THE AFFINITY OF IMIPENEM (*N*-FORMIMIDOYLTHIENAMYCIN) FOR THE PENICILLIN-BINDING PROTEINS OF *STAPHYLOCOCCUS AUREUS* —BINDING AND RELEASE—

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Penicillin-binding proteins 1, 2 and 3 in *Staphylococcus aureus* were found to possess common properties. All have very strong affinities for both benzylpenicillin and imipenem (*N*-formimidoylthienamycin), and all have an activity which releases bound imipenem, but not bound benzylpenicillin. Lower molecular weight penicillin-binding protein 4, which has a rather weak affinity for benzylpenicillin and also weak penicillinase activity showed an extraordinarily high affinity for imipenem but no antibiotic-releasing activity.

Four major penicillin-binding proteins (PBPs), PBP-1 to 4, have been isolated from *Staphylococcus aureus*[1]. They presumably function in the biosynthesis of peptidoglycan during the process of cell-duplication and to be the killing site of β-lactams in this bacterium. However, no evidence for these assumptions has yet been provided.** Among the four PBPs, the importance of PBP-3 has been suggested by the isolation of a mutant that is more resistant to methicillin and contains PBP-3 with an altered affinity for this β-lactam[2]. The defect of PBP-1 has been reported not to be lethal for the cell[3,4]. On the other hand, there has been the observation that alteration of all the three higher molecular weight proteins, PBPs-1, 2 and 3, occurred simultaneously with a mutation to methicillin resistance[5]. Probably, these three proteins have similar functions and the lack of one of them may be compensated for by the other. PBP-4, the smallest among the staphylococcal PBPs, has been reported previously to have DD-alanine carboxypeptidase and penicillinase activities[6]. A defect in PBP-4 was also shown not to be lethal for the cell, but it caused formation of a thinner than normal cell wall[4,7].

We previously studied affinities of PBPs in *Escherichia coli* and *Pseudomonas aeruginosa* for imipenem (*N*-formimidoylthienamycin)[8,9] and benzylpenicillin[10]. In this communication we report similarities among the three higher molecular weight PBPs-1, 2 and 3 of *S. aureus*, in binding imipenem and benzylpenicillin, and in possessing the property to release imipenem, but not benzylpenicillin. Affinity and resistance of PBP-4 for imipenem is also reported.

** In *Escherichia coli*, higer molecular weight PBPs-1A, 1B, 3 and probably also PBP-2 possess two enzymatic activities of peptidoglycan synthesis, *i.e.*, peptidoglycan transglycosylase activity which extends the glycan chain, and β-lactam sensitive transpeptidase activity which crosslinks it[6,17~19]. These PBPs are supposed to have functions specific for the process of cellular duplication[13]. On the other hand, in *S. aureus* major peptidoglycan transglycosylase activity is in fractions different from PBPs and these PBPs possibly have transpeptidase activities only[12].

Materials and Methods

Antibiotics

Imipenem was supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J., USA. Benzylpenicillin potassium salt was obtained from Takeda Chemical Industries Co., Ltd., Osaka, Japan, and [^{14}C]benzylpenicillin potassium salt (60 Ci/mol) from the Radiochemical Centre, Amersham, England.

Bacterial Strains and Preparation of Membranes

S. aureus strain SAK132[11], derived from strain Copenhagen, and strain FDA 209P were used in the experiments in this report. Both strains possess PBPs-1 to 4 but strain SAK132 sometimes showed an additional PBP band (PBP-3′) between PBPs 3 and 4 on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Membrane fractions of *S. aureus* strains were prepared by grinding the log phase cells with alumina followed by differential centrifugation as described previously[12].

Assay of Penicillin-binding Activity of PBPs and Binding Affinity of Imipenem

Assay of PBP by binding of [^{14}C]benzylpenicillin at a low concentration (3 μM) of [^{14}C]benzylpenicillin, and separation on sodium dodecyl sulfate/polyacrylamide gel electrophoresis was as described for *E. coli*[13], except that the step of Sarkosyl treatment for precipitation of the outer membrane was omitted[9].

