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Use of Biotinylated β-Lactams and Chemiluminescence for Study and Purification of Penicillin-Binding Proteins in Bacteria

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A new reagent for the detection of penicillin-binding proteins (PBPs) was developed. An *N*-hydroxysuccinimide ester of biotin was used to tag β-lactam antibiotics with free side chain amino groups such as ampicillin (BIO-AMP), 6-aminopenicillanic acid (BIO-APA), and 7-aminocephalosporanic acid (BIO-ACA). Bacterial PBPs from cells or isolated cytoplasmic membranes of *Escherichia coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* were labeled with BIO-AMP, subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred onto nitrocellulose membranes. Electrophoretic PBP profiles were detected on blots, using colorimetric or chemiluminescence systems, on the basis of the interaction of BIO-AMP–PBP complexes and a streptavidin-peroxidase conjugate. The chemiluminescent reaction permitted a high sensitivity of detection, and PBP profiles could be determined within seconds. All PBP profiles were similar to those obtained with a traditional PBP labeling technique with ^{125}I-labeled penicillin V, except that an additional unidentified PBP (approximately 55,000 Da) was labeled with BIO-AMP in *E. coli* and *H. influenzae*. Differences in the intensities of labeling for specific PBPs were observed between the chemiluminescent and radioactive labeling agents and were attributed to the differences in their affinities for PBPs. Similarly, BIO-AMP, BIO-APA, and BIO-ACA produced different PBP profiles. We also investigated the use of BIO-AMP in PBP purification. BIO-AMP–PBP complexes from a mixture of *H. influenzae* proteins were allowed to bind to avidin immobilized on an agarose support in a microcentrifuge tube. After several washes in the presence of salts, PBPs were eluted by boiling and treatment with SDS. The eluted proteins were separated by electrophoresis on SDS-polyacrylamide gels, and biotinylated proteins were identified on blots by a chemiluminescence reaction. Biotinylation of β-lactams is rapid, safe, and inexpensive. Our results demonstrate the feasibility of using biotinylated β-lactams as nonradioactive reagents for the study of PBPs and for the purification of these proteins.

β-Lactam drugs must bind to specific targets located in the cytoplasmic membrane of bacteria to exert their inhibitory effects. These target proteins can be identified by their ability to covalently bind isotope-labeled penicillin and are termed penicillin-binding proteins (PBPs). The enzymatic functions of higher-molecular-weight PBPs are essential in the cross-linking of the bacterial cell wall. β-Lactam antibiotics kill bacteria through inhibition of these high-molecular-weight PBPs as substrate analogs of the acyl-D-alanyl-D-alanine component of peptidoglycan (11, 28).

Radiolabeling of PBPs with ^{3}H-, ^{14}C-, or more recently, ^{125}I-penicillin is the method of choice for the characterization of new β-lactam antibiotics (structure-activity studies) and for the investigation of PBPs (22, 25). Cost, availability, biohazard, and the length of the experimental procedure (several days) are serious limitations to this methodology. Here, we report the development and use of biotinylated β-lactams such as ampicillin, 6-aminopenicillanic acid, and 7-aminocephalosporanic acid as PBP-labeling reagents. Detection of PBPs could be easily achieved by the use of colorimetric or chemiluminescence detection systems through the specific interaction of biotin with a streptavidin-peroxidase conjugate. Our biotinylated β-lactams could recognize all bacterial proteins defined as PBPs in a variety of gram-negative and gram-positive species (*Escherichia coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*), and the chemiluminescence detection system permitted the rapid and sensitive detection of these PBPs. Furthermore, we showed that biotin–β-lactam–PBP complexes could be purified by affinity chromatography by using the ligand avidin immobilized on beaded agarose.

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MATERIALS AND METHODS

Bacterial strains and media. The medium used for the growth of *H. influenzae* Rd was brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 15 μg each of hemin (Eastman Kodak Co., Rochester, N.Y.) and β-NAD (Sigma Chemical Co., St-Louis, Mo.) per ml. Strain Rd was maintained on chocolate agar plates (Quélab Laboratories, Montreal, Quebec, Canada). *S. pneumoniae* ATCC 27336 and *S. aureus* ATCC 25923 were grown in brain heart infusion, whereas *E. coli* ATCC 25922 was grown in tryptic soy broth (Difco Laboratories). The last three strains were also maintained on agar plates.

Preparation of bacterial membranes. The procedure for the isolation of inner membranes from gram-negative bacteria was modified from that of Preston et al. (22). Overnight cultures were diluted 1:10 in broth and were allowed to reach the exponential phase of growth (A_{600} = 0.6). The cells from 1-liter cultures were collected by centrifugation, washed twice in 10 mM sodium phosphate buffer (pH 7.0), and suspended in 20 ml of the same buffer. DNase I (50 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 0.14 mM 2-mercaptoethanol were added to the suspension, and the cells were disrupted by three

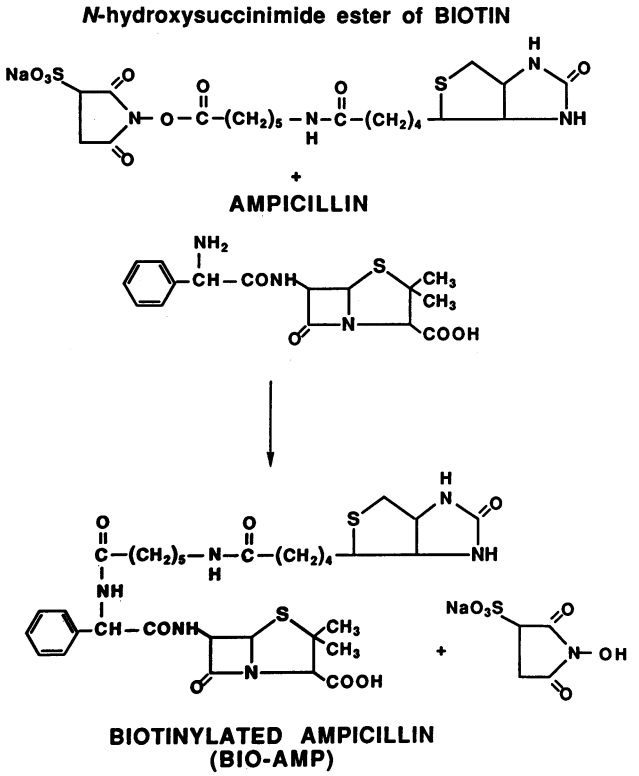

FIG. 1. Biotinylation of ampicillin using an *N*-hydroxysuccinimide ester of biotin.

