Resistance to β-Lactam Antibiotics and Its Mediation by the Sensor Domain of the Transmembrane BlaR Signaling Pathway in Staphylococcus aureus

Staphylococci, a leading cause of infections worldwide, have devised two mechanisms for resistance to β-lactam antibiotics. One is production of β-lactamases, hydrolytic resistance enzymes, and the other is the expression of penicillin-binding protein 2a (PBP 2a), which is not susceptible to inhibition by β-lactam antibiotics. The β-lactam sensor-transducer (BlaR), an integral membrane protein, binds β-lactam antibiotics on the cell surface and transduces the information to the cytoplasm, where gene expression is derepressed for both β-lactamase and penicillin-binding protein 2a. The gene for the sensor domain of the sensor-transducer protein (BlaRS) of Staphylococcus aureus was cloned, and the protein was purified to homogeneity. It is shown that β-lactam antibiotics covalently modify the BlaRS protein. The protein was shown to contain the unusual carboxylated lysine that activates the active site serine residue for acylation by the β-lactam antibiotics. The details of the kinetics of interactions of the BlaRS protein with a series of β-lactam antibiotics were investigated. The protein undergoes acylation by β-lactam antibiotics with microscopic rate constants ($k_2$) of 1–26 s$^{-1}$, yet the deacylation process was essentially irreversible within one cell cycle. The protein undergoes a significant conformational change on binding with β-lactam antibiotics, a process that commences at the preacylation complex and reaches its full effect after protein acylation has been accomplished. These conformational changes are likely to be central to the signal transduction events when the organism is exposed to the β-lactam antibiotic.

Staphylococci are the most common cause of bacterial infections in the United States (1). Among these organisms, methicillin-resistant Staphylococcus aureus has received notoriety since it is currently the scourge of hospitals. Staphylococci have acquired multiple drug resistance genes over the past few decades such that methicillin-resistant S. aureus can usually be treated only by glycopeptides, such as vancomycin (2), or by oxazolidinones, such as linezolid (3). It is disconcerting that for decades such that methicillin-resistant S. aureus can usually be treated only by glycopeptides, such as vancomycin (2), or by oxazolidinones, such as linezolid (3). It is disconcerting that these processes are reported herein for the first time. Furthermore, binding of the antibiotic to BlaRS entails a significant conformational change, a process that is likely to play a role in the signal transduction mechanism from the cell surface to the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—Antibiotics and other reagents were purchased from Sigma, unless otherwise stated. The growth medium was purchased either from Difco Laboratories (Detroit, Michigan) or Fisher Scientific. The chromatography media were either from Bio-Rad Laboratories or Amersham Biosciences. Escherichia coli DH5α was from Invitrogen, and E. coli BL21(DE3) and plasmid pET24a+ were from Novagen. The radioactive sodium bicarbonate (NaH$^{14}$CO$_3$; 58 mCi/mmol) was purchased from Amersham Biosciences, and NaH$^{13}$CO$_3$ was from Cambridge Isotope Laboratories. BOCILLIN FL, a derivative of penicillin V, was purchased from Molecular Probes, Inc.

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S The on-line version of this article (available at http://www.jbc.org) contains supplementary data providing a collection of CD spectra.

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* The abbreviations used are: PBP, penicillin-binding protein; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amineethanesulfonic acid.
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Cloning of the Gene for the Cell Surface Domain of S. aureus BlaR Protein—We used the plasmid pBS58 from S. aureus as the source of the blaR gene (11). We utilized high fidelity Pfu Turbo polymerase (Stratagene) to amplify the sequence that corresponds to the periplasmic portion of the BlaR protein (amino acids 331–385). Two oligonucleotide primers, Dir, 5′-ACATATGGGGAATTTACATTAGATTATTTAAATT-A-3′, and Rev, 5′-TTAATTAGCTTATGAGTATTGTTG-3′, were utilized for this purpose. These primers contain the recognition portion of the BlaR protein (amino acids 331–385), and Ser389AlaRev, 5′-GGTATTC-GGGCAATCCATAACTGATTATTATAATT-3′, and Lys392A-CATATG-GCAATCCATAACTGATTATTATAATT-3′, were used to mutate Lys-392 to Ala. The PCR products were purified using a Zymoclean™ gel DNA recovery kit (Zymo Research, Orange, CA). This fragment was cloned into HIinII sites of plasmid pUC19, and the resulting construct pUC19:blaR was used to transform E. coli DH5α. Transformants were selected on agar plates supplemented with ampicillin. Plasmid DNA from several transformants was isolated, and the presence of the inserted blaR gene was verified by digestion with NdeI and HindIII. Subsequently, the nucleotide sequence of the blaR gene was verified by sequencing of both DNA strands.

