Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus*

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Summary

High-level resistance to β-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* (MRSA) is due to expression of penicillin-binding protein 2a (PBP2a), a transpeptidase that catalyzes cell-wall crosslinking in the face of the challenge by β-lactam antibiotics. The activity of this protein is regulated by allostery at a site 60 Å distant from the active site, where crosslinking of cell wall takes place. This review discusses the state of knowledge on this important enzyme of cell-wall biosynthesis in MRSA.

Keywords

methicillin-resistant *Staphylococcus aureus*; allosteric regulation; conformational change; β-lactam antibiotics; resistance mechanism

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is a global concern in light of broad resistance to the majority of available treatment options. It is a common cause of skin and respiratory system infections. Penicillins, which inhibit cell-wall synthesis, were the first-line treatment of infections by *S. aureus*. But within a few years of penicillin use, 50% of *S. aureus* infections were resistant to the drug due to the catalytic function of β-lactamase, a resistance enzyme that hydrolytically turns over these antibiotics, rendering them inactive. A second generation of semi-synthetic penicillins was introduced in 1959 expressly to circumvent the β-lactamase activity. These antibiotics, which include methicillin and oxacillin, among others, were resistant to the action of the *S. aureus* β-lactamase. However, the first methicillin-resistant *Staphylococcus aureus* (MRSA), strain was identified in the UK in 1961 (1). It became a global problem with two years. This resistance phenotype was due to the acquisition of a gene that encodes for a protein that overcomes the challenge by a broad range of β-lactam antibiotics (including penicillins, cephalosporins and carbapenems; Figure 1), which is the subject of discussion in the ensuing sections. Currently, the drugs of

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choice for treatment of infections by MRSA are vancomycin (2), daptomycin (3), ceftaroline (4) and linezolid (5). Even with controlled use, strains that show reduced susceptibility, or outright resistance, to these drugs have arisen (6–9).

Cell-wall synthesis and the roles of penicillin-binding proteins

Survival of bacteria depends on the health of the cell wall. During growth and division, bacteria biosynthesize cell wall, which is a polymer made up of the peptidoglycan as the principle building unit. The peptidoglycan is composed of repeats of the disaccharide *N*-acetylglucosamine (NAG)-*N*-acetylmuramic acid (NAM) with peptide stems on the NAM unit. The peptide stems of neighboring peptidoglycan strands are the points of crosslinking to give the mature cell wall (10, 11) (Figure 2). The peptide stem varies by organism, but in *S. aureus* it is the pentapeptide L-Ala-γ-D-Glu-L-Lys(Gly)$_5$-D-Ala-D-Ala; the pentaglycyl moiety being linked to the lysine side chain. Synthesis of the peptidoglycan backbone is carried out by the catalytic function of transglycosylases, with transpeptidases—also referred to as penicillin-binding proteins (PBPs)—performing the crosslinking reaction. Both activities could be present in the same protein, but not necessarily. Coordination of these reactions is an elaborate process, which ultimately results in the cell-wall structure (12, 13). As the activity of PBPs is essential for bacterial survival, they are desirable targets of antibiotics, specifically by β-lactams. In *S. aureus*, there exist four native PBPs: PBP1, PBP2, PBP3, and PBP4. There is a fifth PBP in MRSA, namely PBP2a. This is the aforementioned resistance determinant in MRSA, encoded by the gene *mecA*, which was acquired from a non-*S. aureus* origin. Tipper and Strominger argued that PBPs recognize and are efficiently inhibited by β-lactam antibiotics due to the similarity of the β-lactam backbone to the acyl-D-Ala-D-Ala segment of the peptide stem in the peptidoglycan, the physiological substrate of PBPs (14) (Figure 1). PBP2a is a unique transpeptidase that is not inhibited well by β-lactam antibiotics. Hence, it is able to continue peptidoglycan crosslinking in the face of the challenge by these antibiotics, when other PBPs with transpeptidase activity are inhibited.