passages through a French pressure cell (18,000 lb/in^2). Unbroken cells were removed by low-speed centrifugation before the cell extract was loaded onto a discontinuous sucrose gradient (1 part 58% sucrose overlaid with 1 part 52% sucrose in 10 mM sodium phosphate buffer [pH 7.0]) and was centrifuged at 100,000 × *g* for 18 h at 4°C. The 52% sucrose fraction containing the cytoplasmic membrane was collected and was washed twice by centrifugation. Inner membrane preparations were suspended in 50 mM sodium phosphate buffer (pH 7.0) and were stored at −20°C.

Biotinylation of β-lactams. An *N*-hydroxysuccinimide ester of biotin like NHS-LC-biotin (Pierce Chemical, Rockford, Ill.) was linked to primary amines of various β-lactam molecules. The reaction mixture consisted of a fivefold molar excess of NHS-LC-biotin and ampicillin, 6-aminopenicillanic acid, or 7-aminocephalosporanic acid, all from Sigma Chemical Co. (St. Louis, Mo.), dissolved in 0.1 M sodium phosphate buffer (pH 7.2). The mixture was incubated in a microcentrifuge tube with gentle agitation for 30 min at room temperature. The reaction was stopped by the addition of an immobilized ligand with a primary amine like Affi-Gel 102 (Bio-Rad Laboratories, Richmond, Calif.), which is an amino-terminal cross-linked agarose gel with a six-atom hydrophilic arm. The reaction was terminated by incubation for an additional 30 min with gentle agitation at room temperature. The biotinylated β-lactams were separated from the immobilized ligand by centrifugation (Fig. 1). Biotinylated ampicillin (BIO-AMP), biotinylated 6-aminopenicillanic acid (BIO-APA), and biotinylated 7-aminocephalosporanic acid (BIO-ACA) were always freshly prepared before use. The antibacterial activities of the BIO-AMP preparations were also compared with that of ampicillin by a disk diffusion assay and a broth microdilution technique (17).

Radiolabeling of penicillin V. The *p*-(trimethylstannyl)penicillin V reagent was kindly provided by Larry C. Blaszczak (Lilly Research Laboratories, Indianapolis, Ind.) and was iodinated with [^{125}I]Na (specific activity, 16 to 17 mCi/μg of iodine), from Amersham Canada Ltd. (Oakville, Ontario, Canada), by iododestannylation of the tin-substituted penicillin V by a modification of the chloramine-T method described by Blaszczak et al. (2). The ^{125}I-penicillin V (^{125}I-PenV) was stored at 4°C for no longer than 2 weeks before use.

Labeling of PBPs. Radiolabeling of PBPs from isolated cytoplasmic membranes of gram-negative bacteria by using ^{125}I-PenV (37.3 Ci/mmol) was performed by a modification of the method of Preston et al. (22) or as described before (5) for the labeling of intact *H. influenzae* cells. For nonradioactive labeling of PBPs, the procedure was essentially the same as that described above, except that ^{125}I-PenV was replaced with biotinylated β-lactams. PBPs from membranes (40 μg) or from intact *H. influenzae*, *S. aureus*, and *S. pneumoniae* cells collected from 1 ml of a culture (A_{600} = 0.4) and suspended in 50 μl of phosphate-buffered saline (pH 7.0) were labeled by the addition of a final concentration of 4 μg of any of the labeled β-lactams per ml and were incubated for 30 min at room temperature. The samples were then boiled for 5 min in electrophoretic loading buffer consisting of 2.5% glycerol, 5% 2-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) in 1 M Tris (pH 6.8). To achieve lysis, intact cells of *S. aureus* and *S. pneumoniae* were treated with lysostaphin (or lysozyme) and detergents, following the procedure of Ubukata et al. (29), prior to boiling in electrophoretic loading buffer. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (6% stacking gel and 10% separating gel). In some assays designed to determine the specificity of PBP labeling by biotinylated β-lactams, various concentrations of penicillinase (Sigma Chemical Co.) were added to BIO-AMP for 15 min at room temperature before mixing BIO-AMP with bacterial cells or isolated membranes as described above.

Detection of radiolabeled PBPs. After electrophoresis as described above, ^{125}I-PenV-labeled PBPs were visualized by autoradiography after exposure of the dried gels to Kodak X-OMAT film for 1 to 10 days at −20°C.

Streptavidin-biotin detection systems for PBPs. After electrophoresis as described above, proteins were transferred to a nitrocellulose membrane for 25 min at 10 V in the Bio-Rad Trans-blot SD Semi-dry transfer cell (Bio-Rad Laboratories) in transfer buffer (20 mM Tris, 192 mM glycine [pH 8.3] containing 4% [vol/vol] methanol) (Fig. 2, steps A and B). After blotting, the membrane was incubated at room temperature for 1 h in a 0.1% Tween 20 concentration in 20 mM Tris–137 mM NaCl (TTBS; pH 7.6) containing 3% skim milk as the blocking agent. The membrane was then quickly rinsed twice in TTBS before several washes in the same buffer (15, 5, and 5 min at room temperature). A streptavidin-reporter enzyme conjugate was then allowed to bind to biotin–β-lactam–PBP complexes on blots for 1 h at room temperature. In this step, a streptavidin-peroxidase conjugate (Boehringer Mannheim Canada, Dorval, Quebec, Canada), used at a 1:10,000 dilution (0.05 U/ml) in TTBS, served as the reporter molecule. The unbound conjugate was then removed by several washes in TTBS: 15 min at 60°C, 15 min at 37°C, 5 min at 37°C, and three washes of 5 min each at room temperature.

