Penicillin-binding protein 2a (PBP2a) of Staphylococcus aureus is refractory to inhibition by available β-lactam antibiotics, resulting in resistance to these antibiotics. The strains of S. aureus that have acquired the mecA gene for PBP2a are designated as methicillin-resistant S. aureus (MRSA). The mecA gene was cloned and expressed in Escherichia coli, and PBP2a was purified to homogeneity. The kinetic parameters for interactions of several β-lactam antibiotics (penicillins, cephalosporins, and a carbapenem) and PBP2a were evaluated. The enzyme manifests resistance to covalent modification by β-lactam antibiotics at the active site serine residue in two ways. First, the microscopic rate constant for acylation (k_a) is attenuated by 3 to 4 orders of magnitude over the corresponding determinations for penicillin-sensitive penicillin-binding proteins. Second, the enzyme shows elevated dissociation constants (K_d) for the non-covalent pre-acylation complexes with the antibiotics, the formation of which ultimately would lead to enzyme acylation. The two factors working in concert effectively prevent enzyme acylation by the antibiotics in vivo, giving rise to drug resistance. Given the opportunity to form the acyl enzyme species in in vitro experiments, circular dichroism measurements revealed that the enzyme undergoes substantial conformational changes in the course of the process that would lead to enzyme acylation. The observed conformational changes are likely to be a hallmark for how this enzyme carries out its catalytic function in cross-linking the bacterial cell wall.
obtain the K406A mutant two mutagenic primers, MecSAa-α (CCGGTAGCTTTGAGGCATTAAAGCAGAATG) and MecSAa-β (CCGGTAGCTTTGAGGCAACCGTGATATT), which contain the codon GCA (in bold) for alanine instead of that for lysine (AAA) were used. Two other mutagenic primers, SaurY519PD (GCTGATTCAGGTTTGGCGGAAATGATG) and SaurY519PD (GCTGATTCAGGTTTGGCGGAAATGATG), that contain the TTC codon for phenylalanine (in bold) were used to introduce the Y519F derivative of PBP2a. The double mutant enzyme, K406A/Y519F, was produced by introducing a second substitution into the K406A mutant derivative. After mutagenesis the nucleotide sequence for each of the genes producing mutant enzymes was verified, and these genes were cloned between the NcoI and HindIII sites of the pET24d(+) expression vector.

**Expression of Wild-type PBP2a and Its Mutant Variants**

K406A, K406A/Y519F, and Y519F in _E. coli_. The wild-type PBP2a and K406A, K406A/Y519F, and Y519F mutant variants were each expressed using the same method _E. coli_. BL21 (DE3) was transformed with the plasmid pET24d(+), which contained the wild-type and mutant _mecA_ gene in its multiple cloning site. A 3-ml overnight seed culture was used to inoculate 500 ml of the LB medium supplemented with kanamycin (30 μg/ml). Cells were grown at 37 °C with shaking (120 rpm) until the A600 reached ~0.8 (about 6 h) followed by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside to induce expression. The bacterial culture was then incubated at 25 °C for another 20 h. Cells were harvested by centrifugation at 5500 × g for 10 min at 4 °C, and the pellet was suspended in 10 mM Tris/HCl buffer, pH 8 (buffer A).

**Purification of Wild-type and Mutant PBP2a**

The wild-type PBP2a and its mutants K406A, K406A/Y519F, and Y519F were each purified using the same three-step purification protocol with an LP chromatography system (Pharmacia) at 4 °C using the same three-step purification protocol with an LP chromatography system (Pharmacia) at 4 °C. The bacterial culture was then incubated at 25 °C for another 20 h. Cells were harvested by centrifugation at 5500 × g for 10 min at 4 °C, and the pellet was suspended in 10 mM Tris/HCl buffer, pH 8 (buffer A).

The CD spectra of the wild-type PBP2a (6 mg in 25 mM Heps, 1 mM NaCl, pH 7.0) were recorded on a Jasco J-600 instrument (Easton, MD, 5-mm path length) in the absence and presence of 30 μM oxacillin or 30 μM ceftazidime. The _β_-lactams had negligible CD readings compared with the protein. Regardless, the contribution of the _β_-lactam substrate was subtracted in each case. The proteins were incubated with the _β_-lactam antibiotics at 25 °C.