Mechanism of resistance in *Staphylococcus aureus*

Bacteria have evolved numerous strategies to counter the effect of antibiotics, which have been reviewed elsewhere (15–18). *Staphylococcus aureus* produces the PC1 β-lactamase, the product of the *blaZ* gene, which was the original mechanism of resistance to β-lactam antibiotics in this organism. We described above that the β-lactamase hydrolyzes the β-lactam ring, rendering it inactive. Methicillin-resistant *S. aureus* has also acquired the *mecA* gene, which encodes PBP2a (19–23), enabling the bacteria to sustain cell-wall synthesis when other PBPs are inhibited by β-lactam antibiotics. Additional genes that are necessary for methicillin resistance have also been identified, among them *fem* (factors essential for expression of methicillin resistance) and *aux* (auxiliary) factors (24, 25).

As stated earlier, *S. aureus* acquired the *mecA* gene from an unknown source, which might be another staphylococcal organism, as *mecA* homologues have been identified in *Staphylococcus sciuri*, *Staphylococcus vitulinus*, and *Staphylococcus fleurettii* (26–28). Although resistance by the organism is contingent on the presence of the *mecA* gene, some

strains that contain *mecA* exhibit varied phenotypic expression (29, 30). *Staphylococcus aureus* PBP2a is a high-molecular-weight class B PBP and is found only in resistant bacteria. The transpeptidase active site of PBP2a, as in the cases of other PBPs, contains the active-site serine (S403) at the N-terminus of the α2 helix in the sequence motif SXXK. The β-lactam antibiotic binds to the active site and forms a rapidly reversible Michaelis complex (EI) (Figure 3). This complex is converted to a stable covalent adduct by nucleophilic attack by S403 on the β-lactam ring (acyl-enzyme species, E-I). The deacylation reaction is slow, with $t_{1/2}$ values between 26 and 77 hours (31), which renders acylation effectively irreversible. The rate of acylation of PBP2a is slow ($t_{1/2}$ = 3–12 min) and the dissociation constants for all classes of β-lactams (~600–1600 μM) are unfavorable (31), both of which contribute to the manifestation of resistance. These observations were interpreted with X-ray structures for the apo enzyme (32) and the enzyme in complex with antibiotics such as ceftaroline (33) and ceftobiprole (34), showing that the main obstacle to β-lactam acylation of the enzyme is the inaccessibility of the active site.

Structural basis for resistance

Lim and Strynadka published the first crystal structure of soluble PBP2a at 1.8 Å resolution in both the apo (Figure 4A) (PDB ID 1VQQ) and the antibiotic-acylated forms (32). These structures showed the enzyme as an elongated protein with a transpeptidase domain (residues 327–668) and what the authors referred to as a non-penicillin-binding domain (residues 27–326), which includes an N-terminal extension subdomain (residues 27–138). The full-length protein also contains a transmembrane anchor segment (residues 1–23), which can be removed to make the protein soluble for characterization (31, 35, 36). Structures of apo-PBP2a and nitrocefin-acylated PBP2a revealed a closed active site, such that an intact β-lactam could not gain access to the active site. This suggested the need for a conformational change at the active site in order to accommodate the antibiotic. The closed active site can explain resistance, but not the physiological reaction of PBP2a. The enzyme is able to efficiently perform the peptidoglycan-crosslinking reaction, which requires the ability of the active site to accommodate the two strands of the peptidoglycan, which entail a volume in excess of 1000 Å^3. This indicates that the protein should undergo conformational changes in the course of catalysis, which has been documented to be the case, as we will elaborate.

