Structural Analysis of the Role of *Pseudomonas aeruginosa* Penicillin-Binding Protein 5 in β -Lactam Resistance

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Penicillin-binding protein 5 (PBP5) is one of the most abundant PBPs in *Pseudomonas aeruginosa*. Although its main function is that of a cell wall DD-carboxypeptidase, it possesses sufficient β -lactamase activity to contribute to the ability of *P. aeruginosa* to resist the antibiotic activity of the β -lactams. The study of these dual activities is important for understanding the mechanisms of antibiotic resistance by *P. aeruginosa*, an important human pathogen, and to the understanding of the evolution of β -lactamase activity from the PBP enzymes. We purified a soluble version of *P. aeruginosa* PBP5 (designated Pa sPBP5) by deletion of its C-terminal membrane anchor. Under *in vitro* conditions, Pa sPBP5 demonstrates both DD-carboxypeptidase and expanded-spectrum β -lactamase activities. Its crystal structure at a 2.05-Å resolution shows features closely resembling those of the class A β -lactamases, including a shortened loop spanning residues 74 to 78 near the active site and with respect to the conformations adopted by two active-site residues, Ser101 and Lys203. These features are absent in the related PBP5 of *Escherichia coli*. A comparison of the two Pa sPBP5 monomers in the asymmetric unit, together with molecular dynamics simulations, revealed an active-site flexibility that may explain its carbapenemase activity, a function that is absent in the *E. coli* PBP5 enzyme. Our functional and structural characterizations underscore the versatility of this PBP5 in contributing to the β -lactam resistance of *P. aeruginosa* while highlighting how broader β -lactamase activity may be encoded in the structural folds shared by the PBP and serine β -lactamase classes.

The bacterial cell wall is a cross-linked peptidoglycan polymer consisting of a repeating disaccharide unit of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), with NAM bearing a peptide stem (1). The final stages of cell wall biosynthesis and remodeling are performed by the penicillin-binding proteins (PBPs), which consist of the high-molecular-mass (HMM) and low-molecular-mass (LMM) PBP subgroups (2–4). PBPs are the targets of β -lactam antibiotics such as the penicillins, which react with the PBPs to form stable acyl-enzyme adducts, thus inhibiting the physiological role of the enzymes and leading to cell death (5). The widespread use of β -lactam antibiotics, however, has led to the emergence of antibiotic-resistant bacteria exploiting various mechanisms to attain resistance, of which the production of β -lactamases is particularly important in Gram-negative bacteria (6, 7). Similar to PBPs, β -lactams form an acyl-enzyme upon an encounter with serine β -lactamases (8). However, in contrast to most PBPs, β -lactamases catalyze hydrolytic deacylation and thus function catalytically.

Pseudomonas aeruginosa is an opportunistic human pathogen that can exhibit high levels of antibiotic resistance (9–12). PBP5, an LMM PBP, is one of its most abundant PBPs (13–15). The main function of its PBP5 is a DD-carboxypeptidase reaction with the cell wall, which regulates the degree of cross-linking by hydrolytically shortening the peptide stem of the nascent peptidoglycan (16). This role contrasts with the reaction catalyzed by the HMM transpeptidases, which use the full peptide stem in order to cross-link the peptidoglycan components of the cell wall. PBP5 of *P. aeruginosa* (Pa PBP5) is very similar to PBP5 of *Escherichia coli*. In *E. coli*, the PBP5-dependent DD-carboxypeptidase activity is necessary to maintain normal cell morphology, for which the degree of cross-linking matters (17, 18).

