Kinetics of β-Lactam Interactions with Penicillin-Susceptible and -Resistant Penicillin-Binding Protein 2x Proteins from *Streptococcus pneumoniae*: Involvement of Acylation and Deacylation in β-Lactam Resistance

Wei-Ping Lu§*, Erica Kincaid§, Yiping Sun¶ and Mark D. Bauer¶

Anti-Infective Research, Procter & Gamble Pharmaceuticals, Health Care Research Center, Mason, OH 45040§ and Corporate Research Division, Miami Valley Laboratories, The Procter & Gamble Co., P.O. Box

* Author to whom correspondence should be addressed. Phone: 513-622-3925; Fax: 513-622-0085; E-mail: lu.wp@pg.com

Running title: Interactions of PBP2x and PBP2xR with β-lactams
SUMMARY

Kinetic interactions of β-lactam antibiotics such as penicillin-G and cefotaxime with penicillin-susceptible PBP2x from *S. pneumoniae* and a penicillin-resistant PBP2x (PBP2x\(^R\)) from a resistant clinical isolate (CS109) have been extensively characterized. Kinetic evidence for a two-step acylation of PBP2x by penicillin-G has been demonstrated, and dissociation constant, \(K_d\) of 0.9 mM, and acylation rate constant, \(k_2\) of 180 s\(^{-1}\), have been determined for the first time. The millimolar range \(K_d\) implies that the β-lactam fits to the active site pocket of the penicillin-sensitive PBP rather poorly, while the extremely fast \(k_2\) value indicates that this step contributes most of the binding affinity of the β-lactam. The values of \(K_d\) (4 mM) and \(k_2\) (0.56 s\(^{-1}\)) were determined for PBP2x\(^R\). The combined value of \(k_2/K_d\), known as overall binding efficiency, for PBP2x\(^R\) (137 M\(^{-1}\) s\(^{-1}\)) is over 1000-fold slower than that for PBP2x (200,000 M\(^{-1}\) s\(^{-1}\)), indicating a major part played by the acylation steps in penicillin resistance. Most of the decreased binding efficiency of PBP2x\(^R\) comes from the decreased (~ 300-fold) \(k_2\). Kinetic studies of cefotaxime acylation of the two proteins confirmed all of the above findings. Deacylation rate constants (\(k_3\)) for the third step of the interactions were determined to be \(8 \times 10^6\) s\(^{-1}\) for penicilloyl-PBP2x and \(5.7 \times 10^4\) s\(^{-1}\) for penicilloyl-PBP2x\(^R\), corresponding to over 70-fold increase of the deacylation rate for the resistant PBP2x\(^R\). Similarly, over 80-fold enhancement of the deacylation rate was found for cefotaxime-PBP2x\(^R\) complex (\(k_3 = 3 \times 10^{-4}\) s\(^{-1}\)) as compared with that (3.5 \(\times 10^{-6}\) s\(^{-1}\)) of cefotaxime-PBP2x complex. This is the first time that such a significant increase of \(k_3\) values was found for a β-lactam resistant PBP. These data indicate that the deacylation step also plays a role, much more important than previously thought, in PBP2x\(^R\) resistance to β-lactams.
INTRODUCTION

Penicillin-binding proteins (PBPs) are enzymes involved in the final reactions of bacterial peptidoglycan synthesis, and are the targets of β-lactam antibiotics, which exert their action by acylating an active site serine of PBPs (1, 2, 3, 4). *Streptococcus pneumoniae*, a major human pathogen of the upper respiratory tract, contains five high molecular mass (HMM, with molecular mass over 60 kDa), essential PBPs (5, 6, 7). Among them, PBP2x and PBP2b have been identified as the primary β-lactam resistance determinants based on genetic and biochemical evidence (6, 8, 9, 10). Such resistance in PBP2x is largely due to the development of altered or mosaic forms of the PBP as a result of mutation, genetic exchange and recombinational events (11, 12).

Kinetic interaction of PBPs with β-lactams has been a subject of investigation since the elucidation of these proteins as the targets of the β-lactam antibiotics (1, 13, 14, 15). Such studies may shed light on the mechanism of action and of resistance at the molecular or enzymatic level, which should help to design better β-lactams or non-β-lactam PBP inhibitors to confront bacterial resistance to β-lactam antibiotics. It is generally accepted that the interaction of a β-lactam (I) with a PBP follows a three-step reaction mechanism (13) as shown by Scheme 1,

\[
PBP + I \overset{K_d}{\rightleftharpoons} PBP/I \overset{k_2}{\rightarrow} PBP - I \overset{k_3}{\rightarrow} H_2O \rightarrow PBP + I^*
\]

Scheme 1

where \(K_d\) is the dissociation constant for the formation of the reversible Michaelis complex (PBP/I), \(k_2\) is the first-order rate constant for acylation of PBP, or the formation of an ester bond between an active site serine of PBP and the carbonyl carbon of the β-lactam, and \(k_3\) is the rate constant for deacylation of PBP-I to generate free PBP and the hydrolyzed β-lactam \((I^*)\). Such interaction is essentially a single turnover process since the step of deacylation is extremely slow \((k_3 \ll k_2)\), and \(k_{-2}\) is virtually zero because of the covalent
bond formation and the resultant irreversible β-lactam ring opening. Kinetic evidence for this mechanism and kinetic constants for the individual steps have been obtained for low molecular mass (LMM), nonessential PBPs such as Streptomyces R61 DD-carboxypeptidase (13). The first HMM PBP being studied in such detail was β-lactam-resistant PBP2a from methicillin-resistant *Staphylococcus aureus* (16). In that study, an electrospray mass spectrometry (ESMS) method was developed to quantitatively determine acyl-PBP adducts formed (16). Combined with a fast reaction technique (quench flow), this method allowed us to determine the mechanism and the individual kinetic constants. Early on, kinetic studies were carried out with HMM PBP2x and PBP2x variants from *S. pneumoniae* by following β-lactam quenching of the intrinsic fluorescence of the PBPs (17,18,19). The combined values of $k_2/K_d$ were derived, but the individual parameters were not resolved.

