BOCILLIN FL, a Sensitive and Commercially Available Reagent for Detection of Penicillin-Binding Proteins

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We describe a new, sensitive, rapid, and nonradioactive method involving the use of the commercially available BOCILLIN FL, a fluorescent penicillin, as a labeling reagent for the detection and study of penicillin-binding proteins (PBPs). This method allowed rapid detection of 30 ng of a purified PBP protein under UV light and of 2 to 4 ng of the protein with the aid of a FluorImager. This method also allowed rapid determination of the PBP profiles of Escherichia coli, Pseudomonas aeruginosa, and Streptococcus pneumoniae. The PBP profiles obtained are virtually identical to those reported previously with 3H-, 14C-, or 125I-labeled penicillin. Using this method enabled us to determine the 50% inhibitory concentrations of the penicillin-sensitive and -resistant PBP2x proteins of S. pneumoniae for penicillin G, thereby allowing a direct evaluation of their relative affinities for penicillin G. Finally, this method also allowed us to compare relative affinities of a PBP2x protein for different β-lactam antibiotics with the aid of fluorescence polarization technology and to monitor a PBP2x protein during purification.

Penicillin-binding proteins (PBPs) are the enzymes that are required for the biosynthesis of the bacterial cell wall (10, 11, 23, 31). PBPs catalyze the final steps of the polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan, an essential component of the bacterial cell wall (10, 11, 23, 31). PBPs are membrane-bound enzymes and targets of β-lactam antibiotics (6, 7, 10, 11, 14, 24, 28–31). The emerging resistance of pathogenic gram-positive bacteria to β-lactam antibiotics is a serious clinical problem (6, 25, 29, 30).

Materials. BOCILLIN FL (Fig. 1), a derivative of penicillin V, is an orange solid with an extinction coefficient of 68,000 and a maximal absorption at 504 nm (Molecular Probes, Inc.). It fluoresces at 511 nm upon excitation at 504 nm (Molecular Probes, Inc.). BOCILLIN FL is stable for months with no apparent loss of its activity when it was stored at −20°C, even after repeated freezing and thawing (this study).

Detection of PBPs from bacterial membrane preparations. For membranes of the detection of PBPs with BOCILLIN FL as a labeling reagent, the following bacterial strains were used: Escherichia coli MC6RP1 (F− thr-1 leu-1 proA dfr-d frd-lys-4) (9), Pseudomonas aeruginosa PAO1 (16), and S. pneumoniae (her) R6, a penicillin-sensitive and encapsulated derivative of Rockefeller University strain R36A that was kindly provided by A. Tomasz (Rockefeller University). E. coli and P. aeruginosa were first grown in Luria-Bertani (LB) medium (Difco Laboratories, Detroit, Mich.), with vigorous shaking, at 33°C overnight. The overnight cultures (10 ml each) were inoculated into 350 ml of fresh LB medium, allowed to grow to an optical density at 600 nm (OD600) of 1.0, and harvested by centrifugation at 4,400 × g for 10 min. The resulting cell lysates were centrifuged at 12,000 × g for 10 min. The supernatant fractions were collected and centrifuged at 150,000 × g for 35 min. The pellets were collected, washed once, and resuspended in the same phosphate buffer (1 ml each). The resulting suspensions were designated as membrane preparations and used for fluorescent BOCILLIN FL binding assays. The protein concentrations of the membrane preparations were determined by using the Bradford protein assay kit (1), with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, Calif.).

S. pneumoniae (her) R6 was grown in 500 ml of brain heart infusion medium (Difco), without shaking, at 37°C. Cells were collected at an OD600 of 0.5 to 0.8, and membranes were prepared as described above.