The profound similarities and profound contrasts between the

mechanisms used by the PBPs and the serine β -lactamases for their function are matters of continuing study. The serine β -lactamases are proposed to have evolved from the acquisition by the PBPs of new structural motifs so as to enable meaningful rates for the hydrolysis of the acyl-enzyme intermediates derived from β -lactam antibiotics (19). This proposal is supported by the shared structural similarities, both in the overall fold and in the active-site configurations of amino acids, between PBPs and β -lactamases. All PBPs and all serine-based β -lactamases contain a key Ser-X-X-Lys motif, where Ser is the residue that experiences acylation by the β -lactam and Lys shuttles a proton during this acylation. Additional conserved active-site motifs for both classes of enzymes include the Ser-X-Asn and Lys-Thr-Gly triads. Mechanistic understanding has benefited from microbiological (20), kinetic (21–23), structural (including complexes with various ligands along the reaction coordinate) (24–28), and computational (29, 30) studies of *E. coli* PBP5 and PBP6. In addition to the catalytic serine, the lysine in the Ser-X-X-Lys motif has an essential function as a general base in both the acylation and deacylation steps (23). Both the Ser and Asn residues of the Ser-X-Asn motif contribute to ligand binding through direct hydrogen bonds with

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the peptide stem of the peptidoglycan (28). The protonated lysine of the Lys-Thr-Gly motif provides an electrostatic anchor for the negatively charged substrate carboxylate (23). In addition, it has been suggested that an active-site loop (residues 74 to 90 of *E. coli* PBP5) plays a role in catalysis. Deletion of this loop abolishes *E. coli* PBP5's DD-carboxypeptidase activity (25). Ser83 on the corresponding loop in PBP6 is in close proximity to the leaving group of the substrate in the structure of the Michaelis complex, suggesting that it may stabilize the leaving group in the acylation reaction (28).

These amino acids find counterparts in serine-dependent β -lactamases, although their roles may not be the same. The serine in the Ser-X-Asn motif may play a more direct role in the proton relay process during acylation (31). Additional catalytic motifs, such as Glu166 of class A β -lactamases (a residue located on the so-called Ω -loop), assist in the promotion of the hydrolytic water used in deacylation. The two classes of enzymes are closely related to each other, and the evolution of their activities may involve both subtle changes of catalytic roles for existing residues and significant accommodation of new amino acids.

Initial studies on the PBP ensemble of *P. aeruginosa* demonstrated that its PBP5 has sufficient β -lactamase activity so as to confer intrinsic β -lactam resistance (14, 32, 33). This observation identifies Pa PBP5 as sharing with *E. coli* PBP5 possible transitional structures on the evolutionary pathway to β -lactamase activity. However, the details of the DD-carboxypeptidase and β -lactamase activities of Pa PBP5 are unstudied. Here, we report the structural and kinetic characterization of a soluble version of *P. aeruginosa* PBP5 (Pa sPBP5). The crystal structure of apo-Pa sPBP5 (2.05-Å resolution) reveals a protein fold that is highly similar to the related *E. coli* PBP5 and PBP6 structures and yet incorporates attributes that more closely resemble features seen previously only in the class A β -lactamases. Kinetic analyses show that Pa PBP5 accepts penicillins, cephalosporins, and carbapenems as substrates. In contrast, *E. coli* PBP5 does not accept carbapenems. Possible mechanistic features underlying this exceptional contrast with respect to carbapenems as a substrate for one (Pa PBP5) but not the other (*E. coli* PBP5) are suggested by computational evaluation of the predicted carbapenem-derived acyl-enzyme structures.

MATERIALS AND METHODS

Cloning of *P. aeruginosa* sPBP5. Chromosomal DNA from *P. aeruginosa* PAO1 (Gene ID 878956) was isolated by using a DNeasy tissue kit (Qiagen). A Pa sPBP5 gene excluding both its 24-residue-long N-terminal signal peptide and its 17-residue-long C-terminal anchor was amplified by using two custom-synthesized primers (PA-PBP5Nde [5'-ATACATA TGGCTGAATCCATGGTTCCGGCGCCG-3'] and PA-PBP5Hind [5'-CGCAAGCTTAGAAACCACCTCCTCGACGGCGTT-3']). These primers contain recognition sequences for the restriction endonucleases NdeI and HindIII (underlined). The primers were used for cloning of the PCR product into the corresponding sites of the expression vector pET24d(+) (Novagen). Recombinant plasmid was transformed into *E. coli* JM83. The nucleotide sequences of sPBP5 from several transformants were verified by the sequencing of both DNA strands. The plasmid was used to transform *E. coli* BL21(DE3) for protein expression.