(i) Colorimetric detection. The detection of peroxidase–streptavidin–biotin–β-lactam–PBP complexes on nitrocellulose membranes was performed by using the horseradish peroxidase conjugate substrate kit from Bio-Rad Laboratories. The substrates were 4-chloro-1-naphthol and hydrogen peroxide, which formed a water-insoluble bluish black oxidized

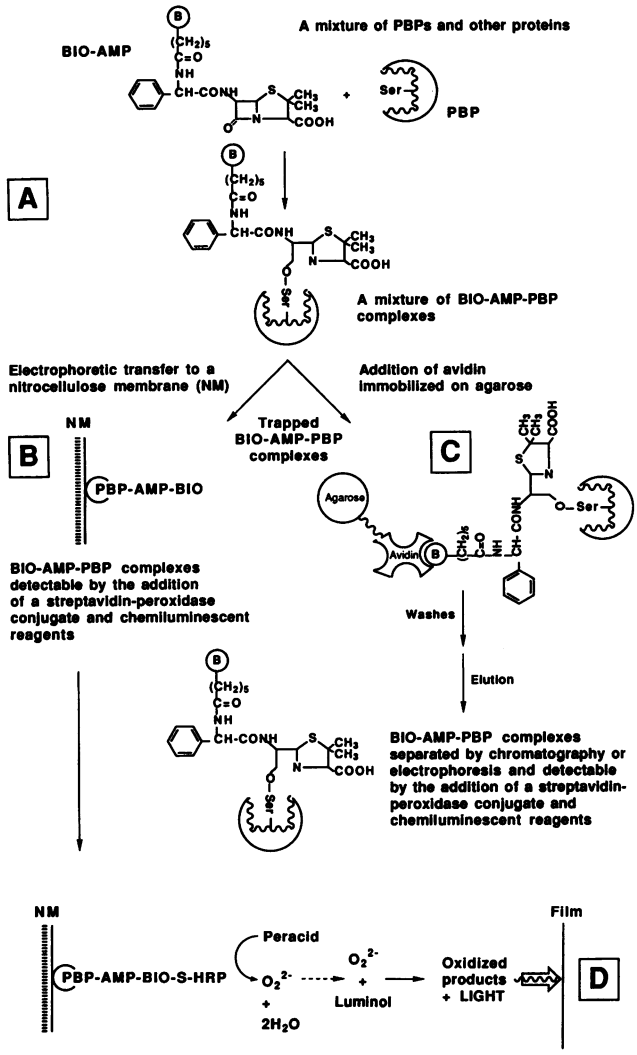

FIG. 2. Steps involved in the formation of BIO-AMP–PBP complexes (A) and then trapping of the complexes by electrophoretic transfer to a nitrocellulose membrane (B) or by the addition of avidin immobilized on agarose (C). Detection of BIO-AMP–PBP complexes is then possible by the addition of a streptavidin-horseradish peroxidase (S-HRP) conjugate and chemiluminescent reagents (D).

product in the presence of the peroxidase. After the appearance of the reaction product, the blots were rinsed with water and air dried, and the PBP electrophoresis profiles were analyzed.

(ii) Chemiluminescence detection. The chemiluminescence reaction of cyclic diacylhydrazides such as luminol was used in the present study as a more sensitive assay for the detection of peroxidase–streptavidin–biotin–β-lactam–PBP complexes on nitrocellulose membranes. The oxidation of luminol catalyzed by the peroxidase in the presence of hydrogen peroxide provokes a subsequent light emission that can be visualized on a film. We used the ECL Western blotting (immunoblotting) detection reagents from Amersham (Amersham Canada Ltd.) and followed the recommendations of the manufacturer. The nitrocellulose membrane was flooded with the substrates for 1 min at room temperature, the excess reagent was drained, and the membrane was covered with Saran Wrap and immediately exposed to a hyperfilm-ECL (Amersham) for 5 s to 1 min before film development and visualization of the PBP electrophoresis profiles (Fig. 2, step D).

Affinity of β-lactams for PBPs. The relative affinities of the labeled β-lactams for individual PBPs were determined by competition assays (21). In competition experiments, the membrane preparations or intact cells were incubated with various concentrations of a competing β-lactam (unlabeled penicillin V or ampicillin) for 10 min, prior to the addition of 10 μg of ^{125}I-PenV or BIO-AMP per ml for an additional incubation period of 45 min at room temperature. The samples were subjected to electrophoresis as described above, and the gels were dried for the detection of radiolabeled PBPs or the proteins were transferred to a nitrocellulose membrane for the detection of biotinylated PBPs. The concentration of the competing β-lactam needed to block 50% of the subsequent binding of the labeled β-lactam to a particular PBP (IC_{50}) was determined by scanning the PBP profiles obtained on films with a Bio-Rad model 620 video densitometer.

