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Biological Characterization of a New Radioactive Labeling Reagent for Bacterial Penicillin-Binding Proteins

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Radiolabeled penicillin G is widely used as the imaging agent in penicillin-binding protein (PBP) assays. The disadvantages of most forms of labeled penicillin G are instability on storage and the long exposure times usually required for autoradiography or fluorography of electrophoretic gels. We investigated the utility of radioiodinated penicillin V as an alternative reagent. Radioiodination of *p*-(trimethylstannyl)penicillin V with [^{125}I]Na, using a modification of the chloramine-T method, is simple, high yielding, and site specific. We demonstrated the general equivalence of commercially obtained [^{3}H]penicillin G and locally synthesized [^{125}I] penicillin V (IPV) in their recognition of bacterial PBPs. Profiles of PBPs in membranes from *Bacteroides fragilis*, *Escherichia coli*, *Providencia rettgeri*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Enterococcus faecium* labeled with IPV or [^{3}H]penicillin G were virtually identical. Use of IPV as the imaging agent in competition experiments for determination of the affinities of various β-lactam antibiotics for the PBPs of *E. coli* yielded results similar to those obtained in experiments with [^{3}H]penicillin G. Dried electrophoretic gels from typical PBP experiments, using IPV at 37.3 Ci/mmol and 30 μg/ml, exposed X-ray film in 8 to 24 h. The stability of IPV on storage at 4°C was inversely proportional to specific activity. At 37.3 Ci/mmol and 60 μg/ml, IPV retained useful activity for at least 60 days at 4°C. IPV represents a practical and stable reagent for rapid PBP assays.

Investigations of the binding of β-lactam antibiotics to their subcellular targets, the penicillin-binding proteins (PBPs), in bacteria usually involve acylation of the PBPs with a radiolabeled penicillin. Although several different radioisotopes incorporated in a wide variety of penicillin structural analogs have been used in such experiments, radiolabeled benzylpenicillin is currently the reagent of choice. Detection of PBPs by labeling with [^{14}C]benzylpenicillin has the disadvantage of requiring 4 to 12 weeks to produce adequate exposure of X-ray films, even with fluorographic enhancement (5, 10). [^{3}H]penicillin G (HPG), prepared by the method of Rosegay (8), is a commonly used reagent for PBP experiments. Although this material reduces the minimum exposure time to 5 to 14 days (depending on the specific activity of the preparation), relatively high cost, chemical instability, and occasional lack of availability from commercial sources detract from its utility in the regular study of PBPs. A new labeling reagent for PBPs that is stable, inexpensive, readily available, high in specific activity, and equivalent to benzylpenicillin in detection of PBPs would be highly desirable. Masson and Labia (6) have described the synthesis of [^{125}I]penicillin X which has high specific activity and requires short exposures for autoradiography, but is highly unstable (5). Blaszczak et al. (2) have recently described the synthesis and chemical characterization of *p*-[^{125}I]phenoxymethylpenicillin (IPV), which seems to satisfy all of the above requirements. In this report, we describe the biological characterization of IPV as a tool for studying the interactions between bacterial PBPs and β-lactam antibiotics.

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MATERIALS AND METHODS

Chemical reagents. The *p*-(trimethylstannyl)phenoxymethylpenicillin was prepared in the Lilly Research Laboratories as described by Blaszczak et al. (2). Iododestannylation of the tin-substituted penicillin was performed by utilizing a variation of the standard chloramine-T method of Masson and Labia (6). Specific activities of the resulting IPV (Fig. 1) varied according to the ratio of [^{125}I]Na/NaI used in the reaction. For routine use, IPV was prepared with a specific activity of 37.3 Ci/mmol. HPG, 17 Ci/mmol, was purchased from Amersham Corp., Arlington Heights, Ill. Ampicillin, amdinocillin, cefotaxime, imipenem, and aztreonam were obtained from their respective manufacturers. Cephaloridine, cefuroxime, cephalexin, benzylpenicillin (penicillin G), and phenoxymethylpenicillin (penicillin V) were obtained from Eli Lilly & Co.