To overexpress the cell surface domain of the BlaR protein (BlaRS) in cytoplasm, we released the corresponding DNA fragment from the plasmid pUC19:blaR by HindIII and NdeI digestion and inserted the fragment into the HindIII/NdeI sites of the expression vector pET24a (+). The DNA from the ligation mixture was used to transform E. coli DH5α, and the plasmids from several transformants were analyzed by electrophoresis on a 1% agarose gel and further purified using a Zymoclean™ gel DNA recovery kit (Zymo Research, Orange, CA). This fragment was cloned into HIinII sites of plasmid pUC19, and the resulting construct pUC19:blaR was used to transform E. coli DE3. Transformants were selected on agar plates supplemented with ampicillin. Plasmid DNA from several transformants was isolated, and the presence of the inserted blaR gene was verified by digestion with NdeI and HindIII. Subsequently, the nucleotide sequence of the blaR gene was verified by sequencing of both DNA strands.

**Mutational Alteration of Ser-389 and Lys-392 in the BlaR S Protein**—The wild-type BlaR protein (BlaRS) protein resulted in quenching of protein fluorescence at 320 nm, when excited at 295 nm. Fluorometric measurements were performed in a Spex Industries (Metuchen, NJ) Fluoromax luminescence spectrophotometer. The slit width was kept at 1 nm for excitation and 1.5 nm for emission. Experiments were carried out at 25 °C in 100 mM sodium phosphate buffer (pH 7.5) and concentrated on an Ultrasave-4 centrifugal filter unit (Millipore) passed through a Bio-Spin 6, Bio-Rad column that was equilibrated with the same buffer (pH 7.5) used for all experiments (with bicarbonate supplement). A 30-μg portion of each protein was reconstituted in the pH 7.5 buffer supplemented with 10 mM unlabeled sodium bicarbonate and 4 mM 13C-labeled sodium bicarbonate (total volume 40 μl) and was incubated at room temperature for 15 min. A 2-μl portion from the reaction mixture was diluted with 28 μl of the pH 7.5 buffer, which was supplemented to give a final concentration of 10 mM unlabeled sodium bicarbonate, and the solution was passed through a desalting column (30-mg Bio-Spin 6; Bio-Rad) column equilibrated with the same buffer as the initial sample. The same procedure did not tamper with the quality of the protein since we measured pseudo-first-order rate constants for acylation of the protein by nitrocenin with and without this treatment, which were the same. **Modification of BlaR by NaH14CO3**—A 50-μl portion of the 100 μM protein solution (either wild-type BlaR or the K392A mutant variant) was diluted in 4 ml of degassed 25 mM sodium acetate buffer (pH 4.5) and concentrated on an Ultrasave-4 centrifugal filter unit (Millipore) down to ~100 μl. Buffer exchange was performed twice with the sodium acetate buffer and then twice with degassed 100 mM sodium phosphate buffer (pH 7.5; hereafter referred to as the “pH 7.5 buffer”). Concentration of protein was measured at the end of this procedure spectrophotometrically. A 30-μg portion of each protein was reconstituted in the pH 7.5 buffer supplemented with 10 mM unlabeled sodium bicarbonate and 4 mM 13C-labeled sodium bicarbonate (total volume 40 μl) and was incubated at room temperature for 15 min. A 2-μl portion from the reaction mixture was diluted with 28 μl of the pH 7.5 buffer, which was supplemented to give a final concentration of 10 mM unlabeled sodium bicarbonate, and the solution was passed through a desalting column (30-mg Bio-Spin 6; Bio-Rad) column equilibrated with the same buffer as the initial sample. The same procedure was used for the measurement of the radioactivity, along with a control experiment, for which protein was withheld from the solution.