Binding of imipenem was measured by the competition assay, because no radiolabeled compound was available. In this method, membranes were incubated with the unlabeled antibiotic at various concentrations for an appropriate time (10 minutes) at 30°C to allow binding to PBPs and then an excess of [^{14}C]benzylpenicillin was added to label the rest of the free PBPs. Concentrations of the unalabled antibiotic for 50% inhibition of the binding of [^{14}C]benzylpenicillin to PBPs were estimated.

Results and Discussion

Rate of Binding of [^{14}C]Benzylpenicillin to *S. aureus* PBPs and Heat-sensitivity

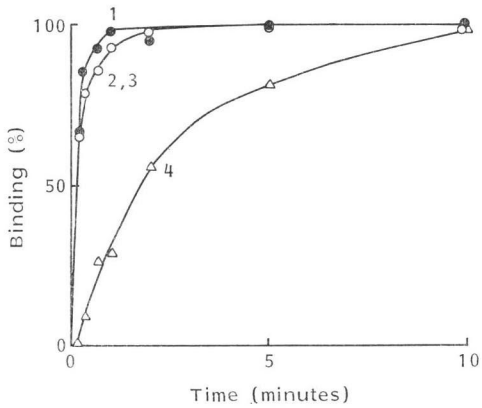
Results are shown in Fig. 1. The rates of binding of [^{14}C]benzylpenicillin to *S. aureus* PBPs-1, 2 and 3 were very high. About 50% saturation of the proteins by [^{14}C]benzylpenicillin was obtained by incubation at 30°C for several seconds, and about 100% at 2 minutes. These rates of binding of [^{14}C]-benzylpenicillin observed for *S. aureus* PBPs-1, 2 and 3 were about 50 to 2,000 times higher than that observed with higher molecular weight PBPs of *E. coli*[14] or *P. aeruginosa*[15]. Since PBPs are supposed to be the killing targets of penicillin, the very high rate of binding of [^{14}C]benzylpenicillin may elucidate the reason for the high killing potency of penicillin for *S. aureus*. In contrast to the higher molecular weight PBPs in *S. aureus*, the lower molecular weight PBP-4 showed a moderate rate of binding of [^{14}C]benzylpenicillin. In the above experiment, PBPs-2 and 3 were located too close together so that the quantitative estimation of the binding of [^{14}C]benzylpenicillin had to be performed for the combined radioactivities of PBPs-2 and 3, but they apparently behaved in a similar manner as seen on a fluorogram. PBP-1 showed slightly faster binding to [^{14}C]benzylpenicillin than PBPs-2 and 3. A more significant difference between PBP-1 and PBPs-2 and 3 was its greater heat sensitivity as shown in Fig. 2. PBP-1 was the most heat labile of the four staphylococcal PBPs, PBP-4 was intermediate, and PBPs-2 and 3 were the most stable.

Affinities of Imipenem and Benzylpenicillin for *S. aureus* PBPs

The results shown in Table 1 indicate that *S. aureus* PBPs-1, 2 and 3 (measured together) have

Fig. 1. Time course of the binding of [^{14}C]benzylpenicillin to staphylococcal PBPs.

Membranes (particulate membrane enzymes) were obtained from *S. aureus* strain SAK132 by grinding with alumina followed by extraction with 0.01 M sodium phosphate buffer, pH 7.0, differential centrifugation between 5,000 (10 minutes) and 100,000×*g* (30 minutes), washing once with the same buffer solution, and suspending in 0.05 M sodium phosphate buffer, pH 7.0 (22.8 mg protein/ml). The binding experiment was carried out with 228 μg membranes (as protein) and 3 μM [^{14}C]-benzylpenicillin in a total volume of 30°C[15]. The radioactivity bound to the proteins was measured by cutting the corresponding portions of the dried gel and counting in a liquid scintillation counter using toluene-PPO-POPOP system. Numbers indicate *S. aureus* PBPs.



