Penicillin-Binding Protein 2a from Methicillin-Resistant *Staphylococcus aureus*: Kinetic Characterization of Its Interactions with β-Lactams Using Electrospray Mass Spectrometry

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ABSTRACT: Penicillin-binding protein 2a (PBP2a) is the primary β-lactam resistance determinant of methicillin-resistant *Staphylococcus aureus* (MRSA). *Mec*A, the gene coding for PBP2a, was cloned with the membrane-anchoring region at the N-terminus deleted. The truncated protein (PBP2a*) was overexpressed in *Escherichia coli* mostly in the soluble form accounting for ∼25% of soluble cell protein and was purified to homogeneity. The purified protein was shown to covalently bind β-lactams in an 1:1 ratio as determined by electrospray mass spectrometry. A novel method based on HPLC-elctrospray mass spectrometry has been developed to quantitatively determine the formation of the covalent adducts or acyl-PBP2a* complexes. By using this method, combined with kinetic techniques including quench flow, we have extensively characterized the interactions between PBP2a* and three β-lactams and determined related kinetic parameters for the first time. The apparent first-order rate constants (k_a) of PBP2a* acylation by benzylpenicillin showed a hyperbolic dependence on the concentration of benzylpenicillin. This is consistent with the mechanism that the binding of the penicillin to PBP2a* consists of reversible formation of a Michaelis complex followed by formation of the penicilloyl-PBP2a* adduct, and allowed the determination of the individual kinetic parameters for these two steps, the dissociation constant K_d of 13.3 mM and the first-order rate constant k_2 of 0.22 s^{-1}. From these values, the second-order rate constant k_2/K_d, the value reflecting the overall binding efficiency of a β-lactam, of 16.5 M^{-1} s^{-1} was obtained. The fairly high K_d value indicates that benzylpenicillin fits rather poorly into the protein active site. Similar studies on the interaction between PBP2a* and methicillin revealed k_2 of 0.0083 s^{-1} and K_d of 16.9 mM, resulting in an even smaller k_2/K_d value of 0.49 M^{-1} s^{-1}. The rate constants k_3 for deacylation of the acyl-PBP2a* complexes, the third step in the interactions, were measured to be $<1.5 \times 10^{-5}$ s^{-1}. These results indicate that the resistance of PBP2a to penicillin inactivation is mainly due to the extremely low penicillin acylating rate in addition to the low association affinity, but not to a fast rate of deacylation. Acylation of PBP2a* by a high-affinity cephalosporin, Compound 1, also followed a saturation curve of k_a versus the compound concentration, from which $k_2 = 0.39$ s^{-1}, $K_d = 0.22$ mM, and $k_2/K_d = 1750$ M^{-1} s^{-1} were obtained. The 100-fold increase in the k_2/K_d value as compared with that of benzylpenicillin is mostly attributable to the decreased (60-fold) K_d, indicating that the cephalosporin fits much better to the binding pocket of the protein.

High molecular mass (HMM) penicillin-binding proteins (PBPs)[1] are the enzymes responsible for the final stages of bacterial peptidoglycan synthesis and are the targets of β-lactam antibiotics (*1*). β-lactam antibiotics exert their inhibitory activity by acylation of nucleophilic serine residues at the active sites of HMM PBPs (*2*). The β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) is primarily due to the production of a unique HMM PBP, PBP2a (PBP2′), which has low affinity for all of the currently marketed β-lactams and can apparently take over the enzymatic functions of peptidoglycan synthesis under conditions when the normal staphylococcal HMM PBPs are inactivated by a β-lactam (*2*). The latter conclusion is supported by several lines of genetic and biochemical evidence (*3*−*5*). PBP2a has been utilized by a number of pharmaceutical companies as a target to discover new β-lactam antibiotics with enhanced affinity to the protein and thus with potent activity against MRSA, and several such β-lactams have been reported (*6*). *Mec*A, the gene coding for PBP2a, has been cloned and sequenced (*7*−*9*). Recombinant PBP2a has been expressed and purified (*10*−*12*). Direct evidence that the serine of S403XXK motif of PBP2a is the β-lactam-binding site has been presented on the basis

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[1] Abbreviations: PBP, penicillin-binding protein; PBP2a*, recombinant, soluble PBP2a from MRSA; MRSA, methicillin-resistant *Staphylococcus aureus*; ESMS, electrospray ionization mass spectrometry; pcHPLC, perfusion capillary high-performance liquid chromatography; PAGE, polyacryalmide gel electrophresis; IC_{50}, inhibitor (β-lactam) concentration at which a half amount of PBP is acylated by the inhibitor; MIC, minimal bacterial inhibition concentration.

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of peptide mapping of penicilloyl-PBP2a* by electrospray mass spectrometry (*13*).

There have been relatively few purified PBPs whose interactions with β-lactams have been extensively characterized. R61 DD-carboxypeptidases, a low molecular mass, nonessential PBP from *Streptomyces* strain R61, were studied by following the quenching of the enzyme's intrinsic fluorescence by β-lactams (*14*). A three-step reaction mechanism was proposed for β-lactams such as benzylpenicillin, and the values of the first-order rate constants (k_2) and the dissociation constants (K_d) were determined. A similar study was carried out with HMM PBP2x from *Streptococcus pneumoniae* by measuring β-lactam quenching of the intrinsic fluorescence of the PBP (*15*). The values of k_2/K_d were derived, but the individual parameters were not resolved.

Characterization of the interactions between PBP2a and β-lactams may shed light on the mechanism of the protein's extremely low affinity toward marketed β-lactam antibiotics. The information may help design better PBP2a inhibitors, which could lead to novel, more effective antibacterial agents against MRSA and other bacteria. The binding of β-lactams to purified PBP2a has been studied (*12*, *16*). Very recently, a more detailed kinetic study of PBP2a interactions with β-lactams has been reported, in which the values of k_2/K_d were determined for β-lactams including benzylpenicillin using spectroscopic and indirect methods (*17*). But the mechanism of such interactions has not been elucidated, and the individual kinetic parameters such as k_2 and K_d have not been determined. In the present studies, we developed a novel method of electrospray ionization mass spectrometry to directly and quantitatively determine a PBP and acyl-PBP adducts. With this method, we extensively characterized the kinetic mechanism of the interactions between PBP2a* and several β-lactams including benzylpenicillin and determined, for the first time, the related microscopic parameters, K_d, k_2, and k_3. We also report here new and improved procedures developed in the studies to clone, overexpress, and purify the recombinant, soluble PBP2a*.