**Determination of the Dissociation Constant of Carbon Dioxide and the BlaR Protein—Binding of carbon dioxide to Lys-392 of the BlaR protein resulted in quenching of protein fluorescence at 320 nm, when excited at 295 nm. Fluorometric measurements were performed in a Spex Industries (Metuchen, NJ) Fluoromax luminescence spectrophotometer. The slit width was kept at 1 nm for excitation and 1.5 nm for emission. Experiments were carried out at 25 °C in 100 mM sodium phosphate buffer (pH 7.5). Aliquots of concentrated protein were diluted in the same buffer, were added to the protein solution (1 μl) to provide the desired carbon dioxide concentration. The measurement of the fluorescence signal was carried out after 2 min from the NaHCO3 addition, which was monitored over 2 min. The experimental data were fit using the GRAFIT software (Erithacus Software, Middlesex, UK) for a single binding site model by the following quadratic equation,

\[
\Delta F/F_0 = F_{	ext{max}} F_0 / (|E| + |I|)^2 - |E| |I| / (|E| + |I|)^2 \quad \text{Eq. 1)}
\]

where \(F_0\) is the initial intrinsic fluorescence of the protein, \(\Delta F\) is the change in fluorescence, \(F_{\text{max}}\) is maximum change in fluorescence after saturation by carbon dioxide, [I], is the concentration of the total protein, \(|E|\), is the enzyme concentration in the assay. The experiment was carried out two times, and analysis of the data was according to literature methods (12, 13). The same experiment was attempted.

**Signal-transduction system for the onset of β-lactam antibiotics resistance in staphylococci.** The green band is the cytoplasmic membrane, which displays BlaR and PBP 2a on its external surface. The β-Lactamase (BlaZ) is excreted to the milieu in staphylococci.
with the mutant K392A BlaR protein.

**Circular Dichroism Spectroscopy**—The CD spectra of the wild-type BlaR and the K392A and S389A mutant proteins (2.0 μM each in 10 mM sodium phosphate, pH 7.0, supplemented with 50 mM sodium bicarbonate), were recorded on a Jasco J-600 (Easton, MD) instrument (5-nm path length) in the absence and presence of various β-lactam antibiotics. The contribution of the substrate was subtracted in each case. The concentrations of the β-lactams were generally 2-fold higher than the respective K<sub>a</sub> values. Prior to recording the spectra of the proteins with a β-lactam antibiotic, the proteins were incubated with the β-lactam antibiotics for 15 min at 25 °C.

**Determination of the Kinetic Parameters for Interactions of β-Lactam Antibiotics with the BlaR Protein**—The BlaR protein experiences acylation of the active site serine, and this acyl-protein species slowly undergoes deacylation according to Reaction 1.

\[
B + 1 \rightarrow B-I \rightarrow P + B + \text{acyl-Blar}
\]

**REACTION 1**

where the ratio \( k_2/k_1 \) equals \( K_b \), \( B \) represents the BlaR protein, \( B-I \) is the non-covalent preacylation complex, \( P \) is the covalent acyl-protein species, and \( P \) denotes the product of hydrolysis of the β-lactam antibiotic.

The first-order rate constants for protein acylation (\( k_2 \)) were determined for different β-lactam compounds using a Cary 50 UV spectrophotometer (Varian Inc.) equipped with an SFA-20 stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK) at room temperature. The parameters for the reaction between the BlaR protein and nitrocefin were determined directly by monitoring the formation of the acyl-protein species, and \( P \) denotes the product of hydrolysis of the acylated species at 500 nm and by fluorescence detection after incubation with BOCILLIN FL. In subsequent experiments, the protein at 1.0 μM was incubated with BOCILLIN FL (5 μM) for 30 min at 37 °C. The reaction was quenched with SDS sample buffer, and the sample was analyzed by SDS-polyacrylamide gel electrophoresis.