**RESULTS AND DISCUSSION**

PBP2a has been cloned and studied by others (20–25). We have cloned this protein for our studies as well. The _mecA_ gene was PCR-amplified from the chromosomal DNA of _S. aureus_ ATCC706986 without the 69 base pairs in the 5′-region encoding the 23-amino acid-long N-terminal membrane anchor. The gene was cloned between the NcoI and HindIII sites of the expression vector pET24d(+), and PBP2a was produced intracellularly after induction with isopropyl-β-D-thiogalactopyranoside. The gene was recoloned into the XbaI and HindIII sites of the smaller plasmid pUC19 to produce mutant variants of the _mecA_ gene efficiently. Subsequent to mutagenesis, the corresponding genes were recoloned back between the NcoI and HindIII sites of the pET24d(+) vector, and the enzymes were produced intracellularly by isopropyl-β-D-thiogalactopyranoside induction. PBP2a was purified to apparent homogeneity in three chromatographic steps. We typically obtained ~40 mg of pure protein from a liter of culture.

We have evaluated the kinetics of interactions of three cephalosporins (nitrocefin, cepfime and ceftazidime), two penicillins (ampicillin and oxacillin), and one carbapenem (imipenem) with PBP2a (Table I). PBP2a, as with virtually all other known PBPs, undergoes acylation with its peptidoglycan substrate at an active site serine (Ser-403) for its transpeptidase activity (cell wall cross-linking). The acyl enzyme species then undergoes the transpeptidation reaction with another...
The Basis for Resistance to β-Lactams by PBP\textsubscript{2}a of MRSA

### Table I

| β-Lactams | $k_2$ | $k_3$ | $K_d$ | $k_2/K_3$ |
|-----------|-------|-------|-------|-----------|
| Nitrocefin | $3.7 \pm 0.3$ | 7.2 | 2±0.1 | $192 \pm 24$ | $19.0 \pm 3.0$ |
| Cefepime | 1.5 | 5.9 | 0.5 | 1818 | 145 | 0.3 | 0.1 |
| Ceftazidime | 1.0 | 0.1 | 3.2 | 0.2 | 671 | 116 | 1.5 | 0.3 |
| Ampicillin | 3.4 | 0.1 | 3.2 | 0.1 | 668 | 124 | 5.0 | 1.0 |
| Oxacillin | 1.6 | 0.1 | 2.5 | 0.1 | 180 | 25 | 9.0 | 1.0 |
| Imipenem | 1.7 | 0.1 | 3.3 | 0.3 | 603 | 93 | 2.8 | 0.4 |

### Table II

| Enzyme | Inhibitor | $k_2$ | $K_d$ | $k_2/K_d$ |
|--------|-----------|-------|-------|-----------|
| K406A | Nitrocefin | 4.5 | 0.1 | 200 | 70 | 22.0 | 9.0 |
| Ceftazidime | 0.8 | 0.1 | 510 | 100 | 1.8 | 0.3 |
| Oxacillin | 1.2 | 0.5 | 800 | 80 | 1.5 | 0.5 |
| Y519F | Nitrocefin | 280 | 40 | 230 | 50 | 1200 | 300 |
| Ceftazidime | 150 | 10 | 1400 | 220 | 250 | 20 |
| Oxacillin | 260 | 20 | 590 | 15 | 440 | 22 |
| K406A/Y519F | Nitrocefin | 5.2 | 1.1 | 200 | 60 | 27 | 10 |
| Ceftazidime | 1.9 | 0.2 | 930 | 200 | 2.0 | 0.5 |
| Oxacillin | 1.2 | 0.3 | 1100 | 60 | 1.1 | 0.2 |

strand of the peptidoglycan. β-Lactam antibiotics subvert this process by undergoing the enzyme acylation process, but the resulting complex is often stable such that the enzyme is inactivated, and the organism is deprived of its vital function.

The processes for interactions of β-lactam antibiotics with PBP\textsubscript{2}a were sufficiently slow that the need for stopped-flow rapid kinetics was obviated. It is noteworthy that manifestation of resistance is because of both a slow rate of enzyme acylation ($k_2$ effect) as well as an absence of high affinity of the enzyme for β-lactams in general ($K_d$ effect). The $t_{1/2}$ of enzyme acylation was in the range of 3 to 12 min with these antibiotics. This contrasts dramatically to $t_{1/2}$ values of low milliseconds for typical penicillin-sensitive PBPs (26, 27). The elevated dissociation constants for the pre-acylation complexes ranged between 180 and 1618 $\mu$M, resulting in second-order rate constants ($k_2K_d^2$) of 1–19 $s^{-1}$.