Purification of PBPs. Biotinylated PBPs could be purified by using the specific avidin-biotin interaction (Fig. 2, steps A and C). PBPs were first biotinylated as described above. Membrane samples were then boiled for 5 min in a denaturation buffer containing 1% SDS and 5% 2-mercaptoethanol. The biotinylated proteins were added to avidin immobilized on agarose beads (Sigma Chemical Co.), and the mixture was incubated for 1 h at room temperature with gentle agitation. The mixture was centrifuged, and the supernatant was removed. The affinity resin pellet was subjected to several washes in which buffers of different compositions were added to the pellet and the mixtures were incubated with agitation for 10 min at room temperature. After each wash, the mixture was centrifuged as described above and the supernatant was removed. The washing procedure was based on the method of Schryvers (24) and consisted of the following steps: (i) twice with 50 mM Tris-HCl–100 mM NaCl buffer (pH 8.0) containing 10 mM EDTA, (ii) twice with 50 mM Tris-HCl–1 M NaCl buffer (pH 8.0), and (iii) twice with 50 mM Tris-HCl–100 mM NaCl buffer (pH 8.0). After the final washing step, the pellet was suspended in elution buffer (8% SDS and 5% 2-mercaptoethanol in 200 mM Tris-HCl [pH 6.8]), and the mixture was heated at 100°C for 5 min to elute the bound biotinylated PBPs before acetone precipitation. The fractions were monitored for the presence of biotinylated PBPs by electrophoresis on SDS-polyacrylamide gels, transfer of proteins on nitrocellulose membranes, and incubation with the streptavidin-peroxidase conjugate as described above (Fig. 2, step D).

RESULTS

The biological activity of BIO-AMP was evaluated by disk diffusion and determination of its MIC for *H. influenzae*. In comparison with ampicillin, BIO-AMP produced a smaller zone of inhibition against the test organism in the disk diffusion assay (30 and 23 mm, respectively) but showed an MIC (0.25 μg/ml) equivalent to that of ampicillin in the broth microdilution test. The difference observed in the disk susceptibility test results may be related to a slower diffusion or a greater nonspecific binding of BIO-AMP in solid medium. Alternatively, the observed biological activity in susceptibility tests could have been provided by residual ampicillin in BIO-AMP preparations. Nevertheless, the inhibitory activities of BIO-AMP preparations occurred concurrently with the ability of BIO-AMP to bind covalently to PBPs, the cellular targets of traditional β-lactams (see below).

Figure 3 shows the PBP profiles of *H. influenzae* detected by chemiluminescence after electrophoresis and transfer of BIO-

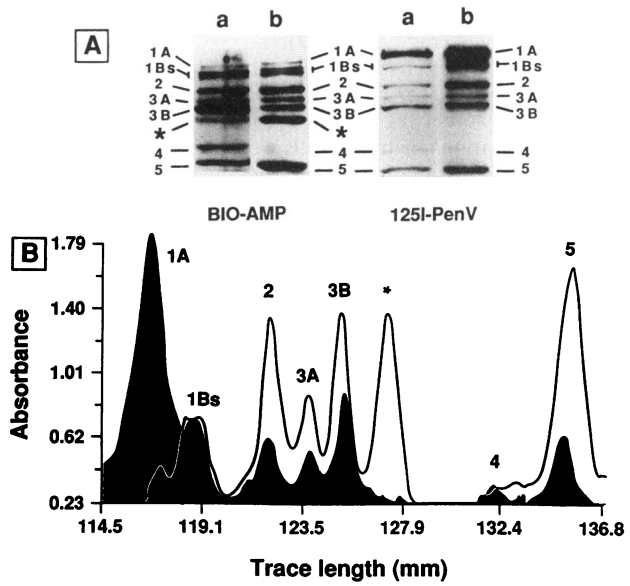

FIG. 3. (A) PBP profile of *H. influenzae* cells (lane a) and isolated inner membranes (lane b) detected by chemiluminescence and autoradiography. Whole cells and inner membranes (40 μg) were labeled with 4 μg of BIO-AMP or ^{125}I-PenV per ml for 30 min. Samples were separated by electrophoresis on SDS-polyacrylamide gels (10%), and the proteins were transferred to a nitrocellulose membrane for detection of biotinylated PBPs or the gels were dried for detection of radiolabeled PBPs. The PBPs of *H. influenzae* (numbering system of Parr and Bryan [19]) are shown in the center and sides of the panel. (B) Comparison of PBP profiles of isolated inner membranes obtained on films after a 1- to 10-s chemiluminescence reaction (BIO-AMP [□]) or a 5-day autoradiography (^{125}I-PenV [■]) recorded by densitometry.

AMP–PBP complexes on nitrocellulose membranes. BIO-AMP was able to bind to PBPs of intact cells (Fig. 3A, lane a) as well as to all PBPs from isolated membranes (Fig. 3A, lane b), although some differences in the intensity of labeling were noted for specific PBPs. This variation in the PBP profiles of intact cells compared with those of isolated membranes was also noted previously when ^{35}S-benzylpenicillin was used as the labeling agent and may be due to decreased drug penetration in whole cells (15). The PBP profiles obtained with BIO-AMP for whole *H. influenzae* cells and isolated membranes were very similar to those determined with ^{125}I-PenV, except that ^{125}I-PenV seemed to bind relatively more to PBP 1A and less to PBP 1Bs than BIO-AMP did (Fig. 3B). This was in accordance with the calculated IC_{50}s of the parent compounds penicillin V and ampicillin for *H. influenzae* PBP 1A (2.0 and 8.9 μg/ml, respectively). BIO-AMP led to a relative increase in labeling of PBP 2 and PBP 3 in profiles of *E. coli* compared with that observed on the PBP profiles obtained with ^{125}I-PenV (Fig. 4). This increased binding of BIO-AMP to PBP 2 and PBP 3 may be related to the IC_{50} of ampicillin for these PBPs, which were reported to be about 10 times less than those calculated for penicillin V (6).