In the work presented here, we characterized interactions of the normal or penicillin-sensitive PBP2x with pen-G and cefotaxime, and determined the individual kinetic constants for the PBP. We also studied a penicillin-resistant PBP2x (PBP2x$^R$) from *S. pneumoniae* strain CS109, a clinical isolate showing high level resistance not only to penicillins but also to almost all cephalosporin antibiotics including the extended-spectrum cephalosporins such as cefotaxime (20,21). In these comparative studies, we wanted to understand not only the extent of the reduction of overall β-lactam affinity to the resistant PBP2x, but also the degree to which each one of the three reaction steps was affected. The implications of these findings in terms of the mechanisms of β-lactam action and resistance, and the role of each step in resistance development are discussed.

---

1 **Abbreviations:** PBP, penicillin-binding protein; PBP2x$^R$, penicillin-resistant PBP2x; MRSA, methicillin resistant *Staphylococcus aureus*; ESMS, electrospray ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; MIC, minimal bacterial inhibition concentration.
EXPERIMENTAL PROCEDURES

Cloning of a truncated \( \text{PBP2x}^R \) gene by PCR and construction of an expression vector. Chromosomal DNA of \( S. \text{pneumoniae} \) strain CS109 (Serotype 23) obtained from the CDC was prepared as described (22), except that the cells were grown on a Tryptic Soy agar plate with 5% blood under 5% \( \text{CO}_2 \). This DNA was used as the template for PCR. Based on the published \( \text{PBP2x}^R \) sequence (21), a forward primer 5'-CATGCCATGGGGACAGGCACTGCTTTGGA-3' with a \( \text{NcoI} \) restriction site (underlined) and a backward primer 5'-TGGGAAGCTTGTCAACAATTCAGCCTG-3' corresponding to the C-terminus of \( \text{PBP2x} \) with a \( \text{Hind III} \) restriction site (underlined) added were used to amplify the gene by PCR. In the forward primer, the \( \text{NcoI} \) restriction site was introduced before codon Gly49, resulting in the \( \text{PBP2x}^R \) coding for a truncated \( \text{PBP2x}^R \), of which the first 48 amino acids at the N-terminus, including a 19 amino acid region coding for the membrane-spanning segment, were deleted. The PCR reaction mixture contained 0.8 \( \mu \text{M} \) each of the primers, 2.5 units of TaqDNA polymerase (Perkin-Elmer), 0.2 mM each dNTP, 10 mM Tris/\( \text{HCl} \), pH 8.3, 50 mM KCl, 0.15 mM \( \text{MgCl}_2 \), and the template (2 \( \mu \text{l} \)) in a final volume of 100 \( \mu \text{l} \), and was subjected to 30 cycles of PCR amplification (95 °C, 2 min; 60 °C, 1 min; 72 °C, 1 min), using a Hot Start technique (Perkin Elmer). The resultant PCR fragment was ligated into the pCR2.1 vector using T4 DNA ligase (TA Cloning Kit, Invitrogen). This construct was transformed into TOP10F' competent cells (Invitrogen), and colonies were selected for analysis based on their appearance on X-Gal plates. Plasmid DNA was isolated from the cell using the Qiagen mini-prep procedure. The pCR2.1 vector has BamH1 and EcoRV restriction sties contained within the multiple cloning region which flank the insert. The plasmid DNA was digested with these enzymes and fractionated on a 1% agarose gel. A 2-kb band was excised and gel-purified. This DNA was then digested with Ncol and HindIII. T4 ligase was used to ligate the product into PET24-d, an expression vector (Novagen) that had been digested with the same restriction enzymes. The \( \text{PBP2x}^R \) in the resultant vector (pPB52-10 (30)) was sequenced in-house.

Expression of \( \text{PBP2x}^R \) in \( E. \text{coli} \). The plasmid pPB52-10 (30) was transformed into \( E. \text{coli} \) 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 30 \( \mu \text{g/ml} \) Kanamycin was
inoculated with an inoculum culture and the cells were grown in a shaker at 37 °C until the OD$_{600}$ reached ~ 1. IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) was added at a final concentration of 1 mM into the culture to induce expression. Three hours later the culture was harvested by centrifugation at 10,000 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 liters of medium were used.

**Purification of the recombinant PBP2x$^R$ to homogeneity.** A three-step purification protocol, different from the published procedures (17, 23), was developed, which offered 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 25,000 g for 45 min. The resultant cell-free extract (~ 3 g protein) was loaded onto a Q-Sepharose HP (Pharmacia) column (5 x 10 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 Buffer A followed by elution with a linear gradient of 0 to 1 M NaCl in Buffer A at ~ 10 ml/min. PBP2x$^R$ eluted at ~ 0.5 M NaCl as judged by SDS-PAGE. The fractions (0.4 to 0.6 M NaCl) containing PBP2x$^R$ were combined. The combined solution was brought into 1 M (NH$_4$)$_2$SO$_4$ and loaded onto a Phenyl-Sepharose column (5 x 7 cm), which had been equilibrated with 1 M (NH$_4$)$_2$SO$_4$ in Buffer A. The column was then eluted with a linear gradient of 1 M to 0 M (NH$_4$)$_2$SO$_4$ in Buffer A. PBP2x$^R$ eluted around 0.5 M (NH$_4$)$_2$SO$_4$. 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 (5 x 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 ~ 450 to 520 ml containing purified PBP2x$^R$ were pooled and concentrated. Occasionally, some portion (up to 20 %) of the protein was purified as a dimer, which eluted earlier on the Superdex column than the major protein peak. The dimer form of the protein showed same molecular weight as did the monomer determined by mass spectrometry or on SDS-PAGE. However, its acylation rate by pen-G decreased ~ 80%, although it still could be fully acylated. This indicates that
denaturation can occur with the recombinant PBP protein during the expression or the purification and one should always check the kinetic activity for a preparation to ensure that it is fully active.