EXPERIMENTAL PROCEDURES

Cloning of a Truncated mecA Gene by PCR and Construction of an Expression Vector. Chromosomal DNA of a MRSA clinical isolate (strain MI339) obtained from Public Health Research Institute of New York City was prepared as described (*18*) and used as the template for PCR. Based on the published *mec*A sequence (*8*), a forward primer 5′-GGGTTTGGTATATATT*CCATGG*CTTCAAAAGATAAAG-3′ with a *Nco*I restriction site (italic) and a backward primer 5′-TATC*AAGCTT*CTGC TTTTGTTATTCATCTATATC-3′ corresponding to the C-terminus of *mec*A with a *Hind*III restriction site (italic) added were used to amplify the gene by PCR. In the forward primer the *Nco*I restriction site (italic) was introduced before codon Ala24, resulting in the *mec*A gene coding for a truncated PBP2a, of which the membrane-spanning segment consisting of the first 23 amino acids at the N-terminus was deleted (*10*). The PCR reaction mixture contained 2 μM each of the primers, 2.5 units of TaqDNA polymerase (Perkin-Elmer), 0.2 mM each dNTP, 10 mMTris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and the template (10 μL) in a final volume of 100 μL, and was subjected to 25 cycles of PCR amplification (94 °C, 0.5 min; 45 °C, 0.5 min; 72 °C, 1 min). The resultant PCR fragment was digested with *Nco*I and *Hind*III and fractionated on a 1% agarose gel. A distinct 2 kb band was excised and ligated using T4 ligase into PET 21d (Novagen) which had been digested with the same restriction enzymes. The *mec*A in the resultant expression vector (pTMCA) was sequenced in-house and by the Biotechnology facility, University of Illinois, Champaigne-Urbana.

Expression of PBP2a in Escherichia coli.* The plasmid pTMCA was transformed into *E. coli* expression strain BL21/DE3 (Novagen) following the manufacture's protocol. The conditions for culture growth and induction were basically according to the instruction of Novagen. LB medium containing 100 μg/mL ampicillin was inoculated with an inoculum culture, and the cells were grown in a shaker at 37 °C until the OD_{600} reached ~1. The temperature of the culture was then rapidly decreased to ~30 °C, and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added into the culture to induce expression. Three hours later the culture was harvested by centrifugation at 10000*g* for 10 min at 4 °C. The cells were then washed once and suspended in 50 mM Tris/HCl buffer, pH 8. For large scale culture, six four-liter flasks each containing 2 L of medium were used.

Purification of the Recombinant PBP2a to Homogeneity.* A three-step purification protocol was developed, which offers the advantages of large scale, high yield, and efficiency. All operations were performed on an FPLC purification system (Pharmacia) at 4 °C. Cell-free extract was prepared by sonication of the cells for ~10 min at maximum output using a 550 Sonic Dismembrator (Fisher) followed by centrifugation at 25000*g* for 45 min. The resultant cell-free extract (1000−2000 mg of protein) was loaded onto a Q-Sepharose HP (Pharmacia) column (5 × 9 cm) at 10 mL/min, which had been equilibrated with Buffer A (10 mM Tris/HCl buffer, pH 8). The column was then washed with ~200 mL of Buffer B (10 mM Tris/HCl, pH 8.9) followed by elution with a linear gradient of 0−0.3 M NaCl in Buffer B at 9 mL/min. PBP2a* eluted at ~0.15 M NaCl as judged by SDS−PAGE. The fractions (0.15−0.2 M NaCl) containing PBP2a* were combined. The combined solution was brought into 1.5 M $(NH_4)_2SO_4$ and loaded onto a Phenyl-Sepharose column (2.6 × 6 cm), which had been equilibrated with 1.5 M $(NH_4)_2SO_4$ in Buffer A. The column was then eluted with a linear gradient of 1.5−0.5 M $(NH_4)_2SO_4$ in Buffer A. PBP2a*, bound to the resin very weakly, eluted between 1 and 1.3 M $(NH_4)_2SO_4$. The protein was pooled and concentrated to ~50 mg/mL with Amicon Centriprep (cut off 30 kDa) tubes or an Amicon centrifiltration cell. The resultant protein preparation was loaded on a Superdex 75 column (2.6 × 75 cm, Pharmacia), which had been equilibrated with Buffer A containing 0.2 M NaCl, and then eluted with the same buffer at 1.0 mL/min. The fractions at ~135−145 mL containing homogeneous PBP2a* were pooled and concentrated.

Determination of Molecular Mass of PBP2a and Its Amino Acid Sequence through Peptide Mapping.* These were done by either microbore high-performance liquid chromatography-electrospray mass spectrometry (microbore HPLC-ESMS) (*13*) or perfusion capillary HPLC-ESMS as detailed below. The N-terminal sequence was analyzed by the standard Edman degradation method.

Preparation of Samples for Measurement of PBP2a Acylation by Electrospray Mass Spectrometry*. For kinetic studies of the binding of a β-lactam to PBP2a*, a typical reaction mixture contained ~10−15 μg of PBP2a* and the β-lactam in a final volume of 20 μL of 10 mM phosphate buffer, pH 7.2, containing 50 mM NaCl (Buffer C). The reaction was carried out at 37 °C and terminated at various time intervals by adding 5 μL of 5% HCOOH before the sample was analyzed by electrospray mass spectrometry (pcHPLC-ESMS, described below). When high concentrations (>2 mM) of a β-lactam were used, a larger volume (~100 μL) of 1% HCOOH was used to stop the reaction and to dilute the β-lactam. The mixture was then transferred into an Amicon microcon 30 tube and spun at ~10000*g* for ~5 min at room temperature to concentrate the protein sample and to remove the excessive β-lactam which interfered with the subsequent electrospray mass spectrometry measurement. After the recovery spin the protein solution was collected and its volume was adjusted to ~25 μL with 1% HCOOH before analysis by pcHPLC-ESMS.

Rapid Quench Experiments. At benzylpenicillin concentrations of 2 mM or above, the rate constants of its binding to PBP2a* reached ~0.02 s^{-1} or higher, and the reactions of initial data points had to be terminated within 15 s. To ensure the accuracy of the reaction time, we performed rapid quench experiments. These were carried out in a quench flow apparatus built by KinTek Instruments (University Park, PA) (*19)*. Typically, PBP2a* (25 μg in 20 μL of Buffer C) and benzylpenicillin (various concentrations in 20 μL of Buffer C) were loaded in the two tubing loops. The reactions were started by rapidly mixing the two reactants from the two tubing loops and then quenched with 3% HCOOH (final concentration) after time intervals ranging from 0.5 to 60 s. The collected reaction mixtures (~150 μL) were then concentrated in Amicon microcon 30 tubes as described above before pcHPLC-ESMS analysis.

Preparation of Samples for Determination of acyl-PBP2a Deacylation*. To study the deacylation reactions, we prepared acyl-PBP2a* samples by incubating PBP2a* (~10 mg/mL) with 0.5 mM benzylpenicillin for ~3 min, with 5 mM methicillin for 30 min, or with 50 μM Compound 1 for 1 min at 37 °C followed immediately by centrifugation/washing three times with 3 × 1 mL Buffer C in Amicon microcon 30 tubes to remove the free β-lactams. The centrifugation was performed at 11000*g* for ~15 min each time at 15 °C. The centrifugation/washing step lasted ~40 min. The resulting PBP2a* preparations with at least 85% of acylation, as determined by pcHPLC-ESMS, were used in the deacylation studies. The acyl-PBP2a* preparations, diluted in Buffer C to ~1 mg/mL, were incubated at 37 or 27 °C. At various time intervals an aliquot (20 μL) of the sample was removed and mixed with 5 μL of 5% HCOOH to stop the reaction before analysis by pcHPLC-ESMS to determine the percentage of PBP2a* remaining acylated.