Preparation of bacterial membranes. Bacterial membranes for PBP studies were prepared from cultures grown to logarithmic phase at 35°C with shaking and harvested by centrifugation. The general procedure for the gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Enterococcus faecium*) was as follows. After two washings with 10 mM sodium phosphate buffer at pH 7.0, cells were suspended in the same buffer containing 100 μg of lysozyme per ml, 4 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and incubated at 35°C for 1.5 h. The cell suspension was subjected to five 2-min pulses of ultrasound in an ice bath. Unbroken cells were removed by low-speed centrifugation. Membrane material in the supernatant was centrifuged for 1 h at 135,000 × *g*, suspended in 50 mM sodium phosphate buffer at pH 7.0 at 12 mg of protein per ml, and stored at −70°C.

Membranes from the gram-negative bacteria (*Echerichia coli*, *Providencia rettgeri*, and *Bacteroides fragilis*) were

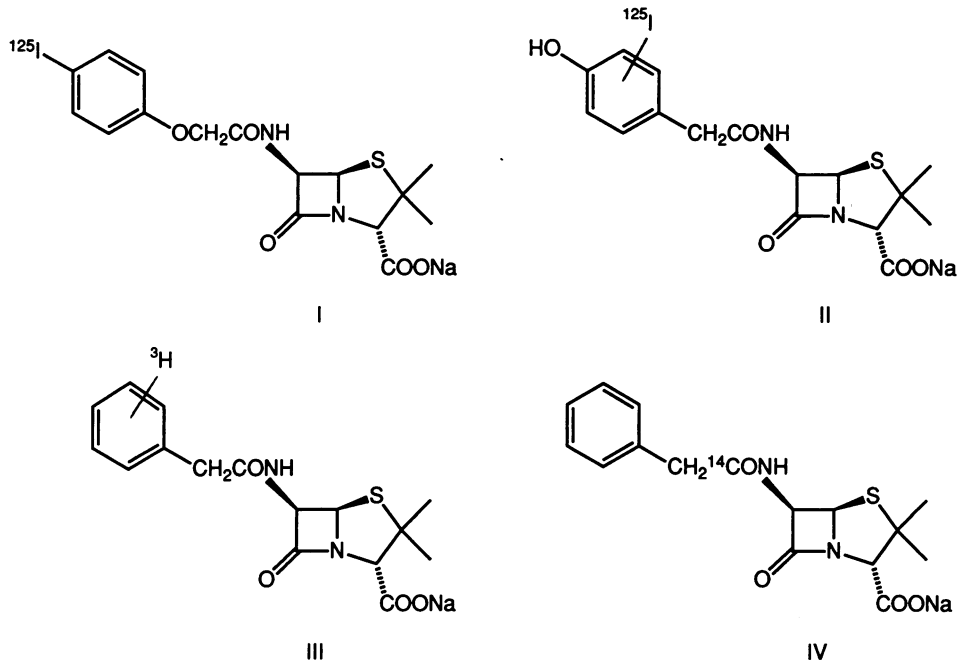

FIG. 1. Chemical structures of IPV (I), [^{125}I]penicillin X (II), HPG (III), and [^{14}C]penicillin G (IV).

prepared as follows. Washed cells were suspended in the same buffer containing 50 μg of DNase I per ml, 1 mM phenylmethylsulfonyl fluoride, and 0.14 mM 2-mercaptoethanol and broken by passing three times through a French press at 16,000 lb/in^2 (SLM Aminco, Urbana, Ill.). After centrifugation to remove unbroken bacterial cells, supernatants were loaded onto a discontinuous sucrose gradient composed of 9 ml of 58% sucrose overlaid with 9 ml of 52% sucrose in 10 mM sodium phosphate buffer, pH 7.0, and centrifuged for 18 h at 135,000 × *g*. The 52% sucrose fraction containing the inner membrane was collected and centrifuged at 135,000 × *g* for 1 h. Membrane preparations suspended in 50 mM sodium phosphate buffer, pH 7.0, at 12 mg of protein per ml were stored at −70°C.