Expression and purification of PBP2x. E. coli strain Sure DH5α harboring plasmid pCG31 encoding PBP2x from *S. pneumoniae* R6 (24), kindly supplied by Prof. R. Hakenbeck, was used to express the protein according to the published method (24). The protein was purified by a newly-developed four-step procedure similar to that above for PBP2xR except that PBP2x was eluted out at around 0.8 M (NH₄)₂SO₄ in the step of Phenyl-Sepharose chromatography. In addition, another step of cation exchange chromatography on a SP-Sepharose column (5 x 10 cm) was inserted between the step of Phenyl-Sepharose chromatography and the last step of gel filtration. The protein solution collected from the (NH₄)₂SO₄ gradient was diluted ~ 15 fold with Buffer A before loading on the SP-Sepharose column, which had been equilibrated with Buffer A. The column was then eluted with a linear gradient of 0 to 1M NaCl in ~ 900 ml of Buffer A at 7 ml/min. The protein fractions were pooled and concentrated to ~ 50 mg/ml before loading on the Superdex 75 gel filtration column.

Preparation of samples for measurement of PBP2xR acylation by ESMS. For kinetic studies of the binding of a β-lactam to PBP2xR, a typical reaction mixture contained the β-lactam and ~ 10 to 15 µg of PBP in a final volume of 20 µl of 10 mM phosphate buffer, pH 7.2, containing 50 mM NaCl (Buffer B). 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 (ESMS) as described below. When high concentrations (> 0.5 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 to an Amicon microcon 30 tube and spun at ~ 10,000 g for ~ 5 min at room temperature to concentrate the protein sample and to remove the excess β-lactam which interfered with the subsequent ESMS measurement. After the recovery spin, the protein solution was collected, and its volume was adjusted to ~ 25 µl with 1% HCOOH before analysis by ESMS.
Preparation of samples by a rapid quench method for determination of PBP acylation by ESMS. All samples of PBP2x acylation were prepared by a quench flow method using a KinTek quench flow apparatus as described before (16) because of the PBP’s extremely fast acylation reaction. This method was performed to prepare PBP2xR samples when pen-G or cefotaxime concentrations were 0.2 mM or above, where the rates (k_a) of the acylation reached ~ 0.02 s⁻¹ or higher (under these conditions, the reactions of initial data points had to be terminated within 15 seconds). Typically, PBP (25 µg in 20 µl of Buffer B) and a β-lactam (various concentrations in 20 µl of Buffer B) were loaded in the two tubing loops. The reactions were started by rapidly mixing the two reactants from the two tubing loops for time intervals ranging from 5 msec to 15 sec followed by quenching with 3 % HCOOH (final concentration), which brought the pH of the mixture to below 2. The collected reaction mixtures (~ 150 µl) were then concentrated in Amicon microcon 30 tubes as described above.

Preparation of samples for determining deacylation of acyl-PBP complexes. Acyl-PBP2x complexes were prepared by first incubating PBP2x (~ 10 mg/ml) with 50 µM pen-G or cefotaxime for ~ 5 min, or PBP2xR (~ 10 to 20 mg/ml) with 0.5 mM pen-G or cefotaxime for ~ 30 min at room temperature to completely acylate the PBPs. All the reactions were performed in Buffer B (50 mM Phosphate, pH 7.2 with 50 mM NaCl) in a final reaction volume of 50 to 200 µl. The next step was to remove the unbound β-lactam from the reaction mixtures by one of the following two separation methods. In the first, this was done by centrifugation/washing three times with 3 x 1 ml Buffer B in an Amicon microcon 30 filtration tube. Each centrifugation was performed at ~ 11,000 g for ~ 15 min at 4 °C. This procedure lasted a total of ~ 40 min. The second one was the so-called spun column method essentially as described before (25, 26). In this method, a pre-dried spun column was made by adding ~ 600 µl of Sephadex-G50 equilibrated in Buffer B into a column made of a 1 ml-syringe column, followed by centrifugation on a swinging bucket benchtop IEC clinical centrifuge at ~ 800 g for 1 min. An above reaction mixture (~ 100 µl) was loaded onto the column immediately after the acylation reaction was complete, followed by centrifugation at the same speed for 1 min. The effluent containing the PBP with bound β-lactam was collected, and small molecules including the free β-lactam remained bound on the Sephadex-G50 gel. The obtained acyl-PBP preparations (~ 10 to 20 mg/ml)
were stored at –20 °C before using. For the deacylation reaction, the acyl-PBP2xR or -PBP2x preparations, diluted in Buffer B to ~1 mg/ml, were incubated at 37 °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. The percentage of PBP remaining acylated for each sample was then determined by ESMS analysis.

Quantitative determination of acyl-PBP complex by electrospray mass spectrometry (ESMS). The ESMS analysis was essentially as detailed previously (16). An on-line pcHPLC-ESMS system consisting of a perfusion capillary column (0.3 x 50 mm, poros RII/H, LC-Packings, CA) and an electrospray mass spectrometer (Sciex API-III LC/MS/MS triple quadrupole instrument, PE Sciex, Ontario, Canada) was used to determine the ratio of acyl-PBP and free PBP. The mass spectrometer scanned over a mass range of 700-1700 amu, using a 1.0 msec dwell time and 0.4 amu step size. The system offers the advantages of sensitivity (using 5 to 20 µg protein), speed (~8 min of total running time) and accuracy (a typical mass precision of better than 0.01%). The quantitative calculation of percentage of PBP acylated from the data determined by ESMS was as described previously (16).