Circumvention of the problem by new β-lactam antibiotics

While PBP2a has low affinity for most β-lactam antibiotics, it has higher affinity for ceftaroline, the most recently approved cephalosporin. (Figure 4D). Ceftaroline is the active form of the prodrug ceftaroline fosamil. It is resistant to the *S. aureus* β-lactamase activity. However, ceftaroline binds to *S. aureus* PBP1, PBP2, PBP3, and PBP2a (IC_{50} <1 μg/mL in most cases) avidly, with PBP4 showing lower affinity (37, 38). It has favorable low MICs (0.5–1 μg/mL) against MRSA strains (37, 39, 40). It has been approved by the FDA for use in the treatment of community-acquired pneumonia and acute bacterial skin and skin structure infections. Current treatment is an intravenous dosage of 600 mg every 12 h for 5–14 days depending on the infection. A second new cephalosporin, ceftobiprole (Figure 4D), seems to have comparable properties. It has activity against MRSA and vancomycin-

resistant strains of MRSA. It has been approved for use in Europe against hospital- and community-acquired pneumonia, but has not been approved by the FDA for use in the United States. It has MIC values in the range of 0.5 – 4 μg/mL against MRSA (41–43). As with ceftaroline, it is a poor substrate for the PC1 β-lactamase (41), but it is a good inhibitor of all PBPs of S. *aureus*, with IC_{50} values of <1 μg/mL (42).

PBP2a-ceftaroline complex reveals allosteric regulation of resistance

The active site of PBP2a is impervious to inhibition by β-lactam antibiotics because it exists in a closed conformation, with the active-site serine deep in a tight groove (32). However, it would have to open up to accommodate the two peptidoglycan strands in order to carry out the transpeptidation reaction in cell-wall synthesis. This process requires an active site with a volume of more than 1000 Å^3 (44, 45), much larger than that needed for interaction with an antibiotic. There is precedence that proteins experience conformational change by allosteric modulation, occasionally with the two binding sites separated by a large distance (46–48). In fact, when the PBP2a structure was analyzed with the CASTp server (49)—which identifies surface-accessible and interior pockets that could be associated with substrate binding—two major grooves were identified: one at the active site, where binding of antibiotic or peptidoglycan is known to take place, and another within the non-penicillin-binding domain. This study was prescient. As it turns out, both sites are involved in catalysis (33), as explained below.

We first reported the possibility of an allosteric site in PBP2a in 2005 after observing an increase in the acylation rate of PBP2a in the presence of a synthetic peptidoglycan fragments (50). These results indicated that the active site becomes more available to the antibiotic, and in principle, the process could also enable catalysis, when the task is performed by the allosteric trigger. We proposed the existence of a second peptidoglycan-binding site outside of the active site. Recently, we reported a crystal structure of PBP2a in complex with the synthetic peptidoglycan fragment (PDB 3ZG5) and two structures in complex with ceftaroline (PDB 3ZFZ and 3ZG0) (Figure 4B) (33). The synthetic peptidoglycan fragment was non-covalently bound in the non-penicillin-binding domain. In the complexes with ceftaroline, as expected, we observed that ceftaroline not only acylates the active-site serine, but a second ceftaroline molecule was also found non-covalently bound in the non-penicillin-binding domain, between the N-terminal extension and Lobe 2 (33). This supports the Tipper-Strominger hypothesis (14), that ceftaroline is recognized not only at the active site, but also at a second site as a result of the similarity of its core structure to the peptidoglycan peptide stem. The β-lactam backbone of this ceftaroline molecule interacts with Y105 and Y297, and salt bridge interactions unique to the PBP2a-ceftaroline complex are formed around this second binding site. The function of this domain was unknown prior to this study. We now know that binding at this domain can trigger a conformational change that activates the protein for the transpeptidase activity, indicative of allostery. For the remainder of this review, this domain will be referred to as the allosteric domain (Figure 4).

The allosteric domain is found 60 Å distant from the active site. In the X-ray structure of PBP2a with ceftaroline bound at both the allosteric and active sites, a large conformational

change was seen through the protein when compared to the structure of the apo enzyme (PDB 1VQQ) (33). A smaller conformational change occurred when the peptidoglycan fragment was bound at the allosteric site, but the active site remained closed. Ceftaroline binding at the allosteric site translated to a set of conformational changes that resulted in a twist of the β3 strand near the active site, enabling its acylation with the antibiotic.. This results in an active-site volume increase from approximately 500 Å^3 to 1300 Å^3, allowing not only acylation by the antibiotic, but also accommodation of the peptidoglycan strands for the transpeptidation event.