**Structure-based Computational Modeling**—The Swiss-Model server (www.expasy.ch/swissmod) was used to construct a model for the BlaR protein. The procedure consisted of first carrying out a BLAST (17) search of the ExNRL-3D data base of sequences of known structures for suitable templates. A pairwise sequence alignment of each template to the target sequence was then carried out using the SIM method (18), and sequence identity was determined after each alignment. Only those structures with 25% or greater sequence identity to the target sequence were selected. The ProMod package (19) was then implemented to use the information from the SIM sequence alignments and the Cartesian coordinates from the Protein Data Bank files of the templates to construct a three-dimensional model of the desired protein.

Three sequences of known structures (Protein Data Bank accession codes: 1H8Y, 1EWZ, 1FOF) with 28% or more sequence identity to BlaR were found. The sequence alignment and resulting model generated by the Swiss-Model server were analyzed with the Sybyl 6.7 (Tripos Inc., St. Louis, MO) software package. The BlaR three-dimensional model generated by the Swiss-Model server was locally refined by molecular dynamics simulations using the AMBER 7 suite of programs (20). The active site Lys-392 was carboxylated in silico, for which derivation of the force field parameters was described previously (21). The protein was fully solvated in a box of TIP3P (22) waters, resulting in a total of 37,585 atoms. A short trajectory was carried out with the protein frozen to equilibrate water molecules. This was followed by a series of energy minimization procedures to gradually relax the protein. Subsequently, a total of 200 ps of molecular dynamics simulation was carried out on the system. Snapshots were collected every 2 ps, and an average structure was generated by root-mean-square fitting of all the collected snapshots to the initial structure. The fitted structures were then averaged, and the resulting model was subjected to a 20,000-step conjugate gradient energy minimization procedure.

**RESULTS AND DISCUSSION**

We cloned the sensor domain of the signal transducer protein BlaR from *S. aureus* (spans amino acids 331–581), which we refer to as the BlaR<sub>p</sub> protein. The cloned protein was expressed in the cytoplasm of *E. coli*. The protein was purified to homogeneity in two chromatographic steps and was highly soluble (up to 26 mg/ml). We routinely obtain 50 mg of pure protein from one liter of growth medium. The C terminus of BlaR from *Bacillus licheniformis* has also been cloned (23, 24).

**Carboxylation of Lysine Side Chain in the BlaR Protein**—The BlaR<sub>p</sub> protein is related to the OXA family of β-lactamas, enzymes of resistance to β-lactam antibiotics (24, 25). The active site peptide sequence of Ser-X-X-Lys, which is known as a minimal motif for these proteins that undergo acylation at the serine residue, is present in both (25). The x-ray structures for the OXA-10 β-lactamase (26, 27) reveal that the active site lysine is carboxylated on its side chain (i.e., the carbamate product of reaction with carbon dioxide). The side chain of lysine in the OXA-10 β-lactamase is sequenced in an unusual environment made up of five hydrophobic amino acid side chains (Phe-69, Val-117, Phe-120, Trp-154, and Leu-155) that is believed to lower the pK<sub>a</sub> of the lysine side chain such that it

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exists in the free base form that undergoes reaction with carbon dioxide (26). The side chain of the carboxylated lysine and that of serine are in contact, and the former activates the latter for enzyme acylation by β-lactam antibiotics (26). The requisite amino acids in the Ser-X-X-Lys motif and the five hydrophobic sites, among others, are conserved among the many OXA β-lactamases and the BlaR protein (25). A pertinent question now is whether the sensor domain of the BlaR protein is also carboxylated at the corresponding lysine residue.

A diagnostic test for carboxylation of the lysine side chain is by 13C NMR, which detects a distinctive signal. The 13C NMR experiment indicated that lysine carboxylation is seen in the BlaRS protein, as shown by the presence of a diagnostic resonance at 164 ppm (Fig. 2). The same experiment was carried out with the K392A mutant protein, and unexpectedly, we observed that the NMR signal at 164 ppm was not entirely eliminated (Fig. 2B). The integrations of the carbamate signals in Fig. 2 indicated that the wild type enzyme (Fig. 2A) had approximately two carboxylated lysines to one in the mutant protein. Therefore, under the NMR experiment conditions, two lysines in the wild-type protein exist in the free base forms, which undergo carboxylation in the presence of the 13C-labeled carbon dioxide, one of which is at position 392.