Kinetic equations. The equations used for resolving the mechanism and the kinetic parameters of Scheme 1 were essentially as described previously (13, 14, 16). The kinetics of the formation of acyl-PBP at a given β-lactam (I) concentration is given by eq 1 of exponential rise: \([\text{PBP-I}]_{t}/[\text{PBP}]_{\text{total}} = 1-\exp(-k_{a}t)\). Here \(k_{a}\) is the apparent first-order rate constant and \([\text{PBP-I}]_{t}/[\text{PBP}]_{\text{total}}\) is the ratio of acyl-PBP to total PBP or the percentage of PBP acylated, derived from the ESMS determination, at time \(t\). In practice, \(k_{a}\) was obtained by computer fitting (SigmaPlot) experimental data to eq 1 using nonlinear least squares analysis. Based on Scheme 1 and \(k_{2} >> k_{3}\), \(k_{a}\) is related to \(K_{d}\) and \(k_{2}\) according to eq 2: \(k_{a} = k_{2}[I]/(K_{d} + [I])\). Thus, if a plot of \(k_{a}\) versus [I] follows a hyperbolic curve, the reaction can be best described by the mechanism represented by Scheme 1, and the related constants \(k_{a}\) and \(K_{d}\) can then be derived by computer fitting of the data of \(k_{a}\) versus [I] to eq 2. Under the condition of [I] \(<\ K_{d}\), eq 2 simplifies to eq 2-1: \(k_{a} = k_{2}[I]/K_{d}\), from which the combined value \(k_{2}/K_{d}\) can also be calculated. Since this \(k_{2}/K_{d}\) value was generated from a single determined point, it would be an approximate value, not as accurate as that obtained with eq 2. The first-order deacylation rate constant
$k_3$ for the third step of Scheme 1 is described by eq 3: $[\text{PBP-I}]/[\text{PBP}]_{\text{total}} = \exp (-k_3 t)$, where $[\text{PBP-I}]/[\text{PBP}]_{\text{total}}$ is the ratio of acyl-PBP to total PBP after incubation of acyl-PBP for time $t$.

*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 gels from Bio-Rad were used to detect the PBPs and to monitor their purity during the purification.

*Chemicals.* Penicillin-G (> 99 %) and cefotaxime (> 99 %) were purchased from Fluka.
RESULTS

Cloning, expression and purification of PBP2x<sup>R</sup> from S. pneumoniae CS 109. A truncated, soluble form of PBP2x<sup>R</sup> protein was cloned, expressed and purified to homogeneity as detailed in Experimental Procedures. The protein was expressed in <i>E. coli</i> in soluble form and large quantity. A purification protocol was developed consisting of three steps: anion exchange chromatography (Q-Sepharose), hydrophobic interaction chromatography (Phenyl-Sepharose), and gel filtration (Superdex 75). With this protocol we routinely obtained over 200 mg of PBP2x<sup>R</sup> with more than 95 % purity from ~ 3000 mg of <i>E. coli</i> protein extract. The molecular weight of the purified protein was determined to be 77,080 (± 5) Da by electrospray mass spectrometry (ESMS), which is good agreement with a calculated mass of 77,077 Da for the truncated protein (Gly49 to Asp750). This indicates that the cloned gene has the same sequence as reported (21).

Occasionally, PBP2x<sup>R</sup> protein dimer was obtained, which could still be 100 % acylated by pen-G, but its acylation rate decreased substantially. PBP2x<sup>R</sup> monomer protein was used throughout this work.

Purification and molecular mass characterization of PBP2x. PBP2x from <i>S. pneumoniae</i> strain 6R was expressed and purified, as described in Experimental Procedures, to ~ 95 % purity based on SDS-PAGE. Three protein species with molecular weights of 78,312 Da (75 %), 78,426 Da (15 %) and 78,154 Da (10 %) were determined for the purified protein preparations by ESMS. These values were quite different from the value of 78,981 Da of the truncated PBP2x (Met1 to Asp720) according to the sequence of pCG31 (21). N-terminal amino acid analysis of the PBP2x preparations showed that the protein had been partly digested from the N-terminus. Instead of Met1, the major protein species actually started from Arg6 with a calculated MW of 78,306, which is close to the major species determined by ESMS. The values of molecular mass of the two minor species are consistent with the proteins starting from Lys5 and Val7, respectively. Such digestion may have occurred during the <i>E. coli</i> expression of the recombinant protein or during the purification. Similar heterogeneous behavior of molecular mass has been reported for the same PBP2x protein (23). Upon incubation of the protein with pen-G, all three species increased their molecular mass by 335 Da as determined by ESMS, indicating that all of them were active in binding the β-lactam. The purified
PBP2x hydrolyzed thiolester substrates (S2d and S2c) with specific activity the same as previously reported (17). A much slower (~ 1000-fold) activity for hydrolyzing the two substrates was found for PBP2xR.

**Acylation of PBP2xR by pen-G.** Upon incubation with pen-G, PBP2xR was acylated as indicated by an increase in molecular mass from 77,078 to 77,412 Da as determined by ESMS (Fig. 1A). At a given penicillin (200 µM) concentration, the amount or % of the PBP2xR-pen-G adduct formed increased with the incubation time (Fig. 1). The data points were fit to eq 1, from which the apparent rate constant, $k_a$, was generated (Fig. 1B). With this approach, the time-dependent acylation of PBP2xR was determined at various concentrations of pen-G from 50 µM to 4 mM (Fig. 2A). At each concentration, the reaction followed the single exponential of eq 1, from which the values of $k_a$ were obtained. The $k_a$ values showed a hyperbolic dependence upon the concentration of pen-G (Fig 2B). The simplest model for this saturation behavior is that the penicillin first binds rapidly and reversibly to PBP2xR and then the first-order acylation occurs as shown by Scheme 1 (15,16,27,28). The data on Fig. 2B were fit to eq 2, from which the values for $K_d$ of 4 mM and $k_2$ of 0.56 s$^{-1}$ were derived. From these two values, the value of $k_2/K_d$ was calculated to be 137 M$^{-1}$ s$^{-1}$.