Labeling of PBPs. Visualization of PBPs by labeling with IPV was performed by a method similar to that used by Spratt (10). A 6-μl volume of membrane preparation was incubated with 6 μl of IPV (40 μg/ml or appropriate dilution thereof) for 10 min at 30°C. The binding reaction was quenched by the addition of 12 μl of blue mix (120 mg of cold IPV per ml–20% sarcosyl–polyacrylamide gel electrophoresis sample buffer, 1:2:12). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 8% acrylamide–0.21% *N,N'*-methylene-bisacrylamide. Dried gels were exposed to Kodak X-OMAT film without fluorographic enhancement at 24°C for 8 to 24 h. Gels from experiments with HPG required fluorographic enhancement and 1 to 2 weeks in contact with preflashed X-OMAT film below −70°C. Competition experiments designed to reveal the PBP binding affinities of nonlabeled β-lactam compounds utilized the same procedures with the addition of a 10-min pre-exposure of the bacterial membrane preparation to 2-μl samples of the competitor compound at various concentrations prior to the addition of IPV.

Chemical stability of IPV. Stock solutions of IPV at 74.6, 37.3, and 18.6 Ci/mmol and nonradioactive IPV stored at 4°C were assayed for antibiotic potency at regular intervals over a period of 38 days. A microbiological diffusion assay, using Penassay seed agar (Difco Laboratories, Detroit, Mich.) containing *Staphylococcus aureus* (FDA 209P) at approximately 10^6 CFU/ml, was used to monitor the loss of antibacterial activity of the test compounds.

RESULTS

Stability. The rate of loss of antibiotic activity from stock solutions of IPV stored at 4°C is dependent on the specific activity of the radiolabeled compound (Fig. 2). At a specific activity of 37.3 Ci/mmol at 122 μg/ml in 50 mM sodium phosphate buffer at pH 7.0, approximately half of the antibiotic activity of IPV was lost in 17 days. Use of IPV stored

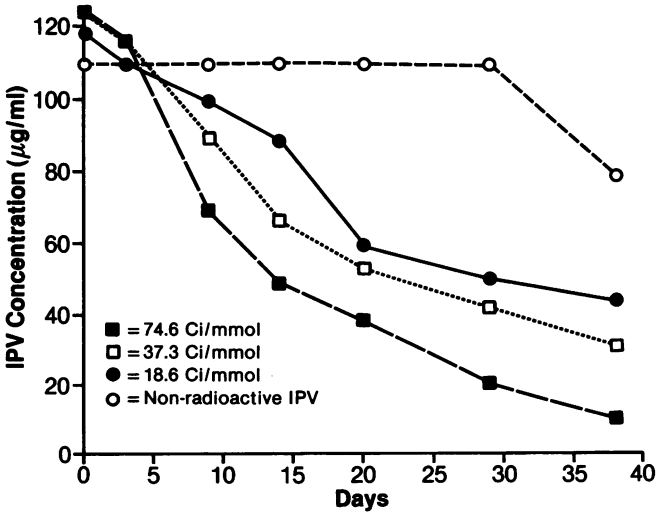

FIG. 2. Stability of IPV. Stock solutions of IPV at indicated specific activity, stored at 4°C, were assayed for antibiotic potency at intervals over a period of 38 days.