We resorted to binding of radioactive carbon dioxide to the BlaRS and the K392A mutant proteins. Analogously to the case of the OXA-10 β-lactamases, the expectation was that the active site carboxylated lysine would be stabilized by specific interactions. On the other hand, the other carboxylated lysine seen in the NMR experiment might have experienced carboxylation in an adventitious process and could be back-titrated by non-radioactive carbon dioxide. Here, each protein was incubated at pH 4.5 to facilitate the decarboxylation of lysine followed by reconstitution of the protein by the radioactively labeled carbon dioxide. The workup was made in the presence of non-labeled carbon dioxide. We were able to measure an average of 0.9 equivalents of radioactive label incorporated per each of the wild-type protein molecule. By the same procedure, no label was introduced into the K392A mutant protein. This argued that carboxylation of the protein was indeed at the Lys-392 position and that there may be another exposed lysine in vivo.

In the case of the OXA-10 β-lactamase, proximity of one of the oxygen atoms of the lysine carbamate to Trp-154 was useful for the dissociation constant of carbon dioxide with the enzyme (26). Tryptophan 475 of the OXA-10 β-lactamase is given for comparison. In B, a similar perspective from the x-ray structure for the OXA-10 β-lactamase is given for comparison.
The rate constants were determined in the presence of 50 mM NaHCO₃ at pH 7.0. The concentration of the protein in the assays was 1 μM. The kinetic parameters for substrates other than nitrocefin were determined in competition experiments with nitrocefin. (See “Experimental Procedures” for more details.)

| Substrate | $k_2$  | $k_3$  | $K_s$  | $k_{cat}/K_m$ |
|-----------|--------|--------|-------|---------------|
| Nitrocefin | 26 ± 6 | 92 ± 10| 24 ± 9| 11 ± 4        |
| FA-penicillin | 1.6 ± 0.1 | 8 ± 4 | 13 ± 2 | 1.2 ± 0.2   |
| Ampicillin | 1.0 ± 0.1 | 9 ± 1 | 23 ± 2 | 0.4 ± 0.1   |
| Oxacillin | 18 ± 1 | 4.8 ± 0.6 | 49 ± 5 | 3.7 ± 0.4   |
| Cefepime | 1.4 ± 0.1 | 12.6 ± 0.3 | 25 ± 3 | 0.6 ± 0.1   |
| Ceftazidime | 4.1 ± 0.5 | 9.1 ± 0.4 | 68 ± 16 | 0.6 ± 0.2   |
| Imipenem | 6 ± 1 | 10 ± 1 | 183 ± 66 | 0.3 ± 0.1   |

Fig. 5. Circular dichroic spectra of the wild-type BlaR protein (2 μM, solid line) and the wild-type BlaRS protein (2 μM) incubated with oxacillin (30 μM, broken line) (A) of the K392A mutant variant of the BlaRS protein (2 μM, solid line) and the K392A mutant (2 μM) incubated with oxacillin (30 μM, broken line) (B) and of the S389A mutant variant of the BlaR protein (2 μM, solid line) and the S389A mutant (2 μM) incubated with oxacillin (30 μM, broken line) (C). All the spectra were corrected for the small contribution from the antibiotic in the mixture.

dissociation constant that was evaluated for the wild-type protein was for carboxylation of residue 392 and is a further validation that this residue is indeed carboxylated in the wild-type protein. As will be described below, carboxylated Lys-392 is the active site base that activates the serine for protein acylation. This is now only the second example of a protein, after the OXA-10 β-lactamases (26), that uses the highly uncommon carboxylated lysine as a basic residue to facilitate reactions in the active site. The few other proteins having carboxylated lysine use the modified amino acid as metal ligand or for hydrogen bonding in the protein structure.

The collective information in the preceding paragraphs made possible the generation of a homology-based computational model for the sensor domain of the BlaR protein (Fig. 4). In comparison with the structure of the OXA-10 β-lactamase, the arrangements of the side chains of serine and the carboxylated lysine and the hydrophobic environment around the lysine, including the proximity of the carboxylated lysine and the tryptophan residue, are preserved.