**Acylation of PBP2x by pen-G.** With the same approaches, pen-G acylation of PBP2x was studied. First, we found that the acylation rate was the same for the three species of the protein with slightly different molecular mass, indicating that those differences had little effect on the kinetics of interacting with pen-G. Therefore, only the major species (78,312 Da) was followed in the kinetic analysis. As shown in Fig. 3, the acylation of PBP2x at various pen-G concentrations was all time-dependent and followed the single exponential of eq 1, from which $k_a$ values were derived. The hyperbolic nature of the plot of $k_a$ versus pen-G concentrations suggests again that the acylation of PBP2x follows the mechanism shown by Scheme 1. From the plot of Fig. 3B, a $k_2$ value of 180 s$^{-1}$ and a $K_d$ value of 0.9 mM were generated, giving the combined $k_2/K_d$ value of 200,000 M$^{-1}$ s$^{-1}$.

**Deacylation of PBP2xR-pen-G and PBP2x-pen-G adducts.** In order to assess whether the deacylation step plays any part in penicillin resistance of PBP2xR, we determined deacylation rate constants ($k_3$) for
penicilloyl adducts of PBP2x and of PBP2x\(^R\). In preparation of the acyl-PBP adducts, complete (100 %) acylation, determined by ESMS analysis, was obtained for PBP2x when either the centrifugation/washing or the spun column method was used to remove the unbound β-lactam. Acyl-PBP2x\(^R\) preparations with lower acylation (80 % or less) were obtained using the centrifugation/washing method, while > 95 % acylation was achieved for the PBP when the spun column method was used. These results can be explained by \(k_3\) values determined for the two complexes and by the difference of the time needed for removing unbound pen-G by the centrifugation/washing method (~ 40 min) and by the spun column method (~ 1 min). The \(k_3\) value for deacylation of penicilloyl-PBP2x at 37 °C was determined to be 8 \(\times\) 10\(^{-6}\) s\(^{-1}\), and a much faster value, 5.7 \(\times\) 10\(^{-4}\) s\(^{-1}\), was found for the PBP2x\(^R\) complex (Fig. 4). At room temperature (22 °C), a \(k_3\) value of 8.5 \(\times\) 10\(^{-5}\) s\(^{-1}\) was obtained for the penicilloyl-PBP2x\(^R\) complex.

**Kinetics of the interactions of cefotaxime with PBP2x\(^R\) and PBP2x.** We also characterized the interactions of the two PBP2x proteins with cefotaxime, one of the so called extended spectrum (or third generation) cephalosporins, since PBP2x\(^R\) of strain CS109 had been identified as one of the cefotaxime resistant determinants of the resistant strain. As shown in Fig. 5, saturation kinetics, indicative of the Scheme 1 mechanism, were also observed for both PBPs. From the plots, the acylation kinetic parameters for PBP2x (\(k_2 = 150\) s\(^{-1}\) and \(K_d = 1.27\) mM) and for PBP2x\(^R\) (\(k_2 = 2.5\) s\(^{-1}\) and \(K_d = 4.3\) mM) were obtained. The resultant \(k_2/K_d\) values of 118,000 M\(^{-1}\)s\(^{-1}\) for PBP2x and of 580 M\(^{-1}\)s\(^{-1}\) for PBP2x\(^R\) indicate ~ 200 fold decrease in the acylation efficiency by PBP2x\(^R\). In addition, cefotaxime-PBP2x\(^R\) displayed a significantly faster deacylation rate than that of cefotaxime-PBP2x (Fig. 6), from which the deacylation rate constant (\(k_3\)) for the PBP2x and PBP2x\(^R\) adducts were determined to be 3.5 \(\times\) 10\(^{-6}\) s\(^{-1}\) and 3 \(\times\) 10\(^{-4}\) s\(^{-1}\), respectively, indicating a substantial deacylation rate increase (85-fold) for the resistant PBP2x\(^R\).
DISCUSSION

The observations of saturation binding kinetics between the β-lactams and PBP2x or PBP2xR are consistent with the two-step acylation mechanism as illustrated by Scheme 1 (13,16,27,28). The first step is a rapid formation of the reversible Michaelis complex, which is followed by the second step of irreversible ester bond formation between the β-lactams and the catalytic site serine (S344) of the proteins. Such a two-step mechanism has been observed for the R61 DD-carboxypeptidase (13) and β-lactam resistant PBP2a of MRSA (16), and this is the first evidence to confirm this mechanism for a penicillin-susceptible HMM PBP such as PBP2x.

The second order rate constants, $k_2/K_d$, referred to as the best measure of inhibitory potency of a β-lactam, have been determined for PBP2x interacting with pen-G (200,000 M$^{-1}$s$^{-1}$) and with cefotaxime (118,000 M$^{-1}$s$^{-1}$) (Table 1), reflecting β-lactam antibiotics’ high efficiency as irreversible inhibitors of the susceptible PBP. Close but different $k_2/K_d$ values were reported before for the same PBP2x interacting with pen-G (58,000 and 99,000 M$^{-1}$s$^{-1}$) and with cefotaxime (162,000 and 209,000 M$^{-1}$s$^{-1}$) (17,18). In contrast to the approach used here, these values were generated by following β-lactam quenching of the intrinsic fluorescence of PBP2x, and calculated based on the determination of a single $k_a$ value and eq 2-1 (17,19).