Interestingly, in contrast to radioactive labeling, an additional PBP (PBP*) of approximately 55,000 Da was detected only in *H. influenzae* and *E. coli* labeled with BIO-AMP (Fig. 3 and 4). Also noteworthy, there were one to three nonspecific low-molecular-mass chemiluminescent bands (approximately 14,000 to 34,000 Da) that appeared on the whole-cell profiles of *H. influenzae* and *E. coli*, even though the cells were not treated with BIO-AMP (bands designated NS in the *H. influenzae* profiles in Fig. 5). The experiment reported in Fig. 5 showed that hydrolysis of BIO-AMP by a penicillinase prevented specific PBP labeling and the appearance of chemiluminescent PBP profiles. The nonspecific bands mentioned above persisted on the PBP profiles of *H. influenzae* cells exposed to the hydrolyzed BIO-AMP reagent, whereas *E. coli* PBP* from isolated cytoplasmic membranes, as well as all typical PBPs of both species, were less labeled with rising concentrations of penicillinase in the assay. Also, the chemiluminescent PBP*s strongly detected on profiles of isolated cytoplasmic membranes from *H. influenzae* and *E. coli* seemed to be specifically labeled with BIO-AMP because they gradually disappeared in the presence of increasing concentrations of unlabeled penicillin V or ampicillin in competition experiments, although the calculated IC_{50}s of such β-lactams for these PBP*s were elevated (>50 μg/ml).

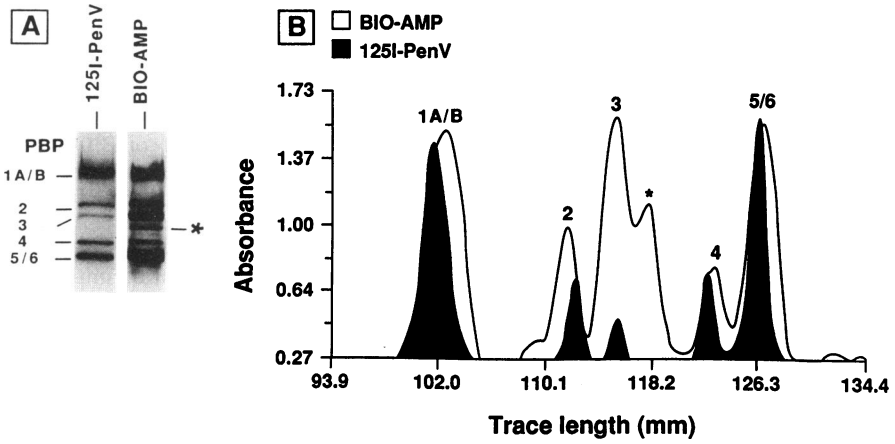

FIG. 4. (A) PBP profiles of *E. coli* detected by chemiluminescence and autoradiography. Inner membranes (40 μg) were labeled with 4 μg of BIO-AMP or ^{125}I-PenV per ml for 30 min. Inner membrane proteins were separated by electrophoresis in an SDS-polyacrylamide gel (10%) system and were transferred to a nitrocellulose membrane for detection of biotinylated PBPs, or the gels were dried for detection of radiolabeled PBPs. The PBPs of *E. coli* are indicated on the left. (B) Comparison of PBP profiles of isolated inner membranes obtained on films after a 24-h autoradiography (^{125}I-PenV) or a 10-s chemiluminescence reaction (BIO-AMP) recorded by densitometry.

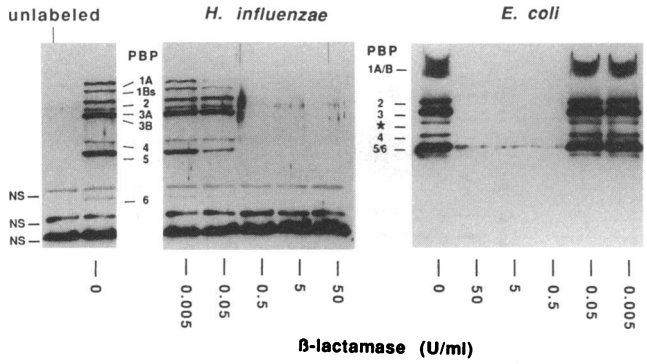

FIG. 5. Chemiluminescence PBP profiles of *H. influenzae* cells and *E. coli* inner membranes. The labeling reagent BIO-AMP was first incubated for 15 min with β-lactamase at the indicated concentrations, and the resulting mixtures were used to label the PBPs for 30 min. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (10%), and the proteins were transferred to a nitrocellulose membrane. PBPs were identified after a 30-s chemiluminescence reaction. NS, nonspecific chemiluminescent bands; unlabeled, *H. influenzae* cells not exposed to BIO-AMP but submitted to the same procedure.

Figure 6 shows that the labeling reagent BIO-AMP is indeed adequate for use in competition experiments with unlabeled β-lactams like ampicillin and penicillin V. The binding of ampicillin and penicillin V to *H. influenzae* PBP 3B in the competition experiments whose results are shown in Fig. 6 was calculated by densitometry and is reported graphically in Fig. 7. The apparant saturation of *H. influenzae* PBP 3B by either ampicillin or penicillin V seemed to require greater antibiotic concentrations when ampicillin or penicillin V was in competition with BIO-AMP than when one or the other drug was in competition with ^{125}I-PenV (Fig. 7), an observation that may be due to the increased sensitivity of the chemiluminescence reaction used to detect PBPs.

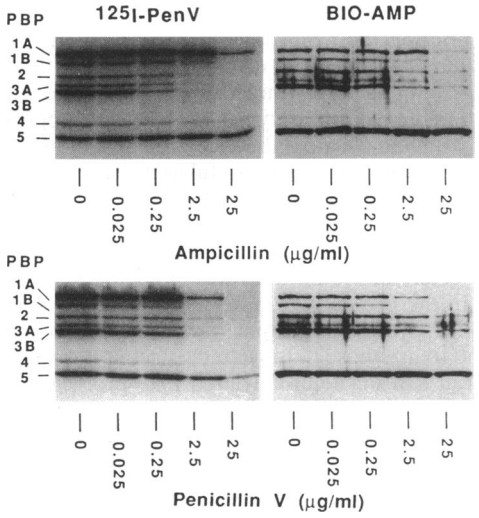

FIG. 6. PBP profiles of *H. influenzae* obtained in competition assays. Cells were first incubated for 10 min with either one of the unlabeled competing β-lactams (ampicillin and penicillin V) at the indicated concentrations. Cells were then labeled with 10 µg of BIO-AMP or ^{125}I-PenV per ml for 45 min. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (10%) before detection of PBPs by a 6-day autoradiography (^{125}I-PenV) or a 15-s chemiluminescence reaction (BIO-AMP). The PBPs of *H. influenzae* are indicated on the left.