Kinetics of Interactions of β-Lactam Antibiotics and the BlaRS Protein—In light of the information that the sensor domain of the BlaR protein has a carboxylated lysine, it is conceivable that the protein at the end of each individual purification protocol would be carboxylated to varying degrees, since the process is reversible. This point was documented by observing typically a 2-fold enhancement of the rate of interactions of the BlaRS protein with β-lactam antibiotics by supplementation of the buffer with sodium bicarbonate (as a source of carbon dioxide). As argued above, the BlaR protein is fully carboxylated in vivo, and the fact that some of the carboxylation of the protein is reversed during the purification is an artifact. Therefore, we have supplemented the reaction mixtures for the kinetic studies with bicarbonate to generate the fully carboxylated and active form of the protein for all kinetic determinations.

BOCILLIN FL, a fluorescent penicillin, was used to further study and analyze the mode of action of BlaRS protein. This molecule modifies BlaRS covalently, as would any β-lactam antibiotic, whereby the protein would migrate through an SDS-polyacrylamide gel to allow quantitative detection by Fluorimager. Titration of the BlaRS protein with BOCILLIN FL revealed saturation and also indicated a one-to-one modification of the protein by the antibiotic. The wild-type and K393A mutant BlaRS proteins were acylated by BOCILLIN FL, as revealed by Fluorimager. The K393A has a residual level of activity (see below) that accounts for this observation. In contrast, incubation of the S389A mutant variant with BOCILLIN FL did not give a fluoregenic band. A previous study based on sequence analysis of the BlaR from B. licheniformis with a class D β-lactamase had suggested that residue Ser-389 (BlaR numbering according to S. aureus) might be the modification site by β-lactams (23). The experiments reported herein clearly reveal Ser-389 to be the serine-active site residue that is acylated.

The kinetics of interactions of several β-lactam molecules (three penicillins, three cephalosporins, and one carbapenem) with the BlaRS protein were investigated (Table I). Stepped-flow kinetics distinguished between a rapid enzyme acylation event and a substantially slower deacylation step. Acylation of the active site proceeded with microscopic rate constants (i.e. $k_{cat}$) of 1–26 s⁻¹, which indicate rapid $k_{cat}$ values for acylation of...
27–690 ms for the β-lactams that we studied. The deacylation rate constants (i.e., $k_d$) for the same β-lactam molecules are listed in Table I, corresponding to $t_{1/2}$ values of ~12–240 min. The $k_d$ for oxacillin would appear to be representative of most of the substrates studied, with a value of $(4.8 \pm 0.6) \times 10^{-5} \text{ s}^{-1}$, which corresponds to a $t_{1/2}$ value of 240 min. In light of the fact that typical strains of S. aureus double their population sizes in 20–30 min under favorable growth conditions, this indicates that a single acylation event per each molecule of the β-lactam signal sensor-transducer protein accounts for the biological consequences per each generation of bacterial growth.

It is a feature of the signal sensor-transducer protein that it is activated by all β-lactam antibiotics (29). Consistent with this information, the dissociation constants (i.e. $K_d$) for various β-lactam antibiotics are in the micromolar range, which are attainable in the milieu where the bacteria grow. The dissociation constants are practically in the same range for the three penicillins and three cephalosporins that we tested, whereas the carbapenem imipenem shows a higher value (Table I). The second-order rate constants (i.e. $k_{\text{ON}}$) for the encounter of the β-lactam molecules and the BlaRS protein were typically $10^4$ to $10^6 \text{ M}^{-1} \text{ s}^{-1}$, indicative of a very favorable process.

As per the computational model and the foregoing evidence, we decided to evaluate the effect of Lys-392 on the kinetics of BlaRS acylation. In the case of the OXA-10 β-lactamase, the mutational change of the corresponding carboxylated lysine resulted in an inactive enzyme that did not experience acylation in the active site by the β-lactam antibiotics (26). Similarly to the case of the OXA-10 β-lactamase, a mutational change of Lys-392 to Ala in BlaRS resulted in a protein that was severely impaired in acylation of the active site serine as evaluated for oxacillin ($k_d = 0.0026 \pm 0.0005 \text{ s}^{-1}$ and $K_d = 43 \pm 14 \mu\text{M}$). The rate constant for acylation was attenuated for the mutant protein by 6730-fold with no change in $K_d$. Since the mutant protein has the same conformation as the wild-type protein by circular dichroic analyses (see below), the attenuation on $k_d$ may be ascribed to poor activation of serine in the mutant protein.

