benzylpenicillin-Labeled Peptides.** The amino acid sequences of PBP 1A and PBP 1B have been obtained from the nucleotide sequences of the *ponA* and *ponB* genes (unpublished data), and the sequence of PBP 3 has been reported by Nakamura *et al.* (22). The sequences of PBPs 1A, 1B, and 3 were scanned for regions that had the amino acid composition of the corresponding penicillin-labeled tryptic peptide. The amino acid compositions of the peptides (Table 1) corresponded exactly to sequences close to the middle of PBPs 1A, 1B, and 3 (Fig. 3), and the residues acylated by penicillin were identified as serine-465, serine-510, and serine-307, respectively.

## DISCUSSION

PBPs 1A, 1B, and 3 of *E. coli* catalyze both a penicillin-insensitive peptidoglycan transglycosylase reaction and a penicillin-sensitive peptidoglycan transpeptidase reaction (2). We show here that inhibition of these PBPs occurs by penicilloylation of a serine residue. By analogy with D-alanine carboxypeptidases (10–13, 25), it is very likely that the same serine residue is also acylated by cell-wall peptides during transpeptidation and that the physiologically important high $M_r$ PBPs are serine peptidases. PBPs 1A and 1B show about 30% sequence identity, but neither protein shows any extensive similarity to PBP 3 (unpublished data). The sequences around the active-site serine residue of the three high $M_r$ PBPs, however, show clear similarities. In all three proteins, the active-site serine residue occurs within the sequence Gly-Ser-Xaa-Xaa-Lys-Pro, and at three nearby positions identical amino acids are present in each high $M_r$ PBP (Fig. 3).

The high $M_r$ PBPs, D-alanine carboxypeptidases, and the class A and class C $\beta$-lactamases show no extensive amino acid sequence similarity (refs. 1, 17, 22, and 23 and unpublished data) although all of these enzymes interact with penicillin by a mechanism that involves acylation of a serine residue. Even around the active-site serine residue, there is little sequence similarity among the four classes of enzymes, except that in all of these enzymes that recognize penicillin [with the apparent exception of the D-alanine carboxypeptidase from *Streptomyces* strain R39 (26)], a lysine follows three amino acids after the acylated serine residue (1, 11, 13, 17, and 23). The functional significance of the conserved lysine is unknown, but presumably this residue has an important role in catalysis or is involved in the binding of the carboxyl group of the peptide substrate and/or the $\beta$-lactam molecules.

The location of the active-site serine residue close to the middle of the high $M_r$ PBPs contrasts with the position of the acylated residue close to the $NH_2$ terminus of the D-alanine carboxypeptidases and $\beta$-lactamases (11, 13, 16, 17, and 23). The latter classes of enzymes catalyze a single reaction, whereas the high $M_r$ PBPs are bifunctional, and the transglycosylase activity of the high $M_r$ PBPs may occupy an $NH_2$-terminal domain that is absent in the D-alanine carboxypeptidases and $\beta$-lactamases (4). Support for this view has been obtained by showing that the $NH_2$-terminal 240 residues of PBP 3 of *E. coli* can be removed without destroying the penicillin-binding activity (27).

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