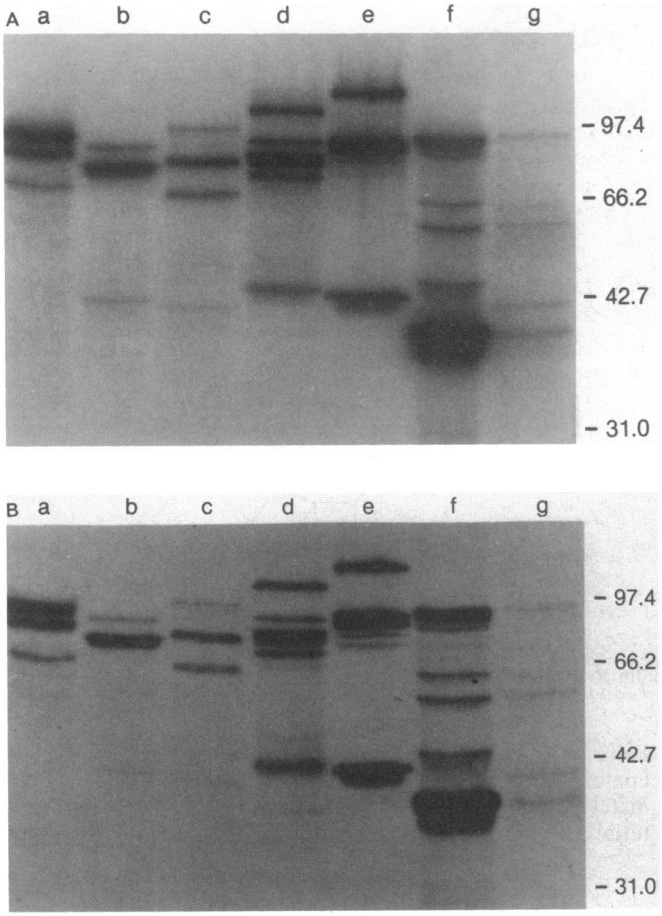

FIG. 3. Labeling of PBPs by IPV and HPG. (A) Autoradiograph of IPV-labeled PBPs developed after 16 h of exposure at room temperature. (B) Fluorograph of HPG-labeled PBPs developed after 10 days of exposure at −70°C. The gel was treated with EnHance. The film was preflashed. Lanes in each film contain images of the PBPs from *B. fragilis* (a), *Staphylococcus aureus* (b), *Streptococcus pyogenes* (c), *Enterococcus faecalis* (d), *Enterococcus faecium* (e), *Escherichia coli* (f), and *P. rettgeri* (g).

at 4°C for times exceeding its half-life resulted in slightly increased background fogging of the X-ray film and the necessity for proportionately longer exposure times.

PBP binding studies. Binding of IPV to the PBPs of *B. fragilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, and *P. rettgeri* was similar to that seen with HPG. In parallel experiments with membranes from the indicated organisms, IPV and HPG detected the same PBPs (Fig. 3A and B).

IPV and HPG saturated the various PBPs of *Escherichia coli* at similar concentrations. PBPs 5 and 6 are saturated by 10 μg of either reagent per ml. The other PBPs, although readily detectable with 10 μg/ml, required 20 μg/ml for saturation by either agent.

IPV at 20 μg/ml saturated the PBPs of *Escherichia coli* in 3 to 6 min. Study of the spontaneous rate of release of bound IPV indicated that little or no IPV was released from PBPs 1 to 4 in 60 min. By contrast, PBPs 5 and 6 released approximately 50% of the bound IPV in 60 min. These results are qualitatively similar to those with HPG.

One of the uses for radiolabeled penicillins is determination of the interaction between bacterial PBPs and various nonradioactive β-lactam compounds in competition experiments. We have evaluated the utility of IPV in such experiments by comparing it directly with HPG. Table 1 contains 50% inhibitory concentrations (I_{50}) for several β-lactam antibiotics measured for ability to compete with IPV or HPG for the PBPs of *Escherichia coli* K-12. Also included for comparison are the analogous data from several published studies that used [^{14}C]benzylpenicillin. We found the I_{50}s determined with IPV or HPG in experiments with the same batch of bacterial membranes to be quite similar to each other and, in most cases, also similar to those reported by other investigators.

DISCUSSION

The data presented here suggest that IPV is a highly useful radiolabeling reagent for the study of bacterial PBPs. Its chemical precursor, *p*-(trimethylstannyl)penicillin V, is a stable solid that is easily manipulated (2). Small aliquots of the precursor can be iodinated easily and inexpensively to produce IPV in quantities sufficient for use over a period of 4 to 6 weeks, after which the process can be repeated.