This allosteric triggering propagates to the active site through a network of salt bridges, akin to dominoes falling from one site to the other (a span of 60 Å) (33). A series of single, double, and triple mutations of the residues implicated in this salt-bridge network was produced and the effect on acylation was tested. Only four of the twelve mutants had no impact on acylation of PBP2a by ceftaroline. Three mutants with substitutions in the allosteric site (K188A, K188A-K219A, and K219A-D367A) show diminished acylation ability (3–4-fold decrease in k_2/K_s (33)). Two mutants (K387A-D635A and D343A-E389A-D635A) were unable to be acylated by nitrocefin. The final three mutants showed a modest decrease in acylation by ceftaroline. As we reported previously, the presence of a synthetic peptidoglycan fragment increases the acylation efficiency of PBP2a (50). When we investigated the ability of this compound to facilitate acylation in the mutants, K287A-D635A was still unable to be acylated and the acylation of D343A-E389A-D635A was diminished compared to wild-type PBP2a. These results indicate that mutations in the salt-bridge network closer to the active site have a larger effect on the signal transmission. It would appear that there are several routes for the propagation of the conformational changes from the allosteric site to the active site through the different lobes of the allosteric domain, with residues near the active site being critical as the bottleneck.

While ceftaroline and a few other β-lactam antibiotics, including ceftobiprole and L-695,256 (51) have good activity against MRSA, some resistance has emerged. Clinical isolates showing decreased susceptibility to ceftaroline, with MIC values of 4 μg/mL, have led to the identification of mutations in PBP2a (52). These include two mutations in the allosteric site, N146K and E150K, and a third mutation outside the allosteric site, H351N. Likewise, strains with mutations around the allosteric site have shown decreased susceptibility to ceftobiprole and L-695,256. These mutations include E150K, E237K, and E239K (53, 54). While it has been suggested that these mutations may disrupt the interaction of PBP2a with other proteins (53), we propose that these mutations either affect binding at the allosteric site or the signal propagation that leads to the opening of the active site.

Comparison of PBP2a in complex with ceftaroline and ceftobiprole

A crystal structure of PBP2a acylated by ceftobiprole was recently published (34) in which structural changes in the β3 strand and the α2 helix, at the N-terminus of which one finds the active-site serine, were similar to those observed in the PBP2a-ceftaroline complex (33). One notable difference between the acyl-enzyme structures with ceftaroline and ceftobiprole is the absence of the E602-R612 salt bridge that is made as a result of interaction with the R1 group of ceftaroline seen in the PBP2a-ceftaroline complex. In addition, the R2 group of

ceftobiprole is stacked between Y446 and M641 in the PBP2a-ceftobiprole structure (34), while upon acylation by ceftaroline, the two residues interact to close the active site (33) (Figure 4A–C). Also absent from the ceftobiprole acyl-enzyme structure is the salt-bridge interaction between K387 and D635 due to a different conformation of D635. This residue appears to play an important role in the conformational change seen in the ceftaroline acyl-enzyme structure, as mutants with an alanine substitution at this position K387A-D635A and D343A-E389A-D635A completely abolished acylation activity (33).

New treatments against MRSA

Despite success in discoveries of new β-lactam antibiotics for treatment of infections by MRSA, resistance is already beginning to emerge. The inexorable emergence of resistance to any antibiotic will always be with us, hence the need for new antibiotics. Recent examples include non-β-lactam antibiotics that target PBP2a and other PBPs (55), new drug scaffolds (56), new antibiotic targets such as the isoprenoid biosynthesis (57), and combination therapy, in which synthetic compounds of various types synergize with conventional antibiotics to fight resistance (58–61). The identification of the allosteric site provides an activation mechanism for PBP2a to facilitate the transpeptidase activity. The allosteric site is an unexploited drug target, as its function is critical to catalysis in cell-wall biosynthesis.