very strong binding affinities for both imipenem and benzylpenicillin. The binding affinity of imipenem for PBP-4 was very high; its 50% saturation concentration was as low as about 0.01 μg/ml. This low saturation concentration of imipenem may probably be due to the resistance of imipenem to penicillinase activity of PBP-4[10]. The results reflect the susceptibility of strains of *S. aureus* to both antibiotics. The minimum inhibitory concentrations of imipenem and benzylpenicillin, determined by an agar dilution method as described previously[8], were 0.013 and 0.025 μg/ml against strain FDA 209P, and <0.013 and 0.013 μg/ml against strain SAK132, respectively.

Fig. 2. Heat sensitivity of *S. aureus* PBPs.

The membrane suspension (340 μg protein in 30 μl 0.05 M sodium phosphate buffer, pH 7.0; for the preparation see the legend to Fig. 1) was incubated for 10 minutes at various temperatures as indicated. Then, 100 μM [^{14}C]benzylpenicillin was added and the binding experiment was carried out for 10 minutes at 30°C. The radioactivity bound to the proteins was measured as described in the legend to Fig. 1. Numbers indicate *S. aureus* PBPs.

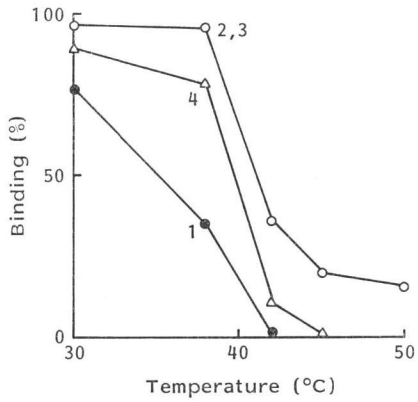


Table 1. Affinities of imipenem and benzylpenicillin for *S. aureus* PBPs as measured by the competition method with [^{14}C]benzylpenicillin*.

PBP	50% saturation concentration**		
	Imipenem (A)	Benzyl-penicillin (B)	A/B
1	0.06	0.03	2.0
2/3	0.09	0.07	1.3
4	0.01	>0.14	<0.07

* The membrane preparations (400 μg protein) of *S. aureus* FDA 209P were mixed with imipenem at 0.02 to 0.58 μg/ml or unlabeled benzylpenicillin at 0.03 to 0.14 μg/ml and incubated for 30°C for 10 minutes. After addition of an excess of [^{14}C]benzylpenicillin mixtures were incubated for an additional 10 minutes at 30°C. Remaining [^{14}C]benzylpenicillin-protein complexes were separated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis and were detected by fluorography. The levels of [^{14}C]benzylpenicillin-protein complexes were measured by a densitometric tracing method.

** Concentration in μg/ml for the 50% inhibition of the binding of [^{14}C]benzylpenicillin to each protein.

Fig. 3. Removal of imipenem from PBPs of *S. aureus*.

The membrane suspension of *S. aureus* strain FDA 209P (400 μg protein in a final volume of 30 μl of 0.05 M sodium phosphate buffer, pH 7.0) was mixed with 0 (A), 0.6 (B), 2.9 (C) and 14.4 (D) μg/ml of imipenem for 10 minutes at 30°C and then was incubated with an excess of [^{14}C]benzylpenicillin (final 100 μM). The [^{14}C]benzylpenicillin-protein complexes were separated by sodium dodecylsulfate/polyacrylamide gel electrophoresis and were detected by fluorography.