For detection of the PBPs of E. coli, P. aeruginosa, and S. pneumoniae (her) R6, reaction mixtures (100 μl each) contained 75 μl of each membrane preparation (≈300 μg of protein) and 25 μl of various amounts of BOCILLIN FL ranging from 0.4 to 50 μM (final concentration). The reaction mixtures were incubated at 35°C for 30 min and denatured with 100 μl each of SDS-denaturing solution (19) at 100°C for 3 min. Then, 5 to 10 μl of each reaction mixture (≈7.5 to 15 μg of protein) was subjected to SDS-PAGE analysis (10% polyacrylamide gel; Bio-Rad Laboratories) (19). The protein gels were rinsed with water immediately after electrophoresis. To visualize the labeled PBPs, the gels were dried, scanned with a FluorImager 575 (excitation at 488 nm and emission at 530 nm) (Molecular Dynamics, Inc., Sunnyvale, Calif.).
Sensitivity of BOCILLIN FL for detection of PBPs and IC$_{50}$ (50% inhibitory concentration) determinations. The penicillin-sensitive and -resistant PB2x proteins from *S. pneumoniae* (b) R6 (a penicillin-sensitive isolate) and 329 (a penicillin-resistant clinical isolate), respectively, were prepared as described previously (33). Protein concentrations were determined as described above (1).

For assessment of the sensitivity of BOCILLIN FL for the detection of PBPs, reaction mixtures (10 µl each) contained 2 to 48 ng of the purified penicillin-sensitive PB2x protein of *S. pneumoniae*, 10 µM BOCILLIN FL, 20 mM potassium phosphate (pH 7.5), and 140 mM NaCl. The reaction mixtures were incubated at 35°C for 30 min, denatured with 10 µl of SDS solution, and subjected to SDS-PAGE as described above. The labeled protein was visualized with a FluorImager as described above or with the naked eye under UV light (290 nm). For quantitation of the protein, the labeled protein was scanned with a FluorImager, and fluorescence intensities of each band were quantified with a Umax XL laser densitometer (Phar-macia LKB Biotechnology, Alameda, Calif.).

Detection of PBPs in bacterial cell membrane preparations. To further evaluate the utility of BOCILLIN FL for the detection of PBPs, we carried out fluorescent BOCILLIN FL binding assays with the membrane preparations of *E. coli*, *P. aeruginosa*, and *S. pneumoniae*. As shown in Fig. 3, PBPs from these organisms were clearly detected when labeled with BOCILLIN FL, and their profiles are very similar to those reported previously with 3H-, 14C-, or 125I-labeled penicillin used as a labeling reagent (14, 21, 22, 26–28, 32). Using a mini-gel (SDS-PAGE) system, we were unable to separate the *S. pneumoniae* PB1a protein from its PB1b protein (Fig. 3, lane 1).

As shown in Fig. 4, the intensities of most PBP bands, except PB4, increased when the *E. coli* membrane preparation was labeled with BOCILLIN FL (Fig. 2A). A linear increase in the fluorescence intensity of the enzyme was observed when the amount of the enzyme increased (Fig. 2B). Thus, these results suggest that the BOCILLIN FL method is at least as sensitive as the 125I-labeled penicillin V method.

RESULTS AND DISCUSSION

Sensitivity of BOCILLIN FL for the detection of purified PB2x proteins of *S. pneumoniae*. To assess the sensitivity of BOCILLIN FL, a derivative of penicillin V (Fig. 1), for the detection of PBPs, we performed fluorescent BOCILLIN FL and 125I-labeled penicillin V binding experiments with purified penicillin-sensitive and -resistant PB2x proteins. When the PB2x proteins were labeled with 125I-labeled penicillin V, 7.5 to 15 ng of the purified PB2x proteins was detectable after overnight exposure of the gels (data not shown). When both proteins were labeled with BOCILLIN FL, 30 to 60 ng of the proteins was detectable with the naked eye under UV light (data not shown). The sensitivity of this reagent for detection of PBPs is similar to that observed with fluorescein-labeled compounds (8). With the aid of a FluorImager, 2 to 4 ng of the penicillin-sensitive PB2x protein was clearly detected when labeled with BOCILLIN FL (Fig. 2A). A linear increase in the fluorescence intensity of the enzyme was observed when the amount of the enzyme increased (Fig. 2B). Thus, these results suggest that the BOCILLIN FL method is at least as sensitive as the 125I-labeled penicillin V method.