The $t_{1/2}$ values for deacylation ($k_3$) of the acyl enzyme species were exceedingly poor, giving $t_{1/2}$ values in the range of 26 to 77 h. Considering that S. \textit{aureus} doubles its population size in 20–30 min under favorable growth conditions, the formation of the acyl enzyme species is irreversible for practical purposes. In essence, the non-covalent encounters between the antibiotics and PBP\textsubscript{2}a are not favorable (high $K_d$), and the rate constants for enzyme acylation are exceedingly poor (slow $k_2$). Hence, formation of the acyl enzyme species would not take place in vivo for these two reasons. The fact that the acyl enzyme species with β-lactam antibiotics is extremely stable is irrelevant to the resistance problem, as the species would simply not form in vivo. Considering that PBP\textsubscript{2}a fulfills the critical physiological needs of the bacterium in the presence of β-lactam antibiotics, the set of events that led to the evolution of this important resistance enzyme to antibiotics is quite remarkable (28).

We hasten to add that the kinetic parameters that we report herein are somewhat different from those reported by Lu \textit{et al.} (29), who used a mass spectrometric approach for analysis of kinetics. Whereas their $K_d$ values are sufficiently high to preclude enzyme acylation when considering the in vivo situation, the corresponding numbers by Lu \textit{et al.} (29) were substantially higher than ours (high millimolar range).

The issue of activation of the active site serine of interest. As will be discussed below, Ser-403 is well sheltered within the active site and its side chain hydroxyl is in contact with the side chain of Lys-406 (8). This arrangement of Ser-X-X-Lys for PBPs and related β-lactamases is understood to be important for the mechanisms of these enzymes (30). We have shown that when the corresponding lysine is mutated to alanine in the OXA-10 β-lactamase, the enzyme cannot undergo acylation by its substrate (31). A similar mutation in the penicillin-binding protein BlaR from \textit{S. aureus} was shown to attenuate the rate of protein acylation by 6730-fold (32). The K406A mutant variant of PBP\textsubscript{2}a underwent extremely sluggish acylation. The effect was mostly on $k_2$, which was attenuated by 80- to 130-fold for the mutant variant (Table II). Whereas the magnitude of the effect is relatively small, this level of attenuation reduces the already sluggish rate of acylation to the range of $10^{-5}$ $s^{-1}$, which is the basal level that was attained for the BlaR protein and not far from the undetectable levels seen for the same mutation in the OXA-10 enzyme. Hence, in the cases of the BlaR and the OXA-10 proteins the acylation rate constants were higher, so the drops in their magnitudes were also larger on mutation. However, the basal level that we have observed for the lysine to alanine mutant variants in all three proteins were essentially the same.

Tyr-519 is another potentially basic residue within the active site. It could potentially provide the activation if it were unprotonated in the side chain and if the side chain were to undergo rotation from the position seen in the x-ray structure. Mutant enzyme variants Y519F and K406A/Y519F gave kinetic properties similar to the wild-type and to the K406A mutant, respectively. Therefore, this tyrosine residue does not play a role in catalysis, and Lys-406 is the basic residue that promotes the active site serine for enzyme acylation (Table II).

It is noteworthy that at least one penicillin-binding protein is now shown to be carboxylated in the side chain of its active site lysine (product of carbon dioxide addition to the lysine side chain amine) (16). In light of the reversibility of lysine carboxylation in proteins, there are known examples of lysine-carboxylated proteins that were identified by x-ray crystallography in their non-carboxylated forms. Hence, there was a possibility that PBP\textsubscript{2}a might be carboxylated at Lys-406. We carried out the diagnostic $^{13}$C NMR experiment for detection of protein lysine carboxylation with PBP\textsubscript{2}a, as reported for other proteins previously (15, 16). The experiment showed that PBP\textsubscript{2}a is not carboxylated at any lysine, and thus the crystal structure depicts the correct structure for Lys-406.

As shown in Fig. 1A, the x-ray structure of PBP\textsubscript{2}a reveals that the active site of the enzyme is not an open cleft. Indeed, the access to the active site is not obvious from the x-ray structure. Lim and Strynadka (17) have shown that the acyl enzyme species with β-lactam antibiotics largely maintains the active site in the same conformation with small movements within the immediate vicinity of the ligand away from that seen in the native enzyme (Fig. 1, B and C). A conformational change to open the active site would appear to be necessary both for the turnover events with the peptidoglycan substrate and for interactions with inhibitors such as β-lactam antibiotics.