Quantitative Determination of acyl-PBP2a Complex by Perfusion Capillary High-Performance Liquid Chromatography-Electrospray Mass Spectrometry (pcHPLC-ESMS).* An on-line pcHPLC-ESMS system consisting of a perfusion capillary column (0.3 × 100 or 50 mm, poros RII/H, LC−Packings, CA) and an electrospray mass spectrometer (Sciex API−III LC/MS/MS triple quadrupole instrument, PE Sciex, Ontorio, Canada) was used to determine the ratio of acyl-PBP2a* and PBP2a*. In this method, 20 μL of the reaction solution, containing 10−15 μg of PBP2a* prepared as above, was injected onto the column and eluted with a gradient of H_2O to 85% CH_3CN in 0.02% trifluoroacetic acid over 2 min at a flow rate of 50 μL/min to separate PBP2a* and its β-lactam complex (acyl-PBP2a*) from other materials in the sample solution such as NaCl, phosphate, and free β-lactam. This desalting step provided a cleaner background for subsequent PBP2a* and acyl-PBP2a* mass determination. The mass spectrometer scanned over a mass range of 700−1700 amu, using a 1.0 ms dwell time and 0.2 or 0.4 amu step size. The system offers the advantages of sensitivity (using 5−20 μg protein), speed (~8 min of total running time), and accuracy (a typical mass precision of better than 0.01%). The signals of free PBP2a* and acyl-PBP2a* were resolved using the program of Reconstruct Hypermass, which constructed a molecular mass spectrum from a *m*/*z* mass spectrum containing multiple charge states (*13*). The peak heights or areas of free PBP2a* and acyl-PBP2a* species were measured from the reconstructed molecular mass spectrum (see Results). The percentage of PBP2a* acylated or the ratio of acyl-PBP2a* to total PBP2a* was defined as acyl-PBP2a*(100)/(acyl-PBP2a* + free PBP2a*). Since the relative ratio of the two signals, instead of an absolute signal of acyl-PBP2a*, was used, this method minimized the error due to PBP2a* protein variation from sample to sample. Such variation may occur during the protein addition and the sample preparation after reaction (e.g., during the steps of concentrating and washing to remove free β-lactam).

Kinetic Equations. It is generally accepted that the interaction between a PBP and a β-lactam (denoted as Pen here) follows a three-step mechanism (*14*, *17*, *20*) represented by Scheme 1,

Scheme 1

$$\text{PBP} + \text{Pen} \overset{K_d}{\rightleftharpoons} \text{PBP/Pen} \xrightarrow{k_2} \text{PBP-Pen} \xrightarrow[H_2O]{k_3} \text{PBP} + \text{P}_1 \text{ (Penicilloate)}$$

where K_d is the dissociation constant for the formation of the Michaelis complex (PBP/Pen), k_2 is the first-order rate constant for the acylation reaction forming a covalently bound β-lactam/PBP complex (PBP-Pen or acyl-PBP), and k_3 is the first-order rate constant for the deacylation of the PBP-Pen complex or hydrolysis of the ester bond of the complex. Reported values of k_3 for acyl-PBP deacylation are generally very slow (<10^{-4} s^{-1}) and are ~100−1000-fold smaller than that of k_2 (*17*, *20*, *21*). The covalent adduct, PBP-Pen, thus is very stable with a half-life of over several hours and can be easily isolated. Therefore, acylation of PBP by a β-lactam is a single-turnover process, equivalent to the acylation of chymotrypsin by a nitrophenyl ester (*22*). Under the condition of [Pen] ≫ [PBP], the kinetics of the formation of acyl-PBP at a given β-lactam concentration is given by eq 1, where k_a is the apparent first-order rate constant and

$$\frac{[\text{PBP-Pen}]_t}{[\text{PBP}]_{\text{total}}} = 1 - \exp(-k_a t) \qquad (1)$$

$[\text{PBP-Pen}]_t/[\text{PBP}]_{\text{total}}$ is the ratio of acyl-PBP to total PBP ($[\text{PBP}]_{\text{total}} = [\text{PBP}]_{\text{free}} + [\text{PBP-Pen}]$) at time *t* or percent of PBP acylated at time *t*, determined by pcHPLC-ESMS. In

practice, k_a was obtained by computer fitting (SigmaPlot) experimental data of the percent of PBP acylated against reaction time to eq 1 using nonlinear least-squares analysis. Based on Scheme 1 and $k_2 \gg k_3$, k_a is related to K_d and k_2 according to eq 2 (*23*, *24*, *25*). Thus, if a plot of k_a versus

$$k_a = \frac{k_2[\text{Pen}]}{K_d + [\text{Pen}]} \quad (2)$$

[Pen] follows a hyperbolic curve, the reaction can be best explained by the mechanism represented by Scheme 1, and the related parameters K_d and k_2 can then be derived by computer fitting of the experimental data of k_a versus [Pen] to eq 2. The first-order deacylation rate constant k_3 for the third step of Scheme 1 is described by eq 3, where [PBP-

$$\frac{[\text{PBP-Pen}]_t}{[\text{PBP}]_{\text{total}}} = \exp(-k_3 t) \quad (3)$$

Pen]$_t$/[PBP]$_{total}$ is the ratio of acyl-PBP to total PBP after incubation of acyl-PBP for time t. The value of k_3 was obtained from computer fitting the data of the percent of PBP remaining acylated versus reaction time to eq 3.

Measurement of [^{14}*C*]*-Benzylpenicillin Binding to Native, Membrane-Bound PBP2a of Staphylococcus aureus by a SDS−PAGE/Autoradiography Method*. Membrane fraction of MRSA strain COL was prepared essentially as reported (*26*). To determine the acylation rate of membrane-bound PBP2a by benzylpenicillin, we preincubated the membrane fraction (5 mg/mL) with nafcillin (final concentration 5 μg/mL) at 37 °C for 10 min. Under these conditions, all other (penicillin-sensitive) PBPs except PBP2a were acylated by nafcillin. Then, [^{14}C]-benzylpenicillin, at a given concentration (from 0.29 to 2.1 mM), was added to label PBP2a. At various time intervals an aliquot (20 μL, 0.1 mg of membrane protein) was removed and mixed with SDS sample buffer. Proteins were then separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as described (*26*). Autoradiography was performed on a phosphor screen using Molecular Dynamics Phosphorimager SI (Sunnyvale, CA). Digital values corresponding to the radioactivity of PBP2a bands were obtained by following the manufacture's instructions and were used to derive kinetic parameters according to the equations above and as detailed in Results. [PBP-Pen]$_t$/[PBP]$_{total}$ was derived from [PBP2a band radioactivity]$_t$/[PBP2a band radioactivity]$_{final}$ essentially as described (*27*).