The microscopic kinetic parameters for acylation of PBP2x by pen-G ($k_2 = 180$ s$^{-1}$ and $K_d = 0.9$ mM) and by cefotaxime ($k_2 = 150$ s$^{-1}$ and $K_d = 1.3$ mM) (Table 1) have been determined the first time for a penicillin-susceptible HMM PBP. These $k_2$ values are very fast and comparable to that (173 s$^{-1}$) of pen-G acylation of the S. aureus PC1 β-lactamase, regarded as one of the most efficient enzymes (31,32). This indicates that the β-lactam acylation reaction is extremely efficient. However, the $K_d$ values for PBP2x are over 100-fold higher than that (8.9 µM) of the PC1 β-lactamase, indicating a very poor initial recognition of the β-lactams by the PBP. The kinetic parameters ($k_2 = 180$ s$^{-1}$ and $K_d = 13$ mM) have been determined for the R61 DD-carboxypeptidase, a LMM penicillin-susceptible PBP (13). It appears that common kinetic features of the β-lactam acylation of these penicillin-susceptible PBPs include rather high, millimolar range $K_d$ values and a very fast acylation rate constant. These observations suggest that the binding potency of β-lactams such
Interactions of PBP2x and PBP2x\textsuperscript{R} with \(\beta\)-Lactams

as pen-G and cefotaxime is mostly due to their extremely efficient ester bond formation with the active site serine of a susceptible PBP. An initial high fit of \(\beta\)-lactams to the PBPs, i.e., low \(K_d\) values, appears not to be required for achieving high potency. Consistent with these conclusions is the recent observation that \(k_2/K_d\) values of a PBP with different \(\beta\)-lactams do not correlate with the interaction energies (which reflect a ligand’s complementarity) between the PBP and the \(\beta\)-lactams based on thermal denaturation studies of \textit{E. coli} PBP5, indicating that a complementary fit of a \(\beta\)-lactam to PBP is not required for acylation (33).

These data are significant in that they may provide insights into why \(\beta\)-lactams such as penicillin are such good broad-spectrum antibacterial agents, being able to hit multiple targets (PBPs) simultaneously and effectively. The high \(K_d\) values indicate a poor fitting to the active sites of PBPs. This property, on the other hand, renders them the flexibility to initially recognize, albeit rather poorly or weakly, a broad range of PBPs with quite different active site pockets. The high potency of the \(\beta\)-lactams to PBPs is due mainly to the high \(k_2\) values of the acylation, which rapidly drive the reaction (Scheme 1) to the right irreversibly. All PBPs contain the three conserved motifs, SXXK, SXN and K(H)T(X)G, in their penicillin-binding domain, a unique feature related to their ability to recognize the D-ala-D-ala configuration (34). This feature sets PBPs apart from other classes of serine proteases and provides for the selectivity of \(\beta\)-lactam for PBPs since penicillin is a structural analogue of D-ala-D-ala. However, apart from the conserved three motifs, the amino acid sequence homology is generally rather low among the PBPs, around 20 to 40\% including the active site regions (34,35). This indicates that the 3-D structures of the active site pockets will probably have significant differences among PBPs, considering there are generally more than 15 amino acid residues involved around a protein active site. It is rather difficult for a conventional or reversible inhibitor to bind tightly to, thus to strongly inhibit, all or most of these enzymes. The \(\beta\)-lactam antibiotics are able to do so thanks to their unique kinetic properties.

The \(k_2/K_d\) values, 580 M\(^{-1}\)s\(^{-1}\) with cefotaxime and 137 M\(^{-1}\)s\(^{-1}\) with pen-G, determined for PBP2x\textsuperscript{R} are 2 to 3 orders of magnitude lower than that for PBP2x and are the lowest among the penicillin-resistant PBP2x variants (18,19). These values also correlate well with the observations that the MIC values for the strain
CS109 increased 250- and 500-fold for pen-G and cefotaxime, respectively, as compared with that (0.016 µg/ml for pen-G and cefotaxime) of the susceptible strain R6, the host of PBP2x, and that PBP2x<sup>R</sup> plays a major role in β-lactam resistance (21). When the individual parameters are compared (Table 1), $k_2$ values for PBP2x<sup>R</sup> decrease 300-fold for pen-G and 60-fold for cefotaxime, and the $K_d$ values increase 5-fold and 3-fold for pen-G and cefotaxime, respectively, reflecting a much smaller loss in the initial recognition affinity by the resistant PBP. It is apparent that the low binding affinity of the β-lactams to PBP2x<sup>R</sup> is mostly attributable to the decreased efficiency of formation of the ester bond and to a lesser extent their poorer recognition of the active site of the resistant PBP. Similarly, an extremely low $k_2$ value (0.22 s<sup>-1</sup>) and a high $K_d$ value (13 mM) were found early on for PBP2a of MRSA (16). These results suggest that for PBP resistance development, decreasing of $k_2$ is preferable. Increasing $K_d$ only offers limited advantages, perhaps because of the susceptible PBP’s already very high $K_d$ values. A $k_2/K_d$ value of 3,300 M<sup>-1</sup>s<sup>-1</sup> was reported for cefotaxime acylation of a penicillin-resistant PBP2x<sup>R</sup> from <i>S. pneumoniae</i> strain Sp328, but, the individual kinetic parameters were not resolved (18). CS109 PBP2x<sup>R</sup> and Sp328 PBP2x<sup>R</sup> were classified into <i>S. pneumonia</i> strain group V and IV, respectively, based on their nucleotide substitutions within conserved amino acid motifs (20). Group V strains are distinguished from other groups by their significantly higher levels of cefotaxime resistance (MIC > 2 µg/ml. This value is 8 µg/ml for strain CS109). In agreement with this is the significant decreased (6-fold) $k_2/K_d$ value for CS109 PBP2x<sup>R</sup> with cefotaxime compared to that for Sp328 PBP2x<sup>R</sup>.