The relatively slow nature of the kinetics of the interactions of β-lactam antibiotics with PBP\textsubscript{2}a indicated to us that these interactions might be studied by circular dichroism spectroscopy to explore the possibility of such protein conformational changes. We carried out these studies with oxacillin (a penicillin) and ceftazidime (a cephalosporin). Incubation of PBP\textsubscript{2}a with either oxacillin or ceftazidime resulted in dramatic conformational changes in the protein (Fig. 2), most readily observed at the minima at 208 and 222 nm, which are because of α-helices. As revealed in Fig. 2, A and C, the helix content decreased on exposure to the antibiotic, and a set of conforma-
typical changes was noted within the first four $t_{1/2}$ values for acylation (for virtually complete protein acylation). These conformational changes continued for the duration of the monitoring for 3 days. In essence, the monitoring of the two wavelengths in the course of the experiments (Fig. 2, B and D) indicated that substantial conformational flexibility exists in the protein. The details of conformational changes were not identical in the two cases, reflecting the differences in the structures of the penicillin and cephalosporin used for these experiments. A fuller understanding of these differences should await structural-biological studies in the future.

Whereas $\sim 30\%$ of the enzymic activity was lost at the end of 3 days of the CD experiment, the conformational state of the enzyme returned largely to the native state in both CD experiments. The relatively subtle conformational change seen for x-ray structures of the acyl enzyme species compared with the native structure (8) would not account for our observations in the CD experiments. Hence, the x-ray structure shows a complex that has settled, conformationally speaking, close to the native state, such as the species that we observed near the middle of the CD determinations ($\sim 700 \text{ min}$ for oxacillin and $1400 \text{ min}$ for ceftazidime). Based on the $k_3$ values (Table I), by the end of the CD experiment, the acyl enzyme species are expected largely to have undergone hydrolysis to return to the native state.

We underscore that these conformational changes are expected to be operative during the typical turnover events by this enzyme as well in light of the closed nature of the active site. A volume in excess of $1000 \text{ Å}^3$ is needed for the sequestration of the two peptidoglycan residues within the active site for the transpeptidase activity (33). The requisite conformational change would be expected to create this space for the catalytic events. Furthermore, these conformational changes must take place substantially more rapidly for the case of the peptidoglycan substrate. Although we cannot predict at the present what may precipitate these conformational changes, it is inherently intuitive that the polymeric peptidoglycan sub-

Fig. 1. Active site of PBP2a from the x-ray structure. A, a stereo view to the active site environment from the x-ray structure of PBP2a is rendered as a solvent-accessible surface (Connolly surface, green), whereas important residues in the active site are shown in a capped sticks representation. A dotted Connolly surface (purple) is used to demonstrate the surface of the regions that cover the active site opening. B, a stereo view of the secondary structures (orange tube representation) and various important residues for the native enzyme structure is depicted. C, the penicillin G/PBP2a acyl enzyme complex is shown. (Penicillin G is shown in yellow, and capped sticks are color-coded according to atom types; oxygen, nitrogen, and carbon are shown in red, blue, and white, respectively.) The perspectives are the same for all three panels.

Fig. 2. Circular dichroic spectra of PBP2a in the presence of $\beta$-lactam antibiotics. A, the far-UV CD spectrum of the wild-type PBP2a (△) during oxacillin turnover at 1 h (○), 24 h (■), 48 h (▲), and 72 h (●). B, change in the molar ellipticity of the wild-type PBP2a at 208 (○) and 222 nm (□) as a function of time during turnover of oxacillin. C, the far-UV CD spectrum of the wild-type PBP2a (△) during ceftazidime turnover at 1 h (○), 24 h (■), 48 h (▲), and 72 h (●). D, change in the molar ellipticity of the wild-type PBP2a at 208 nm (○) and 222 nm (□) as a function of time during turnover of ceftazidime.
strate would bind at a site outside of the immediate active site to initiate the processes.

A pertinent question on activity should be whether truncation by removal of the membrane anchor would affect activity. The conclusion from studies by others is that there is no consequential difference on activity with the loss of the membrane anchor (29). This is also entirely in accordance with the x-ray structure for PBP2a, which indicates that the point of insertion into the membrane by the membrane-spanning portion is quite distal to the catalytic domain (17).

In this study we have described the kinetics of interactions of six β-lactam antibiotics with the PBP2a of S. aureus. We also documented dramatic conformational changes for the protein in the presence of these antibiotics within the time scale for these turnover events. The function of PBP2a would appear to be more complex than previously appreciated. In light of the clinical importance of this protein to resistance to β-lactam antibiotics, a more complete understanding of these processes at the structural level is required. It is with such fuller understanding of these events that we may be able to conceive of strategies for inhibition of this deleterious bacterial enzyme in the near future.

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