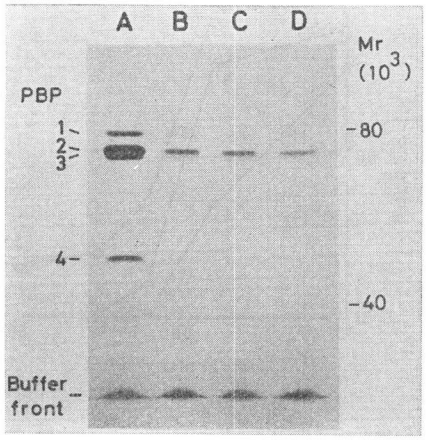
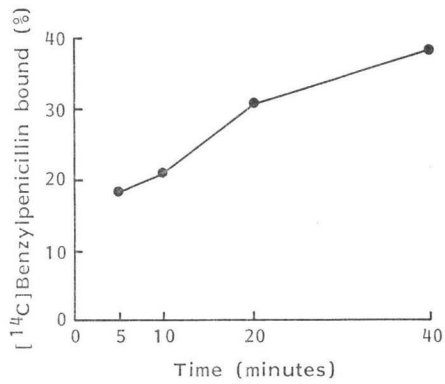


Fig. 4. Incorporation of [^{14}C]benzylpenicillin to the PBPs-1 to 3 of *S. aureus* caused by the removal of bound imipenem from these PBPs.

The membrane preparation of *S. aureus* strain FDA 209P (400 μg protein in a final volume of 30 μl of 0.05 M sodium phosphate buffer, pH 7.0) was mixed with 2.9 μg/ml of imipenem. After incubation for 10 minutes at 30°C, a 10 fold excess amount of [^{14}C]benzylpenicillin was added to the reaction mixture and the mixture was further incubated at 30°C for times indicated. Detection and determination of the radioactivity in the PBPs were described in the legend to Fig. 1. Ordinate shows incorporation of [^{14}C]benzylpenicillin in % of the incorporation without prebinding of the PBPs with imipenem.



Release of Bound Imipenem from *S. aureus* PBPs-1, 2 and 3, and Resistance of Imipenem to the β-Lactamase Activity of PBP-4

A peculiar characteristic of staphylococcal PBPs-1, 2 and 3 is their ability to release bound imipenem. Incubation with a large excess of [^{14}C]benzylpenicillin released bound imipenem from PBPs-1, 2 and 3 and they became saturated with [^{14}C]benzylpenicillin. In the experiment shown in Fig. 3, *S. aureus* membranes were previously treated with 0 (A), 0.6 (B), 2.9 (C) and 14.4 (D) μg per ml of unlabeled imipenem for 10 minutes at 30°C, and then further incubated with an excess of [^{14}C]benzylpenicillin. Slight radioactivity appeared at the position of PBPs-1, 2 and 3 upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis, in a proportion similar to those of the control (A). The amount of [^{14}C]benzylpenicillin that appeared at the position of PBPs-1, 2 and 3 was almost independent of the amount of the unlabeled imipenem present during the preincubation. This result may indicate that the unlabeled imipenem at the above concentrations saturated these PBPs during the preincubation period and then was replaced partially by [^{14}C]benzylpenicillin during the incubation with an excess of the latter compound. The time course of the release of unlabeled imipenem from PBPs-1, 2 and 3 was measured as shown in Fig. 4. [^{14}C]Benzylpenicillin gradually bound to PBPs-1, 2 and 3 at much slower rates than its binding to the PBPs untreated with imipenem (see Fig. 1), this being due to the release of antibiotic from the PBPs. About 37% of the bound antibiotic was removed from the three PBPs by incubation for 40 minutes at 30°C in the presence of an excess amount of [^{14}C]benzylpenicillin. On the contrary, in a similar experiment in which PBPs were first treated with unlabele

benzylpenicillin and then with excess [^{14}C]benzylpenicillin, no release of antibiotic from PBPs-1, 2 and 3 could be observed as judged by binding of [^{14}C]benzylpenicillin to these PBPs.

PBP-4, which possesses penicillinase activity[16] did not show any sign of releasing bound imipenem.

PBPs-1, 2 and 3 have a releasing activity with specificity for imipenem whereas the β-lactamase activity of PBP-4[6] has no activity to imipenem. Study of the product of imipenem released from PBPs-1, 2 and 3 has not yet been carried out.

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