Miscellaneous Techniques. Protein concentration was measured by the Bradford method using a Bio-Rad kit with bovine serum albumin (Sigma) as the standard. SDS−PAGE ready gradient gels from Bio-Rad were used to detect PBP2a* and to monitor its purity during the purification.

Chemicals. Benzylpenicillin (>99%) was purchased from Fluka. [^{14}C]-Benzylpenicillin was bought from Amershame. Methicillin, nafcillin, and cefotaxime were bought from Sigma. Restriction enzymes and T4 ligase were from Promega. Compound 1, a cephalosporin ([6*R*,7*R*]-3-[(2-iodophenyl) thiol]-7-[(phenylacetyl) amino]-8-oxo-5-thial-azabicyclo[4.2.0.]oct-2-ene-2-2carboxylic acid, 552.4 Da), was synthesized in-house.

RESULTS

*Sequence Analysis of the Cloned mecA Gene and the Recombinant, Truncated PBP2a**. A number of *mec*A gene clones were obtained and subjected to DNA sequence analysis. The sequence of a clone (pTMCA-23) was found to be identical to that of *Staphylococcus epidermidis* (WT55) except for one base substitution (*9*). At position 327, T was found instead of C. This, however, was a silent mutation as both codons code for Asp109. Thus, the *mec*A gene (pTMCA-23) cloned from MRSA strain MI339 (for its amino acid sequence see ref *13*) differs from that of *S. aureus* strain 27r by one amino acid (Gly instead of Glu) at position 246 and from that of strain TK784 by 24 amino acids (*10*). Its primary sequence was further verified by peptide mapping of the purified PBP2a* using a combination of CNBr cleavage and microbore HPLC-ESMS analysis as detailed elsewhere (*13*).

Large-Scale Expression and Purification of PBP2a to Homogeneity.* The construct pTMCA-23 was transformed into and expressed in *E. coli*. The recombinant PBP2a* was expressed mostly in the soluble form up to ~25% of the *E. coli* soluble proteins (data not shown). In a typical operation, about 40 mg of soluble PBP2a* was produced per liter of culture on the basis of estimations by ELISA and SDS−PAGE. Thanks to the abundance of the protein in the crude extract, SDS−PAGE was used to monitor the protein during the development of the three-step purification protocol and during the purification. With this protocol the soluble PBP2a* was purified to homogeneity as judged by SDS−PAGE and pcHPLC-ESMS. The final yield of the purification was ~70%. Based on the primary sequence the molecular weight of the truncated PBP2a* of 645 amino acids was calculated to be 73 467 Da. An almost identical value of 73 466 (±3) Da of a single species was determined for the purified PBP2a* by the methods of microbore or pcHPLC-ESMS (Figure 1a,b), verifying the integrity of the recombinant protein. The purified PBP2a* was soluble in Buffer C (10 mM phosphate/50 mM NaCl, pH 7.2) at room temperature or 37 °C.

Acylation of PBP2a by β-Lactams.* Upon incubation of the purified PBP2a* with benzylpenicillin (Chart 1) (0.25 mM) for 10 min at 37 °C, the molecular mass of the protein became 73 802 Da (Figure 1c). The difference between the molecular mass of this species and free PBP2a* (73 467 Da) was 335 Da, which is in excellent agreement with the molecular mass of benzylpenicillin (335 Da), indicating the formation of an adduct of penicillyol-PBP2a* with a 1:1 ratio. Similar results of the formation of acyl-PBP2a* adducts were obtained with other β-lactams such as methicillin, ampicillin, cefotaxime, and Compound 1. It is very unlikely that the observed β-lactam/PBP2a* adducts reflect any partial noncovalent interactions between the β-lactams and PBP2a*. First, washing and concentrating three times of a complex preparation in Amicon microcon 30 tube to remove free β-lactam resulted in no significant decrease of the adducts formed as compared with a control before washing. Second, under the harsh pcHPLC-ESMS conditions used (e.g., 85% acetonitrile with trifluoacetic acid used in HPLC and a relatively high orifice voltage in the mass spectrometer), it is very unlikely for any noncovalent complex to survive (*34*). Upon acylation by a β-lactam in 10 mM phosphate/50 mM

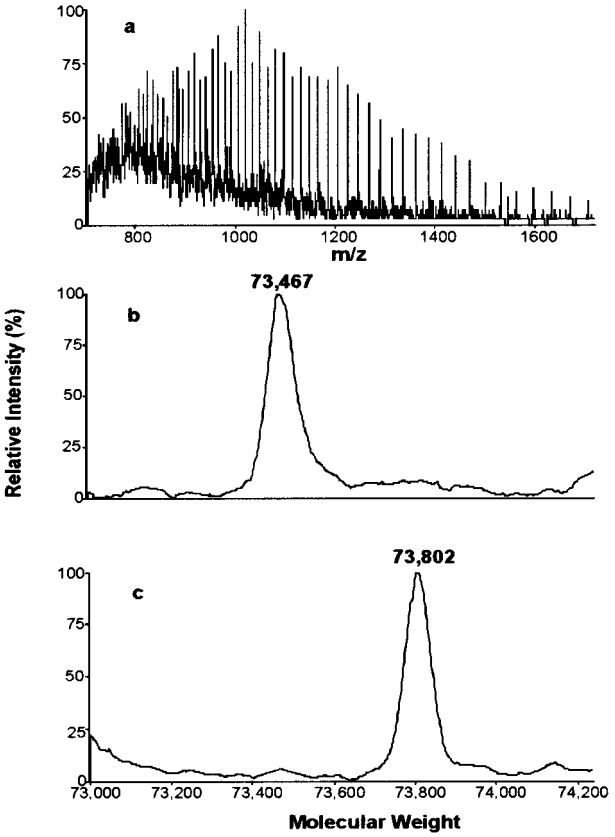
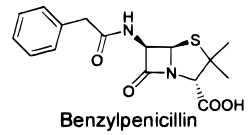
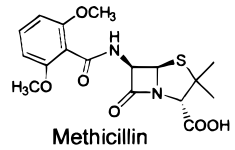
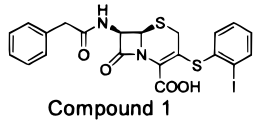


FIGURE 1: Electrospray mass spectra of purified PBP2a*. (a) A raw spectrum of PBP2a*. (b) The spectrum of PBP2a* reconstructed from (a). (c) Reconstructed spectrum of penicilloyl-PBP2a*. The spectra were obtained by pcHPLC-ESMS as described in Experimental Procedures. The acylated PBP2a* sample was prepared by incubation of the protein (20 μg) with 0.5 mM benzylpenicillin at 37 °C for 10 min.

Chart 1: Structures of Beta-Lactams Used







NaCl, pH 7.2 (Buffer C) at 37 °C, the protein gradually precipitated or aggregated. This, however, should not affect the kinetic studies of PBP2a* acylation described below since the precipitation usually occurred ~5−7 min after the protein was completely acylated.

Kinetics of the Acylation of PBP2a by Benzylpenicillin.* The rates of benzylpenicillin binding to PBP2a* were slow at concentrations of benzylpenicillin below 2 mM at 37 °C, which allowed us to follow the formation of acyl-PBP2a* accurately in the time range from 10 s to several minutes or

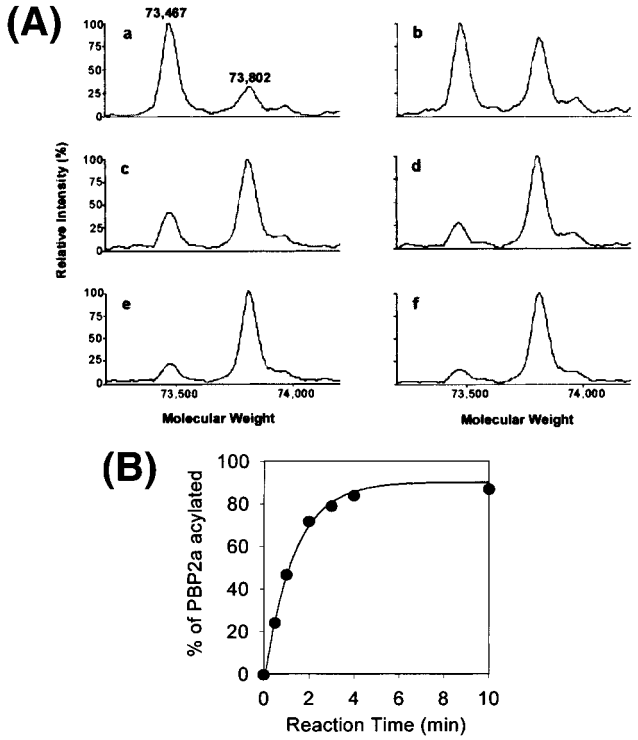


FIGURE 2: Benzylpenicillin binding to PBP2a* as a function of time as determined by pcHPLC-ESMS. (A) Reconstructed electrospray mass spectra of PBP2a* and acylated PBP2a* were recorded after incubation of the protein (15 μg) with 0.75 mM benzylpenicillin for 0.5 (a), 1 (b), 2 (c), 3 (d), 4 (e), and 10 (f) min at 37 °C. In each figure, the peak on the left (73 467 Da) corresponds to PBP2a* and the peak on the right (73 802 Da) represents the penicillioyl-PBP2a* complex. (B) The percentage of penicilloyl-PBP2a* formed versus reaction time. The solid trace represents the best fit to the single exponential according to eq 1 with $k_a = 0.012$ s^{-1}.

longer by manually mixing the reactants and stopping the reactions. A typical set of such measurements in the form of reconstructed molecular mass spectra is illustrated in Figure 2A, in which the extent of PBP2a* acylation increased with time at 0.75 mM benzylpenicillin as shown by the decrease of the peak height of free PBP2a* at 73 467 Da and the concomitant increase of the peak height of acyl-PBP2a* at 73 802 Da. The percentage of acylated PBP2a* at each time point was calculated from the relative peak heights of the two species. Essentially the same results were obtained when the peak areas instead of heights were used for the calculation. These data were plotted in Figure 2B and fit nicely to a single exponential according to eq 1, from which the apparent first-order rate constant ($k_a = 0.012$ s^{-1}) was obtained.

The k_a values were increased more or less linearly with the increase of benzylpenicillin concentration up to ~2 mM (Figure 3). To test whether a saturation binding kinetics was involved, we had to use higher benzylpenicillin concentrations. Under these conditions, data points at much shorter time scale (<10 s) had to be collected. Therefore, a rapid mixing technique, quench flow, was utilized. Consistent data were obtained when either the manual method or the quench flow technique was used in trials with 1 mM benzylpenicillin.

With these approaches, the time-dependent acylation of PBP2a* by benzylpenicillin at various concentrations from 0.1 to 10 mM was determined (Figure 3). At all concentra-

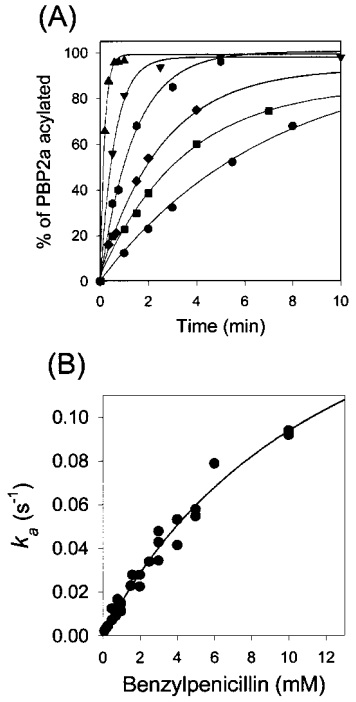


FIGURE 3: Kinetic analysis of the acylation of PBP2a* by benzylpenicillin. (A) Time- and benzylpenicillin concentration-dependent acylation of PBP2a*. Data points were monitored over a sufficiently long period for the reaction to go to near completion (>90% of PBP2a becoming acylated), but the data were only plotted out in the figure for 8 min for clarity. Six representative traces at benzylpenicillin concentrations of (from bottom to top) 0.1, 0.33, 0.5, 0.7, 2, and 6 mM are shown. The solid lines represent the best fits to the single exponential according to eq 1, from which values of k_a, the apparent first-order rate constants, were derived. (B) Plot of the k_a values as a function of the concentrations of benzylpenicillin from 0.1 to 10 mM. The solid line is the best fit to the hyperbola based on eq 2, corresponding to $K_d = 13.3$ mM and $k_2 = 0.22$ s^{-1}.

tions the reaction followed the single exponential of eq 1, from which the values of k_a were derived. The k_a values gradually increased with penicillin concentrations. The increase, which was not linear, approached a plateau at high concentrations of benzylpenicillin (Figure 3B). This hyperbolic dependence of k_a values on the benzylpenicillin concentrations indicates that the binding of benzylpenicillin to PBP2a* involves at least two steps as illustrated by Scheme 1 (*14*, *24*, *25*). The data were fit to eq 2, from which the values for K_d of 13.3 mM and k_2 of 0.22 s^{-1}were derived (Figure 3B). From these two values, the value of k_2/K_d was calculated to be 16.5 M^{-1} s^{-1}. When the concentrations of benzylpenicillin at or below 25 μM were used, the acylation became extremely slow and less than 10% of PBP2a* became acylated after several hours of incubation. This indicates that there is a threshold benzylpenicillin concentration (probably around 50−100 μM), below which PBP2a could not be sufficiently acylated, even though the acylation is an irreversible reaction.

*Deacylation of Benzylpenicilloyl-PBP2a**. Preparations of the penicilloyl-PBP2a* complex, in which unbound benzylpenicillin had been removed and >85% of the protein was in the complex form, were used to measure the kinetics of deacylation at 27 or 37 °C. The solution of penicilloyl-PBP2a* (~1 mg/mL) became cloudy upon incubation for over 7 min at 37 °C, indicating the aggregation of the

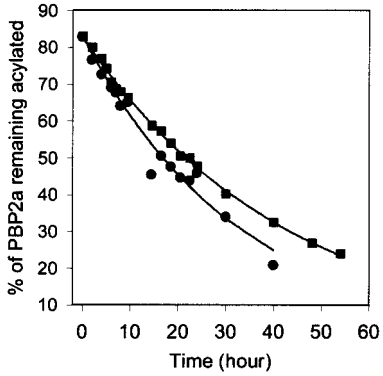


FIGURE 4: Deacylation of penicilloyl-PBP2a*. Penicilloyl-PBP2a* was incubated at 27 (■) or 37 (●) °C for various times before analysis by psHPLC-ESMS. The solid lines represent the best fits of the data to the single-exponential decay according to eq 3 with the deacylation rate constants of $k_3 = 6.5 \times 10^{-6}$ s^{-1} (■) and 8.3×10^{-6} s^{-1} (●).

acylated protein. Significant precipitation was also observed when PBP2a* was incubated with benzylpenicillin (>0.1 mM) for an extended period (over 15 min) under similar conditions (refs *17*, *28*; Lu, unpublished observation). However, the solution of penicilloyl-PBP2a* was clear upon incubation for up to 2 days at lower temperatures such as 27 °C, indicating that the acylated protein remained soluble. Therefore, the descylation reaction was performed at 27 °C. After incubation of the acylated PBP2a* at 27 °C for various times, the extent of the protein remaining in the acylated form was determined by pcHPLC-ESMS. The percentage of PBP2a* in the acylated form decreased exponentially with time according to eq 3 (Figure 4), from which the deacylation rate constant $k_3 = 6.5 \times 10^{-6}$ s^{-1} was obtained. For comparison, a slightly faster $k_3 = 8.3 \times 10^{-6}$ s^{-1} was obtained at 37 °C for the deacylation of the aggregated acyl-PBP2a* complex (Figure 4). Only one deacylated small, nonprotein product with a molecular mass of 352 (±1) Da was detected by pcHPLC-ESMS, consistent with the formation of benzylpenicilloate as the sole low molecular weight product.

Comparison of the Rate of Benzylpenicillin Acylation of PBP2a with That of Its Native, Membrane-Anchored Counterpart.* By using a membrane fraction prepared from MRSA strain COL and the SDS−PAGE/autoradiography method, we also characterized the binding of ^{14}C-benzylpenicillin to the native membrane-bound PBP2a after penicillin-sensitive PBPs in the membrane had been saturated with a nonradioactive β-lactam, nafcillin. As shown in Figure 5, at 0.67 mM benzylpenicillin and 37 °C, the acylation of PBP2a was observed with a k_a value of 0.0092 s^{-1}. Scattered data points were seen, reflecting the difficulties in obtaining highly accurate data with this type of approach (*29*). Nevertheless, this value, $k_a = 0.0092$ s^{-1}, is comparable to that of $k_a = 0.011$ s^{-1} obtained for the purified recombinant PBP2a* at 0.7 mM benzylpenicillin by the method of pcHPLC-ESMS (Figure 5). A similar study of the interaction between native, membrane-bound PBP2a and benzylpencillin (0.88 mM) at 30 °C using a similar SDS−PAGE method was reported (*30*). The data from this study showed a profile close to the above two sets of data (Figure 5). Furthermore, we determined the k_a values at four concentrations of benzylpenicillin from 0.29 to 2.1 mM with the membrane fraction. The data points were not sufficient for an accurate

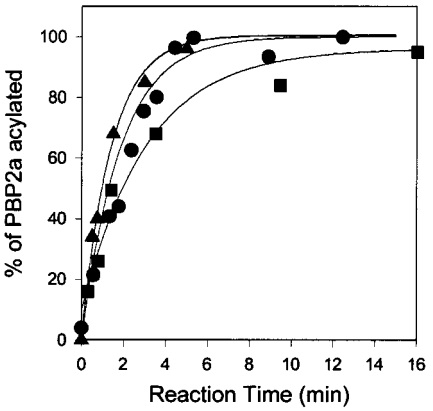


FIGURE 5: Comparison of the rate of benzylpenicillin acylation of purified, recombinant PBP2a* (▲) and native, membrane-bound (●, ■) PBP2a. PBP2a* was incubated with penicillin (0.7 mM) at 37 °C for various times before analysis by pcHPLC-ESMS. In ●, the MRSA membrane was incubated first with nafcillin and then with ^{14}C-benzylpenicillin (0.67 mM) at 37 °C for various times before analysis by SDS−PAGE/autoradiography to quantify the extent of membrane-bound PBP2a acylated. The data of ■ were taken from published results (30), where methods similar to that of ● were used except that benzylpenicillin was 0.88 mM and the temperature was 30 °C. The solid traces represent the best fits to eq 1 with apparent first-order rate constants (k_a) of 0.011 (▲), 0.0092 (●), and 0.005 (■) s^{-1}.

analysis with eq 2. However, under the condition of [Pen] $\ll K_d$, eq 2 is simplified to $k_a = k_2$[Pen]/K_d (*20*). From a plot of k_a versus benzylpenicillin concentration (data not shown), the value of k_2/K_d was derived from the slope to be 16 M^{-1} s^{-1}. This value is very close to the value (16.5 M^{-1} s^{-1}) measured above with the purified PBP2a*. These results indicate that the recombinant truncated PBP2a* is similar to the native, membrane-bound enzyme, with respect to its kinetic behavior of acylation by benzylpenicillin.

Kinetics of the Interaction between PBP2a and Methicillin.* The acylation of PBP2a* by methicillin, studied by the electrospary mass spectrometry-based method, was also dependent on the time and the concentration of methicillin as shown in Figure 6A. The dependence of the apparent first-order rate constant (k_a) on the concentration of methicillin is presented in Figure 6B. The observation of a hyperbolic curve indicates again that the acylation is a two-step process according to Scheme 1. Fitting the data to eq 2 allowed the calculation of the individual kinetic constants $k_2 = 0.0083$ s^{-1} and $K_d = 16.9$ mM, resulting in the value of $k_2/K_d =$ 0.49 M^{-1} s^{-1}. The apparent k_3 values for deacylation of methicilloyl-PBP2a* were determined to be 9.5 and 5.1 × 10^{-6} s^{-1} at 27 and 37 °C, respectively (data not shown). The methicilloyl-PBP2a* complex became precipitated after a few minuntes (∼7 min) of incubation at 37 °C but remained soluble at 27 °C throughout the reaction period (20 h).

Kinetics of the Interaction between PBP2a and Compound 1.* Compound 1 is a cephalosporin described early by R. Woodward and co-workers (*31*). It was recently demonstrated that the β-lactam (named MC02,002) and its derivatives exhibited high activities in binding membrane-anchored PBP2a and against gram positive bacteria including MRSA (*6*). Three or four new β-lactams with PBP2a-binding affinity comparable to that of Compound 1 have also been reported by scientists of several pharmaceutical companies (*6*). To compare the kinetic mechanism and parameters with the

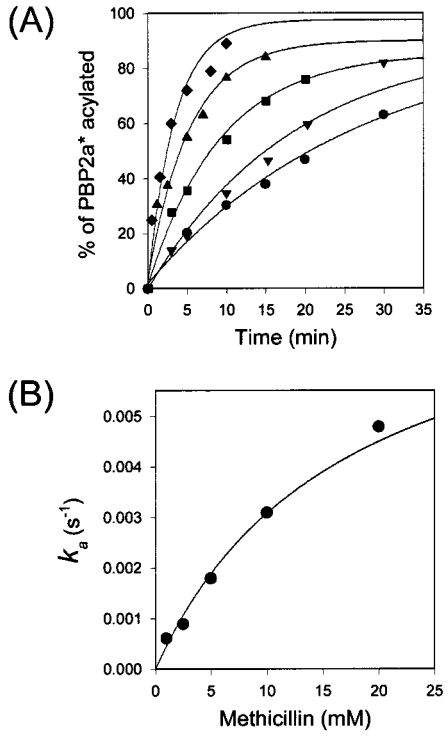


FIGURE 6: Acylation of PBP2a* by methicillin. (A) Primary data of acylation time courses at methicillin concentrations of 1, 2.5, 5, 10, and 20 mM (from bottom to top). The solid traces are the theoretical best fits of each data set to eq 1. (B) The dependence of the apparent first-order rate constant (k_a) on the concentration of methicillin. The curve represents the best fit of the data to eq 2 with $K_d = 16.9$ mM and $k_2 = 0.0083$ s^{-1}.

PBP2a low-affinity β-lactams described above, we characterized the interaction of Comound 1 with PBP2a* using the same approaches. Indeed, very high rates of acylation of PBP2a* by the cepholosporin were observed at relatively low concentrations of the compound from 2 to 100 μM as shown in Figure 7. Again, a saturation curve of k_a values versus the concentration of the cepholosporin was observed, from which $k_2 = 0.39$ s^{-1} and $K_d = 0.22$ mM were obtained (Figure 7B). The resultant second-order rate constant (k_2/K_d) of 1750 M^{-1} s^{-1} is about 100-fold higher than that for benzylpenicillin. The deacylation rate constant for the cephalosporin acylated PBP2a* was determined to be 2.4 × 10^{-5} s^{-1} at 37 °C. Similarly, precipitation of the acyl-PBP2a* complex was observed during the deacylation reaction.

DISCUSSION

We have cloned the β-lactam-resistant PBP2a from MRSA and expressed the recombinant protein. Our approaches described here have resulted in a high expression yield and a soluble and free form of the protein, while previously PBP2a was expressed in low quantities (*16*), in the form of inclusion bodies (*11*), or in a fusion form (*12*). The subsequent development of an effective purification protocol enabled us to obtain a large amount of homogeneous PBP2a*. This allowed us, among other things (e.g., screening for inhibitors against the protein), to extensively characterize the protein in terms of its interactions with several β-lactams with a newly developed method of electrospray mass spectrometry.

Electrospray mass spectrometry has recently been used to characterize reaction intermediates of β-lactam interactions

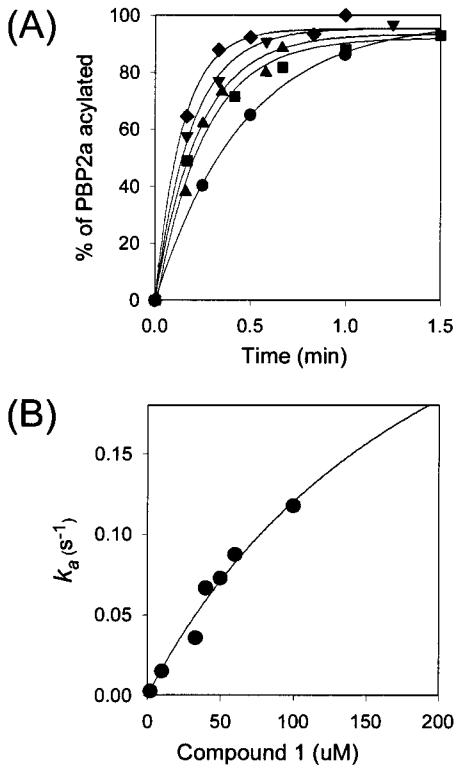


FIGURE 7: Acylation of PBP2a* by Compound 1. (A) Primary data of acylation time courses at Compound 1 concentrations of 33, 40, 50, 60, and 100 μM (from bottom to top). The solid traces are the theoretical best fits of each data set to eq 1. (B) The dependence of the k_a values on the concentration of Compound 1. The curve represents the theoretical best fit of the data to eq 2 with K_d = 0.22 mM and k_2 = 0.39 s^{-1}.

with human leukocyte elastase (*32*, *33*) and with β-lactamases (*34*, *35*). Directly coupled to a fast mixing device, it has also been used to characterize the pre-steady-state kinetics of the formation of a covalent xylobiosyl-enzyme intermediate (*25*). We described here a quantitative electrospray mass spectrometric method by monitoring relative signal intensities within the same mass spectrum. Coupled with a HPLC system, this method (pcHPLC-ESMS) provides a sensitive, accurate, and relatively efficient technique to quantitatively determine the formation of covalent enzyme-ligand adducts such as acyl-PBP complexes.

With this method, we characterized the kinetic interactions of PBP2a* with three β-lactams. The observation of saturation binding kinetics between PBP2a* and the β-lactams can best be interpreted by a two-step mechanism (*14*, *24*) as illustrated by Scheme 1, similar to the acylation of serine proteases such as chymotrypsin (*36*) or the glycosylation of xylanase (*25*). The first step is a rapid formation of the reversible Michaelis complex, and the second step is the irreversible acylation of the catalytic site serine (S403) of the protein. Such a two-step mechanism has been observed for the R61 DD-carboxypeptidase (*14*) and proposed for HMM PBPs (*20*) including PBP2a (*12*, *17*), but this is the first evidence to confirm this mechanism for a HMM PBP.

The apparent second-order rate constant k_2/K_d has been referred to in the literature as the best measure of inhibitory potency of a β-lactam against PBPs (*21*). The value of k_2/K_d (16.5 M^{-1} s^{-1}) determined for benzylpenicillin binding to PBP2a* is in good agreement with that ($\sim$10 M^{-1} s^{-1}) predicated in the literature (*21*, *37*) and that (12 M^{-1} s^{-1}) determined by a competition method with nitrocefin as the reporter (*17*). This value (16.5 M^{-1} s^{-1}) is about 2−3 orders of magnitude smaller than that found for penicillin-sensitive HMM PBPs (*21*) such as PBP2 from *S. aureus* (14 000 M^{-1} s^{-1}) (*38*) and PBP2x from *Streptococcus pneumoniae* (58 000 M^{-1} s^{-1}) (*15*). These data are also consistent with the observation that benzylpenicillin MIC (minimal bacterial inhibition concentration) values ($\sim$50 μg/mL) for MRSA were about 500-fold higher than that ($\sim$0.1 μg/mL) for β-lactam susceptible *S. aureus* (*27*, *30*), which contains no PBP2a.