**FIG. 1. Structure of BOCILLIN FL.**
labeled with BOCILLIN FL concentrations ranging from 1.6 to 25 μM. When concentrations of BOCILLIN FL were lower than 0.8 μM, BOCILLIN FL failed to clearly detect PBPs (data not shown). At concentrations of BOCILLIN FL of 25 μM or higher, the E. coli PBPs appeared to be saturated (data not shown). Thus, we conclude that 1.6 μM BOCILLIN FL is minimal for routine labeling experiments for E. coli.

Comparison of the penicillin-sensitive and -resistant PBP2x proteins of S. pneumoniae (hex) R6 and 328, respectively, by fluorescent BOCILLIN FL binding assays. To further examine the utility of BOCILLIN FL for routine comparisons of PBPs for their affinities for β-lactams, we labeled both purified PBP2x proteins with BOCILLIN FL and determined their IC50s for penicillin G. As shown in Fig. 5, the IC50s of the penicillin-sensitive and -resistant PBP2x proteins for penicillin G were determined to be 22 and 312 μM, respectively. Previously, the Kd values of the penicillin-sensitive and -resistant PBP2x proteins for penicillin V were determined to be 0.11 and 1.28 μM, respectively (33). The affinities of the two PBP2x proteins for BOCILLIN FL have not been determined and may be different. Nevertheless, these results are consistent with our previous finding that the affinity of the penicillin-sensitive PBP2x protein for 125I-labeled penicillin V is about 12-fold higher than that of the penicillin-resistant PBP2x protein (33).

We also employed fluorescence polarization technology to compare the affinities of the penicillin-sensitive PBP2x protein for penicillin G and other β-lactam antibiotics. As shown in Fig. 6, the IC50 of the penicillin-sensitive PBP2x protein for penicillin G was determined to be 7.9 nM. The IC50s of the PBP2x protein for cefotaxime, imipenem, piperacillin, methicillin, ceftazidime, and cephalixin were determined to be 4.4, 5.1, 17, 59, 294, and 1,627 nM, respectively. The Kd/K value or acylation efficiency of BOCILLIN FL for PBP2x can be derived (7a–13) from the Kd/K value of penicillin G for the penicillin-sensitive PBP2x protein of S. pneumoniae, which is known to be 58,000 M−1 s−1 (17, 18). Assuming the IC50 of the penicillin-sensitive PBP2x enzyme for penicillin G is 22 μM, as determined by the gel-based assay (Fig. 5B), the Kd/K value of BOCILLIN FL for this PBP2x is estimated to be 128,000 M−1 s−1. If the IC50 of the PBP2x enzyme for penicillin G is 7.9 nM as determined by fluorescence polarization assay (Fig. 6), the Kd/K value for the enzyme is approximately 232,000 M−1 s−1. Thus, with the IC50 determined either by gel-based assay or fluorescence polarization method, a similar Kd/K value for BOCILLIN FL was derived. On the basis of these two derived Kd/K values for BOCILLIN FL for the PBP2x enzyme, the following Kd/K values can be derived for these antibiotics from their IC50s as follows (12, 13): 58,000 to 128,000, 51,000 to 102,000, 15,000 to 27,000, 4,000 to 8,000, and 160 to 300 M−1 s−1 for cefotaxime, imipenem, piperacillin, methicillin, and cephalixin, respectively. The Kd/K values of these β-lactam antibiotics for the PBP2x enzyme were previously reported to be 162,000, 107,000, 53,000, 4,900, and 1,600 M−1 s−1, respectively (17, 18). Thus, our Kd/K values are in agreement with those reported (17, 18), except that our Kd/K value for cephalixin was significantly lower. Nevertheless, cephalxin had the lowest Kd/K value in both cases (17, 18; this study).