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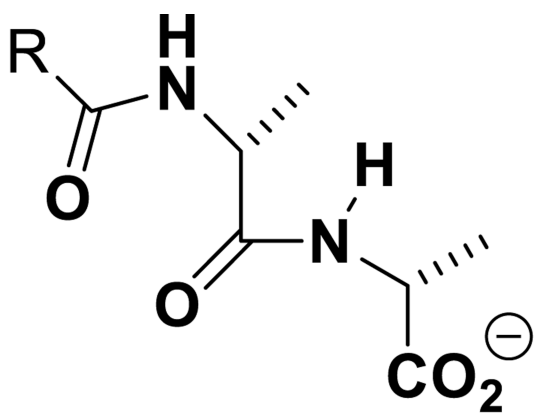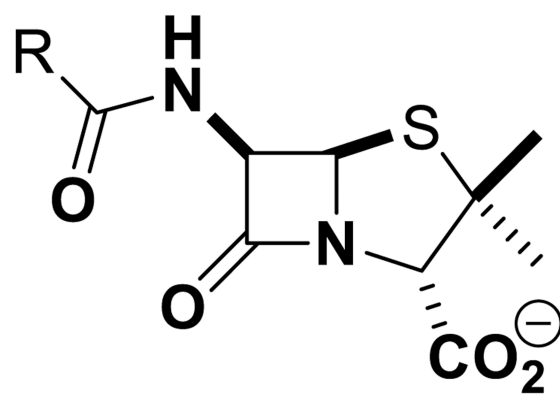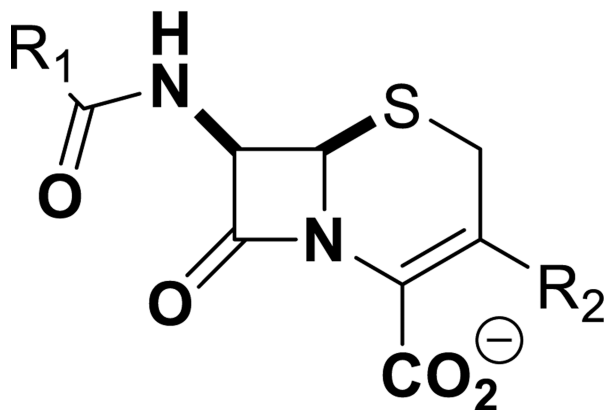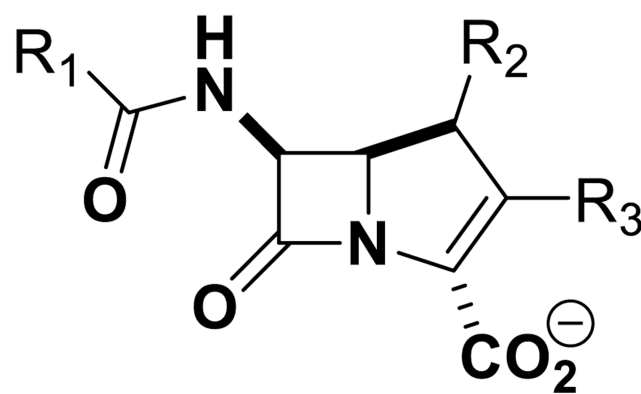
Figure 1.
The core structures of penicillins, cephalosporins, and carbapenems mimic the D-Ala-D-Ala of the peptide stem of the cell wall.
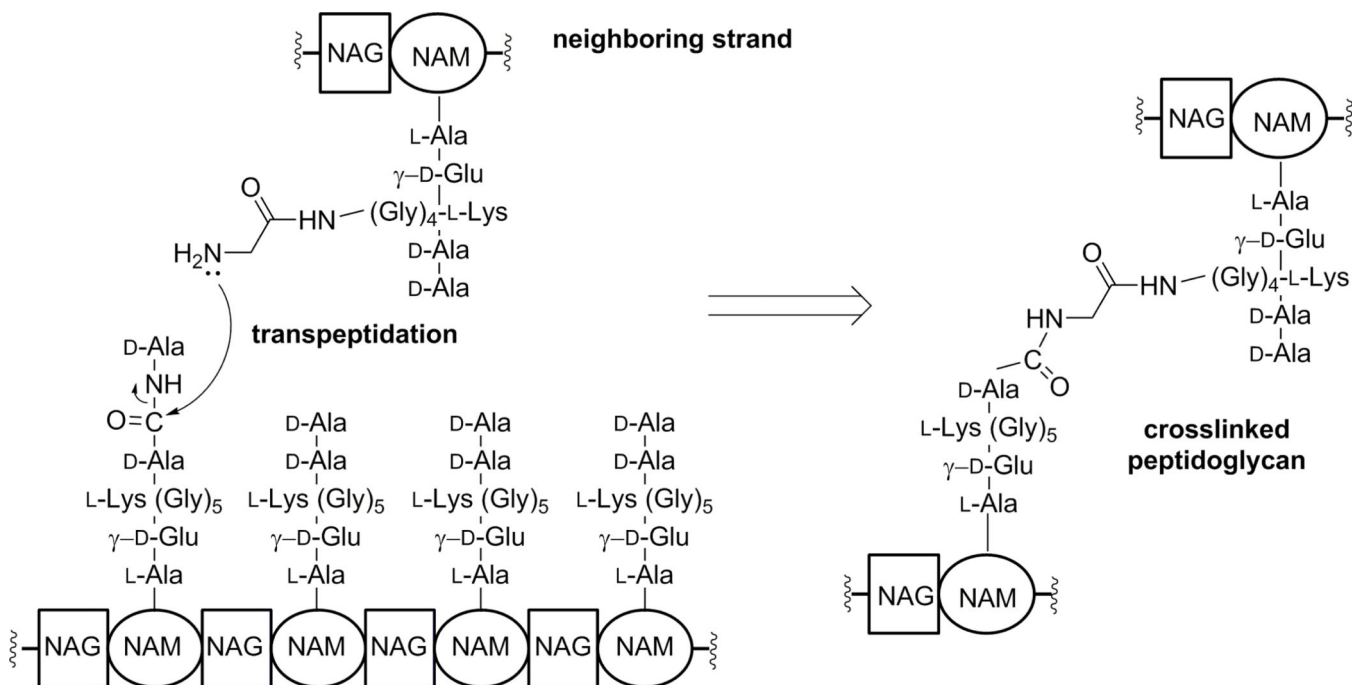
Figure 2.
Crosslinking of peptidoglycan strands in cell-wall synthesis. Elongation of the glycan strand is carried out by PBPs with transglycosylase activity. The transpeptidation reaction, where the terminal D-Ala is displaced and the peptide stems are crosslinked is accomplished by PBPs such as PBP2a in MRSA.
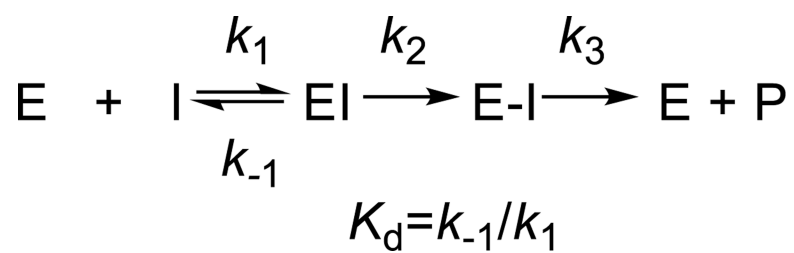
$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_2}{\longrightarrow} E\text{-}I \overset{k_3}{\longrightarrow} E + P$$