The amino acid sequence of PBP2x<sup>R</sup> of strain CS109 is 90 % identical to that of the penicillin-susceptible 6R PBP2x (21). Within the 351-residue penicillin-binding (or transpepetidase) domain (residues 266 to 616 (19)), 36 sites of the CS109 PBP2x<sup>R</sup> have changed (21). Among these changes is the substitution at Thr<sup>338</sup> (by Ala). This substitution occurs in about 80 % of resistant PBP2x<sup>R</sup> proteins and has been confirmed as one of the critical resistance determinants by site-directed mutagenesis (18). Three other substitutions at Glu<sup>552</sup>, Thr<sup>550</sup> and Ser<sup>571</sup> have also been identified as key positions for resistance development based on their frequency of occurrence in resistant PBP2x<sup>R</sup> variants, high resolution structure of a complex between PBP2x and cefuroxime, and site-directed mutagenesis studies (18,19). However, none of these three substitutions exists in CS109 PBP2x<sup>R</sup> (21), one of the PBP2x<sup>R</sup> proteins with the strongest β-lactam resistance activity known to
date. Two of three positions, Glu$^{552}$ and Thr$^{550}$, are located on strand β3, identified as a part of the active site groove playing a critical role in resistance development (19). However, the amino acid sequence of strand β3 in CS109 PBP2x$^{R}$ is identical to that of 6R PBP2x. These data indicate that the substitution(s) on strand β3 may not necessarily be required for PBP2x resistance development in contrast to what has been suggested before (18,19).

The deacylation rate constant, $k_3 = 0.8 \times 10^{-5} \text{ s}^{-1}$, determined here for PBP2x is almost identical to the value reported recently for the same PBP (36). This value, in the range determined for a number of β-lactam sensitive PBPs (29) and corresponding to a half-life of 24 h, means that the acyl-PBP adduct is very stable. Deacylation of β-lactam/PBP complexes has been studied for other β-lactam resistant PBPs. The value, $1.5 \times 10^{-5} \text{ s}^{-1}$, determined for PBP2a of MRSA, indicates that the acyl-PBP2a adduct is stable with $t_{1/2} > 13$ h. This $k_3$ value is also comparable to that of penicillin-sensitive PBPs, indicating little role played by this step in PBP2a’s resistance mechanism (16). No detectable deacylation of penicillin-Sp328 PBP2x$^{R}$ complex was observed upon incubation up to 90 min (indicating a $k_3$ value of $< 1 \times 10^{-5} \text{ s}^{-1}$) in a dilution/SDS PAGE based experiment (23). However, a value of $3 \times 10^{-5} \text{ s}^{-1}$, determined using an ESMS method, was reported recently for the same PBP2x$^{R}$ complex (36). This 3-fold increase is quite significant, although the value is still 19-fold slower than that of CS109 PBP2x$^{R}$ determined here.

A potential way for a PBP to develop β-lactam resistance, as speculated in the literature (30,37, is to increase the deacylation rate, so that the PBP becomes active more quickly to resume peptidoglycan synthesis. We provide here the first evidence that this indeed is the case with CS109 PBP2x$^{R}$. The $k_3$ values for penicilloyl-PBP2x$^{R}$ and cefotaxime-PBP2x$^{R}$ are 71- and 85-fold faster than those of their respective acyl-PBP2x adducts (Table 1). By increasing $k_3$ from $0.8 \times 10^{-5} \text{ s}^{-1}$ for penicilloyl-PBP2x to $5.7 \times 10^{-4} \text{ s}^{-1}$ for penicilloyl-PBP2x$^{R}$, the half-life ($t_{1/2}$) of the PBP adducts is decreased dramatically from 24 h to just 20 min. Similarly, the $t_{1/2}$ value is reduced from 54 h for cefotaxime-PBP2x adduct to merely 40 min for cefotaxime-PBP2x$^{R}$ complex based on their respective $k_3$ values.
The $t_{1/2}$ values of 20 or 40 min suggest that in every 20 or 40 min, 50% of acylated PBP2x$^R$ will become deacylated and active again in the absence or presence of low concentration of the respective $\beta$-lactams.

The percentage of PBP acylated or ratio of acyl-PBP ([PBP-I]) to total PBP ([PBP]$_{\text{total}}$) can be derived from eq 4: $\frac{\text{[acyl-PBP]}}{\text{[PBP]}_{\text{total}}}=\frac{k_a}{k_a + k_3}$ at a given $\beta$-lactam concentration according to the literature (14).

Under normal conditions (i.e., $k_3 \ll k_2$ and/or high $\beta$-lactam concentrations), the contribution of $k_3$ to the ratio of acylated versus total PBP is negligible. However, when $k_2/K_d$ is diminished, thus $k_a$ is decreased, the contribution of $k_3$ becomes significant. For instance, at 6 $\mu$M pen-G, the $k_a$ for acylation of PBP2x$^R$ will be 0.00084 s$^{-1}$ according to eq 2, and just 56% of the PBP will be acylated at the steady state based on eq 4.

It should be noted that the MIC value for the CS109 strain is 6 $\mu$M, and at this concentration, a significant proportion of PBP2x$^R$ being free of acylation is consistent with the notion that PBP2x$^R$ plays a major role in $\beta$-lactam resistance of the organism. Since the deacylation rate relates with a $\beta$-lactam’s half-life in therapy, it is apparent that, combined with decreased values of $k_2/K_d$, these much faster $k_3$ values would reduce significantly the drug’s half-life in therapeutic situations, thus, rendering the bacteria resistant to the $\beta$-lactams.

**Acknowledgments**

We thank Suzanne Paule for help in gene cloning, Feng Wang for N-terminal analysis, Jauh Lin Duh for assistance in protein purification and sample preparations, and Karen Howard-Nordan for critical reading of the manuscript.
REFERENCES


Figure legends

Fig. 1. Penicillin-G binding to PBP2xR as a function of time at a given pen-G concentration as determined by ESMS. (A) Representative reconstructed electrospray mass spectra of PBP2xR and acylated PBP2xR recorded after incubation of the protein with 200 µM pen-G for 5 (a), 15 (b) and 60 (c) sec at 37 °C. In each figure, the peak on the left (77,078 Da) corresponds to PBP2xR and the other one (77,412 Da) represents the penicilloyl-PBP2xR complex. From the relative height of the two peaks, the percentage of protein becoming acylated at each time point was calculated as described before (16). In this case, PBP2xR was 22 % (a), 40 % (b) and 72 % (c) acylated, respectively. (B) Percentage of penicilloyl-PBP2xR formed versus reaction time. The solid trace represents the best fit to the single exponential according to eq 1, from which the apparent first-order rate constant, \( k_a = 2.4 \text{ min}^{-1} \), was derived.