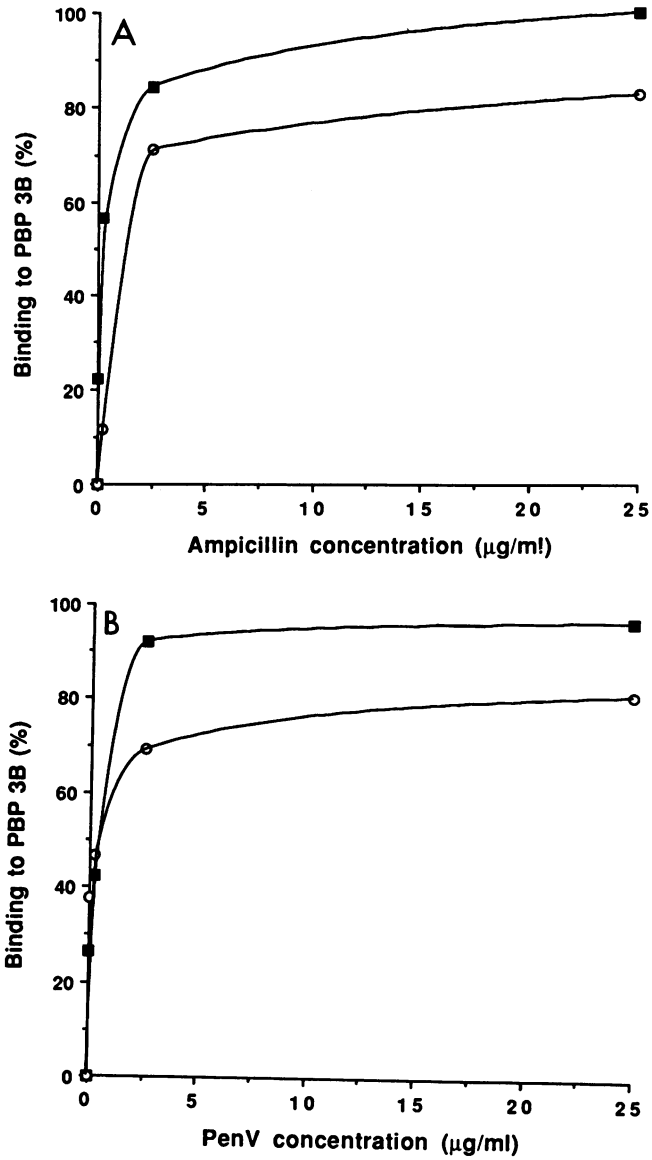

FIG. 7. Relative ampicillin (A) and penicillin V (B) binding to *H. influenzae* PBP 3B in relation to the β-lactam concentrations used in competition experiments shown in Fig. 6. These values were calculated from densitometric analyses of the PBP profiles obtained on films after a 15-s chemiluminescence reaction (○) or a 6-day autoradiography (■).

PBP profiles of gram-positive organisms such as *S. pneumoniae* and *S. aureus* were also obtained by using BIO-AMP as the labeling reagent, although, similar to the results obtained with ^{125}I-PenV, we could not well resolve some PBPs under our experimental conditions (Fig. 8). The PBP profiles obtained with either BIO-AMP or ^{125}I-PenV were very comparable, except that some nonspecific chemiluminescent bands appeared on profiles of *S. pneumoniae* and *S. aureus* (bands designated NS in Fig. 8).

Remarkably, chemiluminescence could very rapidly provide

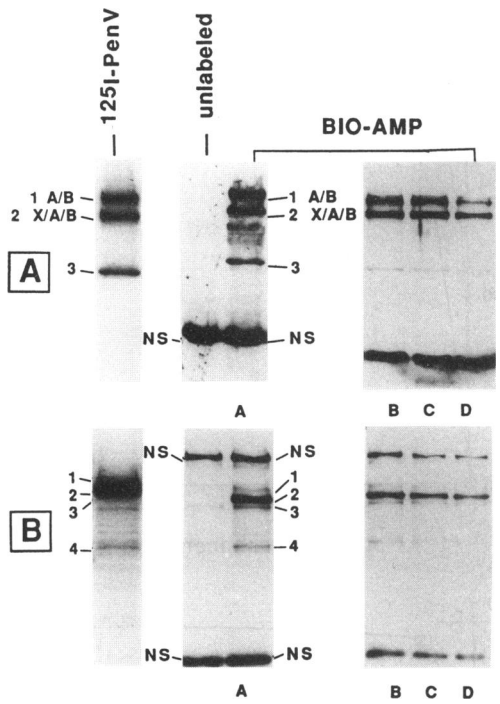

FIG. 8. PBP profiles of *S. pneumoniae* (A) and *S. aureus* (B) detected by autoradiography and chemiluminescence. Cells were labeled with 4 μg of BIO-AMP or ^{125}I-PenV per ml for 30 min. Samples were separated by electrophoresis on SDS-polyacrylamide gels (10%), and the proteins were transferred to a nitrocellulose membrane for detection of biotinylated PBPs or the gels were dried for detection of radiolabeled PBPs. PBPs were identified after a 1- to 10-day autoradiography (^{125}I-PenV) or a 5- to 30-s chemiluminescence reaction (BIO-AMP). The PBPs of *S. pneumoniae* and *S. aureus* are shown on the left and in the center of the panels. Lanes a to d, serial dilutions of samples applied on gels; NS, nonspecific chemiluminescent bands; unlabeled, cells not exposed to BIO-AMP but submitted to the same procedure.