**Conformational Change in the BlaRS Protein**—Signal transduction from one side of the membrane to the other necessitates communication between the surface and the cytoplasmic domains. A means to this communication is by conformational change of the membrane-bound protein after binding to the β-lactam antibiotic. As shown in Fig. 5, the BlaRS protein is prone to significant conformational change on binding to the β-lactam antibiotics. The conformational change commences upon binding to the β-lactam antibiotic at the preacylation complex and reaches its full extent on protein acylation. If the acyl-protein species is allowed to undergo its sluggish deacetylation, the protein returns to the native conformation (data not shown). As indicated earlier, the K392A mutant variant of the BlaRS protein is severely deficient in the acylation step. This mutant variant would not experience acylation by β-lactam antibiotics during the course of the CD experiment. However, the non-covalent binding by a β-lactam antibiotic, for example by oxacillin (Fig. 5B), resulted in a discernable change in the CD spectrum of the protein (similar results were seen with the S389A mutant protein, which does not have the opportunity to give the acyl-protein species; see Supplemental Material). The minima at 208 and 222 nm, which are due to the helices, were enhanced, and the maximum at 195 nm, due to β-sheets, sharpened (Fig. 5B). These data argue for the enhancement of secondary structures (helixicity and β-sheets) in the protein on non-covalent binding by the antibiotic. Upon acylation of the protein by oxacillin (Fig. 5A), these effects were enhanced further, but the native state returned upon deacylation. Similar results and trends were noted for all β-lactam antibiotics shown in Table I (see Supplemental Material), so the effects of the conformational change on the BlaRS protein are shared by all of these antibiotics. In light of the fact that this conformational change is significant and is generally seen regardless of the nature of the β-lactam antibiotic, we believe that it is likely that it plays a role in the signal transduction process. However, we acknowledge the fact that in the whole cell context, other factors may play a role as well.

The essence of signal transduction is the switch between inactive and active forms of a given protein. In the case of the β-lactam signal sensor-transducer, a key question is how binding of the β-lactam antibiotic to the sensor domain facilitates signal transduction. Two classical models for signal transduction have been proposed (30). In one, ligand binding induces the formation of a new conformation in the protein. In the other, an equilibrium mixture of conformational states exists, and the ligand binding shifts the equilibrium in favor of the active form. The data presented here for the BlaRS protein are consistent with either the induced-fit or the population shift model. It is conceivable that the protein switches its conformation on binding by the β-lactam antibiotic during the formation of the preacylation complex, which reaches its maximal effect after acylation of the enzyme in the active site (the induced-fit model). Alternatively, the CD spectrum of the native BlaRS protein may be due to the distribution of several preexisting conformational states, which binding of the β-lactam antibiotics to one would shift the equilibrium in favor of the active structure (the population shift model). The discrimination between these two models for the case of the BlaRS protein should await availability of structural information in the future.

We have described in this report the dynamic nature of the sensor domain of the BlaRS protein from staphylococci. This protein undergoes structural rearrangement on binding to a wide range of β-lactam antibiotics, the implications of which for the signal transduction event remain to be studied by structural biologists. We have shown that β-lactam antibiotics modify the protein covalently and essentially irreversibly within a bacterial population doubling time at Ser-389. The covalent modification of the sensor domain is facilitated by an uncommon carboxylated lysine at position 392 within the antibiotic binding site. The means by which the BlaRS system carries out its signal sensing and transducing processes is unique to Gram-positive bacteria. We have provided herein insights into how this specific protein in S. aureus facilitates the manifestation of resistance to β-lactam antibiotics, a process that remains a challenge in clinical treatment of infections caused by this organism.

**Note Added in Proof**—Knox and colleagues recently determined the x-ray structure for the OXA-1 β-lactamase (Sun, T., Nukaga, M., Moyama, K., Braswell, E. H., and Knox, J. R. (2003) Protein Sci. 12, 82–91). Comparable with the case of the OXA-10 β-lactamase and BlaRS, the active site lysine in this enzyme is also carboxylated.

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