Fig. 2. Kinetics of pen-G acylation of PBP2xR determined by ESMS. (A) Time- and pen-G concentration-dependent acylation of PBP2xR. Five representative traces at pen-G concentrations of (from bottom to top) 0.2, 0.5, 1, 2 and 4 mM are shown. The solid lines represent the best fits (\( k_a \) values) to eq 1. (B) Plot of the \( k_a \) values as a function of the concentrations of pen-G from 0.05 to 5 mM. The solid line is the best fit to the hyperbola based on eq 2, corresponding to \( K_d = 4.1 \text{ mM} \) and \( k_2 = 0.56 \text{ s}^{-1} \).

Fig. 3. Kinetics of pen-G acylation of PBP2x determined by ESMS. (A) Primary data of acylation time courses at pen-G concentrations of 30, 100, 200, 500 and 1000 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 pen-G. The curve represents the best fit of the data to eq 2 with \( K_d = 0.9 \text{ mM} \) and \( k_2 = 180 \text{ s}^{-1} \).

Fig. 4. Deacylation of penicilloyl-PBP2x or penicilloyl-PBP2xR. Penicilloyl-PBP2x (■) or Penicilloyl-PBP2xR (●) was incubated at 37 °C for various time intervals before analysis by 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 \( k_3 = 8 \times 10^{-6} \text{ s}^{-1} \) (■) for PBP2x and \( 5.7 \times 10^{-4} \text{ s}^{-1} \) (●) for PBP2xR, respectively.
Fig. 5. Kinetics of acylation of PBP2x\(^R\) or PBP2x by cefotaxime. (A) Plot of the \(k_a\) values for PBP2x as a function of the concentrations of cefotaxime from 0.1 to 2 mM. The solid line is the best fit to the hyperbola based on eq 2, corresponding to \(K_d = 1.27 \text{ mM}\) and \(k_2 = 150 \text{ s}^{-1}\). (B) Plot of the \(k_a\) values for PBP2x\(^R\) as a function of the concentrations of cefotaxime from 0.05 to 1 mM. The solid line is the best fit to the hyperbola based on eq 2, corresponding to \(K_d = 4.3\) mM and \(k_2 = 2.5 \text{ s}^{-1}\).

Fig. 6. Deacylation of cefotaxime-PBP2x and -PBP2x\(^R\) adducts. Acyl-PBP2x (■) or Acyl-PBP2x\(^R\) (●) was incubated at 37 °C for various times before analysis by 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 \(k_3 = 3.5 \times 10^{-6}\) s\(^{-1}\) (■) for PBP2x and \(3 \times 10^{-4}\) s\(^{-1}\) (●) for PBP2x\(^R\), respectively.

Table 1. Kinetic Parameters of Interactions between HMM PBPs and Penicillin-G/Cefotaxime

<table>
<thead>
<tr>
<th>PBP</th>
<th>β-lactam</th>
<th>(k_2/K_d) (M(^{-1})s(^{-1}))</th>
<th>(k_2) (s(^{-1}))</th>
<th>(K_d) (mM)</th>
<th>(k_3) (s(^{-1}))</th>
<th>(t_{1/2}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBP2x</td>
<td>Penicillin-G</td>
<td>200,000</td>
<td>180</td>
<td>0.9</td>
<td>8 x 10(^{-6})</td>
<td>24</td>
</tr>
<tr>
<td>PBP2x(^R)</td>
<td>Penicillin-G</td>
<td>137</td>
<td>0.56</td>
<td>4</td>
<td>5.7 x 10(^{-4})</td>
<td>0.33</td>
</tr>
<tr>
<td>PBP2a</td>
<td>Penicillin-G</td>
<td>17</td>
<td>0.22</td>
<td>13</td>
<td>1.5 x 10(^{-5})</td>
<td>13</td>
</tr>
<tr>
<td>PBP2x</td>
<td>Cefotaxime</td>
<td>118,000</td>
<td>150</td>
<td>1.27</td>
<td>3.5 x 10(^{-6})</td>
<td>54</td>
</tr>
<tr>
<td>PBP2x(^R)</td>
<td>Cefotaxime</td>
<td>580</td>
<td>2.5</td>
<td>4.3</td>
<td>3 x 10(^{-4})</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Data for PBP2x from penicillin-susceptible *S. pneumoniae* strain 6R and PBP2x\(^R\) from penicillin-resistant *S. pneumoniae* strain CS109 are from this study and for β-lactam-resistant PBP2a from methicillin-resistant *S. aureus* are from early studies (16). All values were determined with the ESMS method. The values of \(t_{1/2}\), half-life of acyl-PBP adducts, were calculated with the equation: \(t_{1/2} = 0.693/k_3\).
Fig. 2

A

% of PB2P2<sup>R</sup> acylated vs. Time (Sec)

B

$\kappa_a$ (min<sup>-1</sup>) vs. Penicillin-G (mM)
Fig. 3

A

% of PBP2x acylated by pen-G

Time (Sec)

B

$K_a$ (sec$^{-1}$)

Penicillin-G (mM)
Fig. 4

% of PBP2x or PBP2x^R remaining acylated

Time (hour)
Fig. 6

- Graph showing the percentage of PBP2x or PBP2xR remaining acylated over time (hour).
- The graph displays a clear decrease in the percentage from 100% at time 0 to near 0% as time increases.
Kinetics of beta-lactam interactions with penicillin-susceptible and -resistant penicillin-binding protein 2x proteins from streptococcus pneumoniae: Involvement of acylation and deacylation in beta-lactam resistance
Wei-Ping Lu, Erica Kincaid, Yiping Sun and Mark D. Bauer

J. Biol. Chem. published online June 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102499200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts