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X-ray Crystal Structure of the Acylated β-Lactam Sensor Domain of BlaR1 from *Staphylococcus aureus* and the Mechanism of Receptor Activation for Signal Transduction

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The semisynthetic methicillin (a penicillin) was introduced in 1959 in response to the challenge of β-lactamase-producing *Staphylococcus aureus*. Shortly after, in 1961, reports emerged first from the United Kingdom, then from elsewhere around the globe, of *S. aureus* with acquired resistance to this new antibacterial agent.[1] This organism is referred to as methicillin-resistant *S. aureus* ("MRSA"). The description of recent MRSA isolates with the acquisition of the enterococcal gene *vanA* conferring resistance to vancomycin, an antibiotic of choice for treatment of severe MRSA infections, has created a new and disconcerting chapter in the evolution of this pathogen.[2−4] As a result, therapeutic options have become limited.

Resistance to β-lactams in MRSA may be due to production of β-lactamase and/or an extra penicillin-binding protein, PBP 2a.[5−7] The structural gene for the class A staphylococcal β-lactamase, *blaZ*, is widely distributed in staphylococci and is usually located on plasmids.[8−10] PBP 2a is encoded by the chromosomal gene *mecA* and is an especially insidious protein, as it confers resistance to all available β-lactam antibiotics.[5,6,11−14] Expression of the β-lactamase and PBP 2a genes of *S. aureus* is regulated by two similar β-lactam sensor/signal transducer systems along with their respective repressor proteins (Figure 1), and either *bla* or *mec* regulatory genes can control production of β-lactamase or PBP 2a. It is worth stating that regulation of this process in clinical strains is largely due to the *bla* system, because often the *mec* regulatory genes contain deletions and mutations.[14−16]

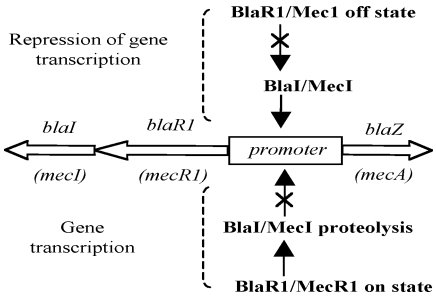

Figure 1. Scheme for the regulation of transcription of the resistance genes in *S. aureus*.

Whereas our understanding of these β-lactam-sensing and signal-transducing systems in *S. aureus* is not complete, the *bla* system has been studied in greater detail. The C-terminal sensor domain of the signal transducer BlaR1 is located on the extracellular surface of the cytoplasmic membrane of *S. aureus*. The protein backbone is understood to criss-cross the membrane four times (as helices[17]), in the process of which a cytoplasmic domain of 186 amino acid residues is created. It is proposed that the cytoplasmic domain, with its zinc protease hydrolytic activity, degrades the BlaI repressor protein, thereby preventing the formation of the dimer, which in turn derepresses expression of the structural and regulatory genes.[14]

Activation of the pathway is induced by acylation of the active-site serine (Ser-389) from the sensor domain of BlaR1 when the bacterium is exposed to a β-lactam antibiotic. This "β-lactam-sensing" function is promoted by the $N\zeta$-carboxylated side chain of Lys392.[18] We present evidence herein that, on acylation of the active-site serine by the antibiotic, the lysine side chain experiences a spontaneous decarboxylation that entraps the acylated receptor species, a process that keeps the sensor in its activated state. Furthermore, we report the crystal structure of the covalent complex of β-lactam sensor domain of BlaR1 and the cephalosporin antibiotic ceftazidime at 1.75 Å resolution. The complex shows the mode of binding of the antibiotic and reveals that the lysine residue has undergone decarboxylation. Quantum mechanical/molecular mechanical (QM/MM) calculations and the interaction networks in the crystal structure shed light on how this process may be achieved and provide insights into the mechanistic features that differentiate the signal-transducing receptors from the structurally related β-lactam resistance enzymes.

The β-lactam-binding domain of BlaR1 from *S. aureus*, residues 331−585, was overexpressed in *Escherichia coli*. The nine methionine residues were substituted by selenomethionine (SeMet) for the purpose of crystallographic phasing. Both the native and the SeMet proteins in complex with ceftazidime at pH 7.5 gave thin monoclinic crystals, which diffracted to 1.75 Å. These crystals dissolved after 5 days, while cubic crystals diffracting to 5 Å resolution, and identical to those grown from the apoprotein, appeared. This process likely results from the slow hydrolysis of the acyl−enzyme species of the complex and highlights the fact that biochemical processes occur in this crystallization medium. Attempts to extend the diffraction pattern of the apoprotein crystals while maintaining neutral or basic pH values, necessary for the maintenance of the *N*-carboxylated lysine, were unsuccessful. The SAD data set collected at the peak of the Se absorption edge on the monoclinic crystal was sufficient for phase determination. The native structure was refined to 1.75 Å resolution with R and R_{free} values of 20.6% and 23.4%, respectively. BlaR1 is a two-domain protein. The first domain (residues 338−387 and 515−585) includes a seven-stranded antiparallel β-sheet, the extended N-terminal

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peptide stretch, and the C-terminal helix. The second domain (residues 388−514) carries the catalytic amino acids and is made of six helices connected by loop regions (see Supporting Information).

The crystal structure of the BlaR1(331−585)−ceftazidime complex revealed the predicted common fold between BlaR1 from *S. aureus*, the class D β-lactamases (OXA-1 and OXA-10), and the apo BlaR1 from *Bacillus licheniformis*.[19] Superimposition of the BlaR1 and OXA-10 structures (root-mean-squared deviation = 1.29 Å over 178 Cα atom pairs of equivalent residues), however, indicated significant local differences. One of them is the new position of the short β-4 strand, which disrupts the dimerization interface observed in the OXA-10 enzyme, in line with the monomeric states of *S. aureus* BlaR1 in its apo and acylated forms deduced from analytical ultracentrifugation measurements (data not shown). The superimposition revealed a remarkable conservation of the active-site geometry for the residues that belong to three sequence motifs in all penicilloyl serine transferases: Ser-389, Lys-392 (Ser-67, Lys-70 in OXA-10), Ser-437, Val-438 and Asn-439 (Ser-115, Ala-116, and Val-117 in OXA-10, respectively), Lys-526 (Lys-205 in OXA-10), and Trp-475 and Met-476 from the loop (Trp-154 and Leu-155 in OXA-10).

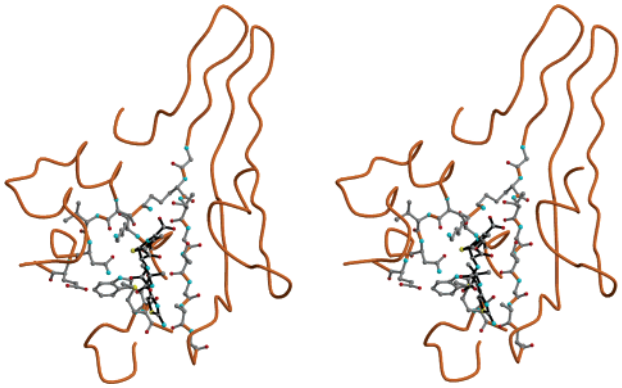


Figure 2. Stereoview of the interface between the two-domain BlaR1 sensor moiety acylated by ceftazidime.

The four sensor domains present in the asymmetric unit of the monoclinic crystals superimpose with a rmsd of 0.34 Å over the 245 commonly observed Cα atoms and are all acylated by ceftazidime at Ser-389 (Figure 2). The oxygen atom of the ester carbonyl is located in the oxyanion hole at hydrogen bond distances (3.0 Å) to the main-chain nitrogen atoms of Ser-389 and Thr-529. The oxygen atoms of the carboxylate group at C4 of the dihydrothiazine ring of ceftazidime are at 2.5 Å from the hydroxyl groups of Thr-527 and Thr-529 located on the β-5 strand that delineates one side of the ligand-binding site. Two features suggest that these threonine residues serve as anchors for the carboxylate of the β-lactam ligand: these two threonines are invariant in all sensor transducers, as well as Gly-565, the first residue from the C-terminal helix, which is the single amino acid sterically compatible with the location of Thr-529. The binding of the carboxylate thus does not involve Lys-526, from the typical K-T/S-G motif 3 of penicillin-binding proteins, or a positively charged side chain that would be structurally equivalent to Arg-244, Arg-349, or Arg-250 in classes A, C, or D of β-lactamases, respectively. Additional polar interactions between the sensor domain and the substituent at C7 of the β-lactam ring, including the thiazole ring, strengthen binding of ceftazidime to the protein (Figure 3).

According to the unambiguous electron density at 1.75 Å resolution, Lys-392 is not *N*-carboxylated in the acyl−enzyme complex. This is a critical point of distinction with class D

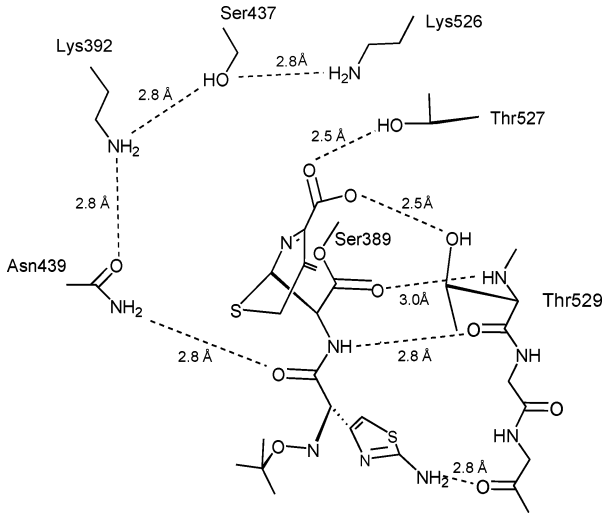


Figure 3. Network of polar interactions in the ligand-binding site of BlaR1 from *S. aureus* in complex with ceftazidime.

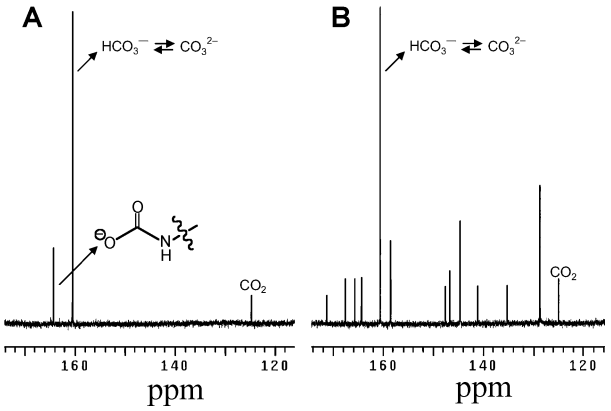
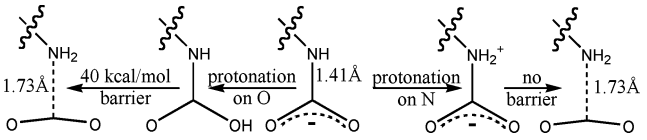


Figure 4. (A) Wild-type sensor domain of BlaR1 (1 mM), labeled by ^{13}C-sodium bicarbonate (from left; δ164.2, 160.5, 124.8 ppm). (B) Ceftazidime added to ^{13}C-labled wild-type sensor domain of BlaR1 (1 mM, the signal at δ164.2 has disappeared and the remaining resonances all are accounted for by ceftazidime; see Supporting Information).

β-lactamases. In their apo forms, class D enzymes and BlaR1 are carboxylated at Lys-70 and Lys-392, respectively.[18,20−22] The assertion that the sensor domain is carboxylated at Lys-392 was borne out by the observation of the diagnostic ^{13}C NMR resonance at 164 ppm of the protein labeled by ^{13}C-bicarbonate (as a source of carbon dioxide), by fluorescence quench of Trp-475 (which interacts with the carbamate), and by mutagenesis at the active site.[18] A carboxylated Lys-392, modeled from the current X-ray structure, would be at exactly the same position as in OXA-10 β-lactamase,[20−22] with one carbamate oxygen atom at 3.1 and 2.9 Å from Ser-389 Oγ and Trp-475 Nζ1, respectively, and the carbamate nitrogen atom at 3.2 Å from Ser-389 Oγ. However, while Lys-70 in class D β-lactamases remains carboxylated in the acyl−enzyme species, as illustrated by the crystal structure of OXA-10 in complex with 6β-hydroxyisopropylpenicillinate,[23] the current structure indicates that BlaR1 undergoes decarboxylation on acylation of the serine. The diagnostic ^{13}C NMR signal in the native BlaR1 is shown in Figure 4A. The three signals from the sealed NMR tube correspond to the carbamate (164), equilibrium between carbonate and bicarbonate and carbon dioxide (left to right, Figure 4A). On addition of ceftazidime and rapid acylation of the protein ($t_{1/2}$ = 170 ms),[18] the signal at 164 ppm disappeared (Figure 4B; full NMR spectra with ceftazidime and with oxacillin, a penicillin, are given in the Supporting Information). The now-decarboxylated Lys-392 is

shown in the X-ray structure hydrogen bonded (2.8 Å) to Asn-439 and to Ser-437, which is at 2.8 Å from Lys-526 (Figure 3).

The X-ray data, kinetic studies, and theoretical calculations provide insights into the mechanistic features that could differentiate resistance enzymes to signal-transducing proteins. The X-ray structure of OXA-10, as well as the modeled carboxylated apo BlaR1 (for pH > 8), indicated that either the nitrogen or the oxygen of the *N*-carboxylated lysine (the carbamate) is in contact with and could promote the active-site serine for acylation. If one of the terminal carbamate oxygen atoms is protonated by promoting proton abstraction from Ser-389 Oγ, decarboxylation would need to occur via the reverse of the carboxylation reaction. QM/MM calculations using the X-ray coordinates of the OXA-10 active site indicated barriers of 20−30 kcal/mol for the carboxylation of the lysine side chain by CO_2 and ca. 40 kcal/mol for decarboxylation. By contrast, protonation at the carbamate nitrogen atom results in a charged nitrogen that leads directly to decarboxylation. QM/MM calculations were started from the optimized carbamate structure from the X-ray coordinates with a proton added to the carbamate nitrogen or oxygen atoms. When the carbamate was protonated on the nitrogen, geometry optimization by QM/MM gradually elongated the carbamate C−N bond without encountering a barrier, resulting in a structure with a neutral CO_2 loosely bound to the lysine nitrogen, similar to the reactant complex for the carboxylation process. This suggests that protonation of the carbamate nitrogen leads to a barrierless decarboxylation of the lysine carbamate, and provides a scenario in which carbamate protonation and serine acylation could lead to *N*-decarboxylation and formation of a stable acylated protein. Kinetic studies on class D OXA-10 β-lactamase indicated that both the lysine carbamate oxygen and nitrogen could abstract proton from serine for the acylation step. When the nitrogen does this function, the carbamate undergoes decarboxylation, and catalysis is arrested at the acyl−enzyme state and resumes only after lysine is recarboxylated.[20]



It would appear that nature has driven this process in the case of the BlaR1 sensor in the direction that the carbamate nitrogen predominantly abstracts the proton, so that *N*-decarboxylation of lysine takes place and the acyl−enzyme species is entrapped. The frequency by which it occurs was evaluated by the parameter the partition ratio (Supporting Information), which indicates that six molecules of ceftazidime are hydrolyzed before full acylation of BlaR1 by the stable species seen in the X-ray structure is realized. This compares to a partitioning process in the order of several hundred turnover events for each arrest of catalysis for the OXA-10 β-lactamase. Hence, BlaR1 shows some residual β-lactamase activity before the entrapment of the acylated receptor.

In the BlaR1 acyl−enzyme complex, the hydrogen bond between the decarboxylated Lys-392 and Asn-439 likely prevents recarboxylation of the lysine residue from taking place. Asparagine 439 belongs to the second sequence motif in penicillin-binding proteins and is invariant in all sensor-transducer proteins of Staphylococci (species *aureus*, *epidermis*, *hemolyticus*), suggesting shared properties of these proteins. The stability of the acyl−protein species ($t_{1/2}$ of 12−120 min depending on antibiotic[18]) ensures that the signal sensing and message transduction take place. This contrasts to the situation of the class D β-lactamase, where the invariant Val-117, at an equivalent position to Asn-439, contributes to the global apolar environment of the ligand binding that favors the documented carboxylated state of the lysine before and after acylation[23] and thus both the acylation and deacylation reactions.

The stability of the acylated BlaR1 species, and the structural alterations upon acylation shown by CD measurements,[18] may suggest a two-state model for the process of signal transduction. This possibility seems to be supported by the finding that the apo-form of the sensor domain of BlaR1 from *B. licheniformis* binds to the extracellular 60 amino acids loop region connecting the transmembrane helices 2 and 3, an interaction abrogated in the presence of antibiotics.[24] Deciphering the identity and the function of the residues that contribute to the signaling state of the receptor, and their specific interactions with the effectors, should inspire the design of new drugs against the mechanism for activation disclosed herein, which finds no precedent in the literature. It should be of interest to explore the generality of this "*N*-decarboxylation switch" in other biological systems.

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Note Added in Proof. While this manuscript was in review, a paper on crystallography of BlaR1 appeared on the Web: Wilke, M. S.; Hills, T. L.; Zhang, H. N. C. *J. Biol. Chem.* **2004** (published online ahead of print).

Supporting Information Available: Procedures for the preparation of BlaR1, crystallization, structure determination, NMR, QM/MM, and partition ratio experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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