information on PBP profiles. After electrophoresis, Western blotting, and treatment of the membrane with a blocking agent, a highly sensitive means of detecting PBPs was possible within seconds. An equivalent sensitivity in PBP detection with ^{125}I-PenV as the labeling agent usually took an extended exposure time of dried gels during autoradiography (1 to 10 days). A streptavidin-peroxidase-based colorimetric detection assay with 4-chloro-1-naphthol as the substrate was also used for to detect BIO-AMP–PBP complexes on blots (8). By this less efficient approach, maximal sensitivity was achieved with higher concentrations of BIO-AMP in PBP assays (up to 128 μg/ml), and only *H. influenzae* PBPs 3B and 5 from intact cells could be visualized on blots when the usual concentration of labeling agent was used (4 μg/ml) (data not shown).

Basically, any β-lactam that possesses a free primary amino group could be labeled with the *N*-hydroxysuccinimide ester of biotin, and we evaluated the abilities of some other biotinylated β-lactams to covalently bind to PBPs. In comparison with the PBP-labeling capability of BIO-AMP, BIO-APA or BIO-ACA could also label the PBPs of *H. influenzae* (Fig. 9). A striking difference was seen with BIO-ACA, which bound specifically less to PBP 2. The latter observation was in agreement with the general tendency of narrow-spectrum cephalosporins to show less binding than penicillins to PBP 2 (6).

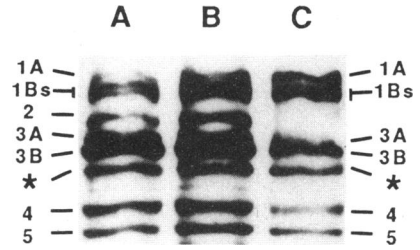

FIG. 9. Chemiluminescent PBP profiles of *H. influenzae* cells labeled with various biotinylated β-lactams. Cells were incubated with 4 μg of biotinylated β-lactam per ml for 30 min and were electrophoresed on SDS-polyacrylamide gels. PBPs were identified on blot by a 20-s chemiluminescence reaction. Lanes: A, BIO-AMP; B, BIO-APA; C, BIO-ACA.

By taking advantage of the specific interaction of biotin with avidin, purification of BIO-AMP–PBP complexes from isolated membranes was attempted following the scheme presented in Fig. 2. *H. influenzae* PBPs from isolated membranes were biotinylated with BIO-AMP, and the proteins were solubilized by heating in the presence of SDS before mixing with an avidin-agarose support in a microcentrifuge tube. After several washes, the BIO-AMP–PBP complexes were released from the beaded support by boiling in SDS before SDS-polyacrylamide gel electrophoresis, Coomassie stainings, and Western blotting to evaluate the purification yield (Fig. 10). The purification yield was evaluated by measuring the relative densities of specific PBP bands from crude and purified PBP extracts on luminescence detection films and by their respective protein contents. This rapid purification technique yielded about a 25-fold purification of PBPs.

DISCUSSION

Biotin (vitamin H), a 244-Da molecule, binds with high affinity to avidin and streptavidin. Because of its low molecular mass, biotin can be conjugated to many proteins or molecules without altering their biological activities. In turn, both proteins, avidin or streptavidin, can be conjugated with a variety of signal-providing reporter molecules such as gold, fluorescent dyes, and many types of enzymes and to solid supports such as agarose with almost no influence on the binding of biotin, making these conjugates very useful as detection reagents or as tools for protein purification (1, 23).

Biotinylation of molecules and proteins can be achieved easily by using several commercially available *N*-hydroxysuccinimide esters of biotin that form amide bonds with primary amines. In the present work, we were successfully able to tag some β-lactam molecules that have free amino groups with biotin and to use those biotinylated β-lactams for the detection of bacterial PBPs. The biotinylated PBP profiles obtained for *H. influenzae* (Fig. 3), *E. coli* (Fig. 4), and *S. aureus* and *S. pneumoniae* (Fig. 8) were comparable to those obtained with the radioactive ^{125}I-PenV reagent used in the present study and to those published previously (4, 7, 13, 25).

β-Lactam antibiotics interact covalently with PBPs at the active-site serine residue. In order to acylate PBPs and produce some inhibitory activity, β-lactam drugs must possess some intrinsic chemical reactivity and present a specific configuration to the enzyme-active site (18). The addition of biotin to the side chain of β-lactams could have seriously impaired the ability of the antibiotic to bind to the active site of PBPs because of steric hindrance. Fortunately, although the results showed that BIO-AMP may have less biological activity than

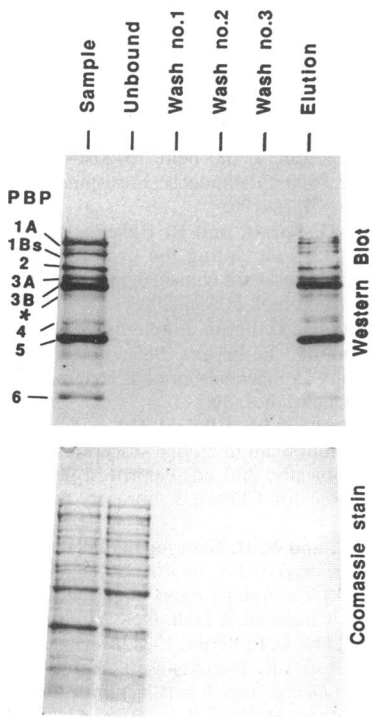

FIG. 10. Rapid purification procedure for *H. influenzae* PBPs. Isolated inner membranes were labeled with BIO-AMP (Sample), boiled and denatured, and mixed with avidin immobilized on agarose beads. The unbound proteins were removed, and the affinity resin was subjected to several washes before the BIO-AMP–PBP complexes were eluted (see text for details). The fractions were monitored for the presence of biotinylated PBPs after electrophoresis on SDS-polyacrylamide gels, transfer of proteins on nitrocellulose membranes, and detection by a 15-s chemiluminescence reaction (Western blot). A replicate gel of fractions was also stained with Coomassie blue.