We treated the K_d values obtained here as a true equilibrium constant for the binding reactions since the condition of $k_2 \ll k_{-1}$ is met (*22*, *24*). This condition was established on the basis of the facts of the relatively high value of K_d (13.3 mM) and the low value of k_2 (0.22 s^{-1}) in the case of benzylpenicillin and the assumption of $k_{+1} > 1 \times 10^5$ M^{-1} s^{-1} (*22*, *39*). The determined association rate constants (k_{+1}) for enzyme-ligand interactions in the literature fall in the range from 1×10^5 to 1×10^8 M^{-1} s^{-1} (*40*). In addition, the time dependence of the acylation followed the single exponential at all benzylypenicillin concentrations, and no lag was seen at low benzylpenicillin concentrations (Figure 3A). A similar observation was made for methicillin and Compound 1 (Figures 6A and 7A). These data strongly support the rapid equilibrium assumption (*41*). The high value of K_d (13.3 mM) for benzylpenicillin thus implies that the β-lactam fits poorly into the active-site pocket of PBP2a. However, it should be noted that this value is in the same range as that for benzylpenicillin binding to the R61 DD-carboxypeptidase (K_d = 13 mM), a small molecular mass nonessential PBP (*14*). The determined value of k_2 = 180 s^{-1} for the latter enzyme was about 3 orders of magnitude higher than that measured for PBP2a (0.22 s^{-1}). These suggest that the low binding affinity of benzylpenicillin to PBP2a is mostly attributable to the low efficiency of formation of the covalent bond and to a lesser extent the poor fit of the penicillin to the active-site pocket of the enzyme. Whether this can be generalized as the mechanism of PBP2a resistance to β-lactams awaits the determination of the microscopic kinetic parameters for other PBPs, especially penicillin-sensitive HMM PBPs. With the methods described here, it is feasible to characterize the kinetics of β-lactam binding to these PBPs in a purified form.

Michaelis−Menten kinetics, that is, the Lineweaver−Burk equation, have been used to analyze the data of acylation of PBP2a upon incubation with various concentrations of penicillin for a fixed time, from which K_m and V_{max} values were derived (*16*). However, according to the kinetic mechanism established here, the binding of penicillin to PBP2a is a single-turnover process or a pre-steady-state reaction rather than a multiple-turnover or steady-state enzyme reaction on which Michaelis−Menten kinetics are based. β-Lactam acylation of a PBP is mechanistically equivalent to the acylation of a β-lactamase consisting of the first two steps of Scheme 1, except that the latter enzyme is kinetically much more efficient with much smaller K_d and faster k_2. The two classes of enzymes are further distinguished by the fact that β-lactamases have k_3 values which are about a million times faster than that of PBPs. The K_m value for a β-lactamase reaction is defined as $K_m = K_d k_3/(k_2 + k_3)$ (*41*), which is obviously improper to use for the

interaction between a PBP and a β-lactam.

Methicillin exhibited an even poorer binding affinity to PBP2a* than did benzylpenicillin, as indicated by a 36-fold decrease in the k_2/K_d value. This is consistent with the observation that the MIC value of methicillin against MRSA was about 32-fold higher than that of benzylpenicillin (*6*, *30*). If the individual parameters are compared for the two compounds, the decrease in the binding efficiency for methicillin is mostly due to the decrease in k_2 (~22-fold), implying that the step of the ester bond formation plays a more important role than the first step of molecular recognition. This observation may be in agreement with the acylating chemistry of methicillin, in which both of the *ortho* positions of the side chain phenyl group are occupied by a methoxy group, resulting in steric hindrance around the carbonyl carbon of the β-lactam ring (*42*). This, therefore, may decrease the accessibility to the carbonyl carbon by the nucleophilic active-site serine of a penicillinase or PBP2a, resulting in a decreased rate of acylation. However, it is conceivable that other factors may contribute to the decreasing rate of acylation. For instance, the long-distance electron-donating effect from the methoxy groups could make the carbonyl carbon in methicillin less electrophilic (as compared to that in benzylpenicillin), thus becoming less susceptible to the nucleophilic attack. Protonation on the β-lactam nitrogen could also be affected by the structural changes in a way that influences the acylation rate.

The ~100-fold increase of the k_2/K_d value (1750 M^{-1} s^{-1}) for Compound 1 binding to PBP2a as compared with that for benzylpenicillin is in good agreement with a much lower (~50-fold decrease) MIC value against MRSA by Compound 1 (~1 μg/mL) (ref *6*, unpublished observation). This is also consistent with the observation that IC_{50} values against PBP2a were 60 and ~0.6 μM/mL for benzylpenicillin and Compound 1, respectively (ref *6*, unpublished data). Nevertheless, the k_2/K_d value for Compound 1 is still significantly (10−100-fold) lower than that ($0.2-5 \times 10^5$ M^{-1} s^{-1}) for benzylpenicillin binding to normal, β-lactam-sensitive HMM PBPs (*21*, *38*), suggesting that there is still room for improvement of the binding potency of such PBP2a high-affinity β-lactams. Comparison of the individual parameters of $k_2 = 0.39$ s^{-1} and $K_d = 0.22$ mM for Compound 1 with $k_2 = 0.22$ s^{-1} and $K_d = 13.3$ mM for benzylpenicillin suggests that most of the enhanced efficiency of acylation is attributed to the decrease (60-fold) in dissociation constant K_d for Compound 1. This indicates that the cephalosporin fits better into the active-site pocket of PBP2a.

The deacylation rate constant of 6.5×10^{-6} s^{-1} for penicilloyl-PBP2a* determined at 27 °C is more reliable than that obtained at 37 °C since the acylated protein became precipitated upon incubation for a few minutes at 37 °C but remained soluble at 27 °C for an extended time. Such precipitation or aggregation, due to some type of conformational or secondary structural change of PBP2a* upon acylation (Lu, unpublished data), could significantly affect the rate of deacylation. Assuming that the reaction rate doubles with each 10 °C increase in temperature, a value of $k_3 = 1.3 \times 10^{-5}$ s^{-1} for the deacylation of penicilloyl-PBP2a* at 37 °C can be calculated. A similar value of 9.5×10^{-6} s^{-1} was determined for the deacylation of methcilloyl-PBP2a* at 27 °C. These values fall in the range of k_3 values ($0.5-10 \times 10^{-5}$ s^{-1}) reported for other PBPs (*21*, *37*), indicating that the acyl-PBP2a* adducts are very stable with a half-life of more than 7 h. It has been postulated that the resistance of a PBP such as PBP2a to a β-lactam could be achieved by either decreasing the value of k_2/K_d or increasing the value of k_3 (*37*, *38*). In the latter case, the bound β-lactam would be rapidly hydrolyzed, regenerating active PBP2a for peptidoglycan synthesis. The kinetic constants determined here and by others (*17*) clearly indicate that the mechanism for PBP2a resistance to β-lactams is due solely to the dramatic rate decrease in acylation and does not involve the step of deacylation.

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