The stability of IPV in solution at 4°C varies inversely with the specific activity of the radiolabel in the reagent. We selected 37 Ci/mmol as the optimal specific activity for minimizing radioautographic exposure times (8 to 24 h) and maximizing stability on storage (4 to 6 weeks). By contrast, Masson and Labia (6) recommend use of [^{125}I]penicillin X solution (2 Ci/mmol) immediately after preparation due to the high rate of radiolysis (half-life, <48 h).

One of the drawbacks associated with the use of radiolabeled β-lactams other than benzylpenicillin as imaging reagents for bacterial PBPs is the likelihood of differences between benzylpenicillin and the alternative compounds in their recognition of proteins in the bacterial membranes (1, 7, 9). In direct comparisons between HPG and IPV in acylation of membrane proteins in representative strains of gram-positive and gram-negative species, we saw no protein saturated by one reagent that was not also saturated by the other within reasonable ranges of concentrations. We also showed that the kinetics of acylation of (and dissociation from) the PBPs by IPV are similar to those of benzylpenicillin.

The similarities between IPV and benzylpenicillin in their interactions with bacterial PBPs suggested that IPV could be used in competition experiments for the determination of the binding characteristics of nonlabeled β-lactam compounds to those proteins. In each of the antibiotic-bacterium combinations we tested, we found close agreement between I_{50}s determined with the two reagents.

IPV offers several distinct advantages over the other reagents in the preparation, handling, and use of chemically sensitive radiolabeled β-lactam molecules: (i) the stannylated precursor of IPV is stable indefinitely in the dry state; (ii) the labeling procedure may be performed in the last synthetic step, under extremely mild conditions, and with a very high degree of site selectivity, thereby obviating the need for any purification of radioactive material; (iii) the approach promises to be quite general with respect to radiolabel and the β-lactam molecule; and (iv) the radiochemical lifetime of IPV approaches the half-life of the ^{125}I-labeled nuclide (60 days). Finally, a typical competition experiment with IPV may be performed for <1% of the cost of HPG and requires only 10% of the film exposure time.

TABLE 1. Comparison of I_{50}s for PBPs from *Escherichia coli* K-12 determined with three different labeling agents

Compound	Label	I_{50} (μg/ml) of β-lactam compounds for PBPs						Reference
		1a	1bs	2	3	4	5/6	
Penicillin G	IPV	0.6	6	2.5	0.9	0.5	20	
	HPG	1.2	5	2.5	1.2	0.6	20	
	CPG[a]	0.5	3	0.8	0.9	1	24	3
Penicillin V	IPV	0.6	2	3.5	2	0.6	40	
	HPG	0.6	2.5	2.5	2	0.6	40	
	CPG	1.4	9.5	7.7	10.5	14	50	3
Ampicillin	IPV	1.2	5	0.9	0.6	0.2	72	
	CPG	1.4	3.9	0.7	0.9	2	140	3
Amdinocillin	IPV	>250	>250	<0.003	>250	>250	>250	
	HPG	>250	>250	<0.003	>250	>250	>250	
	CPG	>250	>250	<0.25	>250	>250	>250	3
Aztreonam	IPV	>1	>1	>1	0.16	>1	>1	
	HPG	10	100	100	0.1	100	100	11
Cephaloridine	IPV	0.15	4	40	8	4	>109	
	HPG	0.3	4	36	8	4	>109	
	CPG	0.25	2.5	50	8	17	>250	3
Cephalexin	IPV	3	256	>256	8	32	>256	
	CPG	4	240	>250	8	30	>250	3
Cefuroxime	IPV	0.12	1.3	4	0.1	1	>36	
	HPG	0.15	2.7	8	0.1	12	>36	
	CPG	0.12	1.6	13.7	0.09	200	>250	3
Cefotaxime	IPV	0.15	0.7	8	<0.05	36	>50	
	CPG	0.05	0.7	5	<0.05	30	>50	3
Imipenem	IPV	0.2	1	<0.03	10	<0.03	0.7	
	CPG	0.2	0.6	<0.1	9.8	<0.1	0.3	4

[a] CPG, [^{14}C]benzylpenicillin.

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