The IC50 of the penicillin-sensitive PBP2x protein for penicillin G determined by the gel-based assay (22 μM) was about 3,000 times higher than that determined by the fluorescence polarization assay (7.9 nM). This difference is not surprising, since the concentration of BOCILLIN FL (labeling reagent) used in the gel-based assay (10 μM) was 5,000 times higher than the concentration used in the fluorescence polarization assay (2 nM). When IC50s are determined by a true competition assay, the use of higher substrate concentrations generates higher IC50s with a fixed amount of enzyme (2, 4, 12). In addition, the molar ratio of BOCILLIN FL to the enzyme for the gel-based assay (5 μM BOCILLIN/FL/2 μM PBP2x) was approximately three times higher than that for the fluorescence polarization assay (1.5 to 2 nM BOCILLIN FL/1.3 nM PBP2x). The concentration of the enzyme used in the gel-based assay (2 μM = 3 μg in 20 μl) was approximately 1,500 times higher than that used in the fluorescence polarization assays (1.3 nM). Despite the very different conditions employed in the two types of assays (gel-based and fluorescence polarization assays), especially the different concentrations of BOCILLIN FL used in the two assays, the derived Kd/K values for BOCILLIN FL were in excellent agreement (see above). Together, these results suggest that BOCILLIN FL can be used to evaluate relative affinities of PBPs for β-lactam antibiotics.

Finally, the standard assay conditions for the determination of IC50s for β-lactam antibiotics include prelabeling enzymes with β-lactam compounds to be tested, followed by incubation with a labeled β-lactam compound (3, 28). In this study, the IC50s were determined by a true competition assay and were thus strictly dependent on the BOCILLIN FL concentrations utilized in the assay (12).

Implications and conclusions. In this study, we describe a new, rapid, and sensitive method for the detection and study of PBPs. We used this method to detect PBPs from membrane preparations of three different organisms and to compare two...
penicillin-sensitive and -resistant PBP2x proteins for their relative affinities for penicillin G. The PBP profiles generated by this method are very similar to those reported before by the method of 3H-, 14C-, or 125I-labeled penicillin (14, 21, 22, 26–28, 32). Using this method, we were also able to demonstrate that a PBP1a mutant of *S. pneumoniae* was indeed missing the PBP1a protein compared with its parent strain (data not shown). The IC50s determined for the penicillin-sensitive and -resistant PBP2x proteins allowed us to directly evaluate their relative affinities for penicillin G and other β-lactam antibiotics. The results of this comparative study are consistent with those of the previous studies with 125I- and 14C-labeled penicillin and also a thioester substrate (17, 18, 33). Finally, this methodology was also successfully applied to the detection of a PBP2x protein during its purification (data not shown). The results of our study, therefore, have validated BOCILLIN FL as a labeling reagent for the detection of PBPs and the evaluation of relative affinities of PBPs for different β-lactam antibiotics.

The use of BOCILLIN FL as a labeling reagent for the detection of PBPs offers several advantages over the radioisotope methods (22, 27, 28). By this method, results can be obtained immediately after the completion of SDS-PAGE, since no gel manipulations are required for detection. Routinely, the 125I-labeled penicillin V and 3H- or 14C-labeled penicillin methods require days to weeks. This BOCILLIN FL method is sensitive, allowing rapid detection of quantities of proteins in nanograms with the naked eye under UV light or with the aid of a FluorImager. The sensitivity of this method is similar to that of the 125I-labeled penicillin V method, which also detected quantities of the proteins as small as nanograms but required overnight exposure. The BOCILLIN FL method does not involve the use of radioactive materials, in contrast to the 3H-, 14C-, and 125I-labeled penicillin methods. Thus, no hazardous materials are produced. Only small amounts of BOCILLIN FL are needed for routine labeling studies. We established that a concentration as low as 1.6 μM the reagent could be used for labeling bacterial membrane preparations.

BOCILLIN FL and 3H-labeled penicillin are both commercially available (28), but the latter methodology typically requires weeks for the development of PBP bands. 125I-labeled penicillin V has considerably shortened the amount of time that is required, but it is not commercially available (27). Fluorescein-labeled and biotinylated penicillins offer similar advantages (5, 8, 15, 20), yet they are not commercially available (5, 8, 15, 20). Also, the use of biotinylated penicillins requires transferring proteins to membranes and blocking and developing the membranes, processes which usually require at least a full day. BOCILLIN FL is a safe and sensitive reagent for the detection and study of bacterial PBPs.

Finally, a comparison of the PBP binding properties of BOCILLIN FL and those of its parent molecule, penicillin V, has not yet been carried out. Therefore, the effects of the addition of the fluorophore on the PBP binding properties of BOCILLIN FL are unknown. Further study is also needed to address the suitability of BOCILLIN FL for kinetic studies of PBPs.

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