$$K_d = k_{-1}/k_1$$

Figure 3.
The minimal scheme for interactions of PBP2a with β-lactam antibiotics. The β-lactam interacts reversibly with the free enzyme before forming the stable acyl-enzyme species (E–I). Deacylation is slow (k_3) and the reaction is essentially irreversible.
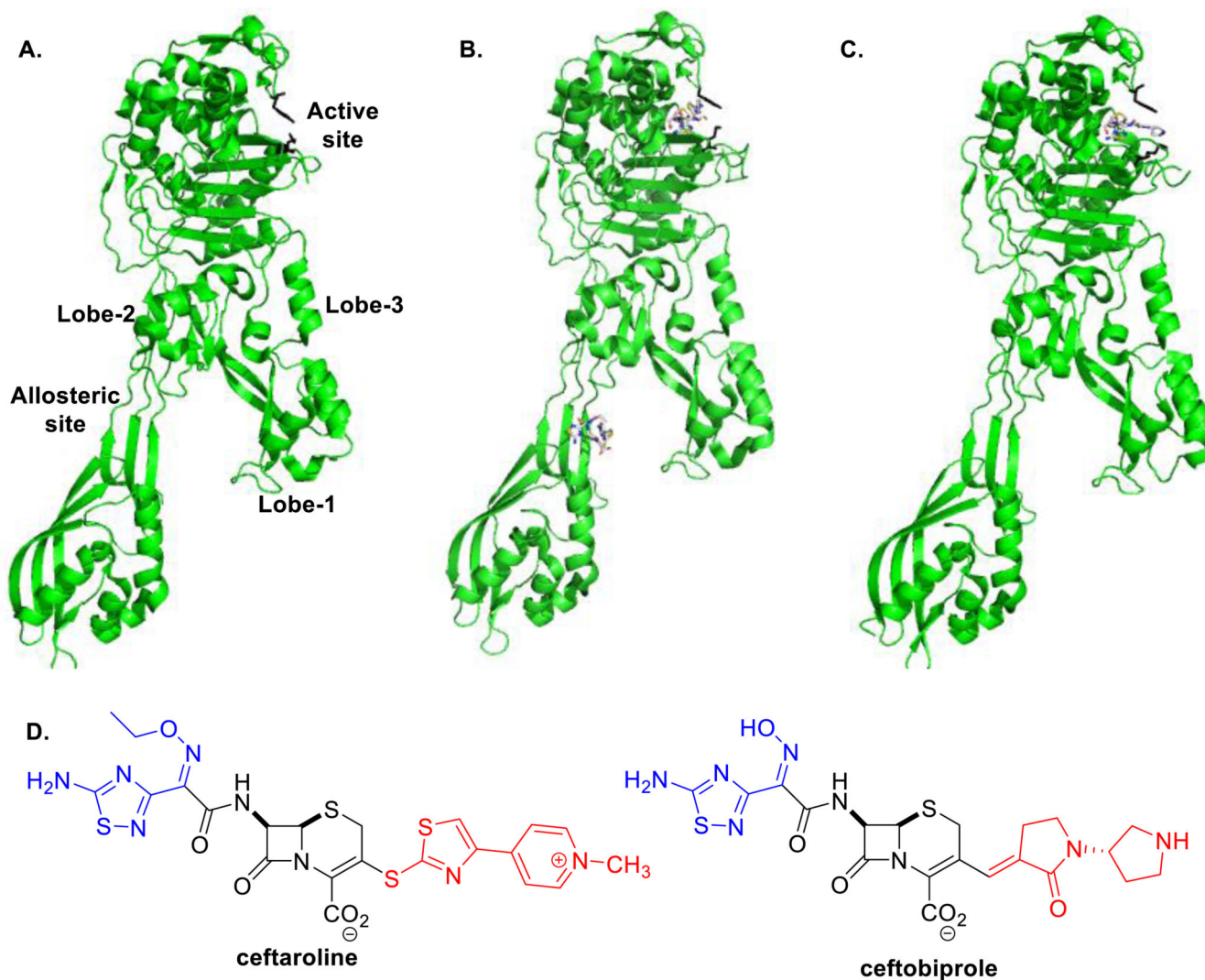
Figure 4.
X-ray structures of PBP2a in **A.** the apo-enzyme form (PDB code 1VQQ) **B.** complex with ceftaroline bound at the active site and allosteric site (3ZG0) **C.** complex of PBP2a with ceftobiprole acylating the active site (4DKI). The side chains of Y446 and M641, which act as gatekeepers of the active site, are shown as black sticks (1 o'clock). **D.** Chemical structures of ceftobiprole and ceftaroline. The R1 and R2 groups are shown in blue and red, respectively.