ampicillin, all biotinylated β-lactams tested in the present study had some ability to bind to PBPs of four different bacterial species. Interestingly, we also showed that BIO-AMP, BIO-APA, BIO-ACA, and ^{125}I-PenV preferentially targeted different PBPs, in accordance with the previously reported affinities of penicillin and cephalosporin molecules (3, 6, 22). Importantly, we were able to demonstrate that the labeling of PBPs with BIO-AMP was specific because it was eliminated by the presence of a penicillinase and was diminished by penicillin V or ampicillin in competition experiments (Fig. 5 to 7).

In the present study, we did not measure the amount of individual PBP acylated by BIO-AMP, nor did we measure the kinetics of acylation. However, because biotinylated β-lactams were not expected to bind more to PBPs in comparison with ^{125}I-PenV because of steric hindrance at the active site, it is reasonable to assume that for at least equivalent amounts of labeled target enzymes, the chemiluminescence reaction from the signal-providing a reporter conjugate streptavidin-peroxidase was much more sensitive than autoradiography. One could virtually see light emission from blots in the darkroom, and luminescence-sensitive films could be exposed by chemiluminescent PBPs within 1 to 30 s.

Nonradioactive methods of PBP detection have long been sought, and some investigators have met with considerable success. For example, some researchers (12, 20) have reported the use of antibodies against the β-lactam determinant to identify β-lactam–PBP complexes of several bacterial species on immunoblots. Such techniques are still used in some studies, but they require antisera specific to a given β-lactam (14). More recently (8), we have reported the use of biotinylated ampicillin, while another group (9) reported the use of an *N*-hydroxysuccinimide-activated fluorescein in an approach to the labeling of PBPs. The report of Galleni and colleagues (10) showed the great sensitivity of their fluorescent probe for PBPs when detection was aided with an automatic DNA sequencer. Similar to that found with our BIO-AMP labeling reagent, Galleni et al. (10) have also reported the detection of additional unidentified PBPs in electrophoresis profiles of *E. coli* in comparison with those obtained by traditional radiolabeling assays. More work is needed to characterize some of these new PBPs, termed PBP*s in our study, which may be nonspecific since they never completely disappeared in competition experiments with high concentrations of penicillin V or ampicillin.

Compared with the traditional radioactive compound-based approach, our convenient biotin-based detection system may still show some technical difficulties. For example, nonspecific detection of some chemiluminescent products was obtained in PBP profiles of whole bacterial cells, and adequate controls should always be included. Furthermore, adequate proportions of biotinylated β-lactams and PBP samples should be used, and balanced chemiluminescent reagents and specific film exposure times for each bacterial species to be investigated should be used. We obtained highly reproducible and specific PBP profiles for each of the bacterial species tested, once the experimental conditions were defined. The stability of BIO-AMP was investigated over a period of 25 days at −20°C. During this period, no more than a 14% decrease in the intensity of labeling of some PBPs was recorded (data not shown). However, we noticed that the *N*-hydroxysuccinimide ester of biotin had to be used within 2 weeks to prevent the appearance of multiple nonspecific bands on PBP profiles. While the yield of BIO-AMP synthesis was not evaluated in the present study, mass spectrometry-fast atom bombardment performed on preparations of the labeling reagent BIO-AMP confirmed the presence of BIO-AMP as a sodium salt and the expected molecular mass of BIO-AMP as a sodium salt (710.26 + 1).

The complex formation between biotin and avidin is noncovalent but is formed rapidly with a high affinity ($K_a = 10^{15}$ M^{-1}). It is not dissociable by the use of extremes of pH, organic solvents, or many denaturing agents. The complex can be released by 6 to 8 M guanidine HCl (pH 1.5) or by boiling in the presence of SDS (23, 24). In the present study, we evaluated BIO-AMP as a tool for PBP purification. The results showed that BIO-AMP–PBP complexes can be isolated from a protein mixture by using avidin immobilized on beaded agarose (Fig. 10). To release the BIO-AMP–PBP complexes from the avidin-agarose solid support, we used harsh conditions (SDS and boiling), but the binding of biotin to avidin can also be released by milder means (23). Some derivatives of biotin possess spacer arms that contain a disulfide bond which can be cleaved by thiols, therefore allowing dissociation of the biotin-conjugated molecule from avidin. Monomeric forms of avidin (which include low-affinity biotin-binding sites) may also provide an affinity support for reversible binding of biotinylated proteins that can be eluted by ligand competition by using a biotin-containing buffer (23).

β-Lactam antibiotics are currently recommended for the treatment of numerous bacterial infections, and despite years of clinical use, molecular analyses on the structure and function of PBPs represent a relatively new field of research that is now in expansion (11, 18, 26). Structural analyses of the β-lactam-binding sites of a wide variety of bacterial PBPs are

now needed to give a rationale for the design of more potent drugs and β-lactams that will be able to overcome PBP-mediated resistance (16, 27). Here, we reported the results of some preliminary work showing the potential of biotinylated β-lactam reagents for use in the rapid purification of PBPs. The PBPs could be separated further and the resulting samples may be suitable for various protein analyses: amino acid sequencing, active-site peptide determination, and construction of amino acid-derived oligonucleotide probes of PBP genes. Such a rapid approach for the purification of PBPs represents an initial and essential step for the molecular characterization of these proteins.

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