Purification of *P. aeruginosa* sPBP5. sPBP5 was purified by using a three-step protocol. Cells were incubated overnight in 5 ml of LB medium supplemented with 30 μ g/ml of kanamycin. The suspension was diluted into 0.5 liter of fresh kanamycin-supplemented (30 mg/liter) LB medium. Growth was continued with mixing (180 rpm) at 37°C until the culture reached an optical density at 600 nm (OD₆₀₀) of 0.8. Isopropyl- β -D-thio-

galactoside was added to a final concentration of 0.4 mM. The culture was incubated for an additional 4 h. Cells were harvested by centrifugation at 5,000 $\times$ g for 15 min. The cells were resuspended in 20 mM HEPES (pH 8.5) buffer (buffer A). Cells were disrupted by 30 cycles of sonication (25 s of burst and 25 s of rest for each cycle). Cell debris was removed by centrifugation (18,500 $\times$ g for 40 min). The supernatant was loaded at 2.5 ml/min onto a Q-Sepharose column (2.5 by 30 cm, with 80 ml of High Q support resin; Bio-Rad) equilibrated with buffer A at 4°C. Proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A at a flow rate of 2.5 ml/min (total volume of 820 ml). sPBP5 was eluted at 0.2 to 0.3 M NaCl, as determined by SDS-PAGE. The protein fractions were combined, concentrated in buffer A, and loaded at a 1.0-ml/min flow rate onto a size exclusion column (2.5 by 100 cm, with 300 ml of Sephacryl S-200; Bio-Rad) equilibrated with buffer A. Pa sPBP5 eluted from the column at 200 to 300 min. The fractions containing sPBP5 were concentrated and brought to 0.9 M (NH₄)₂SO₄ in buffer A. This protein solution (3 ml) was loaded at a 1-ml/min flow rate onto a phenyl-agarose column (2.5 by 25 cm, with 60 ml of phenyl-agarose; Sigma) equilibrated with 0.9 M (NH₄)₂SO₄ in buffer A. The protein was eluted with a linear gradient of 0.9 to 0 M (NH₄)₂SO₄ in buffer A at 1 ml/min (total volume of 780 ml). The fractions containing sPBP5 were eluted at 0.7 to 0.6 M (NH₄)₂SO₄, as identified by SDS-PAGE. The fractions were dialyzed against 20 mM HEPES (pH 8.5) buffer. The yield of Pa PBP5 was approximately 30 mg, as determined by a bicinchoninic acid (BCA) protein assay (Pierce). The protein was concentrated to 40 mg/ml. SDS-PAGE showed that the purity of *P. aeruginosa* sPBP5 was >95%.

Enzyme assays. The DD-carboxypeptidase activity was monitored by using a fluorescence-based assay method described previously (23). Two different assays assessed β -lactamase activity. Penicillin G and ampicillin were assayed by a microiodometric assay (34). The hydrolysis of the other β -lactams was monitored spectrophotometrically: the nitrocefin $\Delta\epsilon_{486}$ was 20,500 M⁻¹ cm⁻¹ (35), the cephalothin $\Delta\epsilon_{262}$ was 7,700 M⁻¹ cm⁻¹ (36), the imipenem $\Delta\epsilon_{295}$ was 11,500 M⁻¹ cm⁻¹, the meropenem $\Delta\epsilon_{297}$ was 10,940 M⁻¹ cm⁻¹, and the doripenem $\Delta\epsilon_{297}$ was 11,460 M⁻¹ cm⁻¹ (37). The pH profiles of k_{cat} and k_{cat}/K_m were fitted computationally to a double-ionization bell-shaped model.

Pa PBP5 crystallization and structure determination. The purified protein was dialyzed at 4°C into 20 mM HEPES (pH 8.0) buffer containing 1 mM dithiothreitol (DTT). Following centrifugation (14,000 $\times$ g for 10 min), the protein concentration was calculated to be 8 mg/ml (0.21 mM) by A₂₈₀ measurement. Crystallization screening was done by using commercial solutions (PEG/Ion Screens and Crystal Screens 1 and 2; Hampton Research) at temperatures of 4°C and 15°C. The crystals used for structure determination were obtained by using 20% polyethylene glycol 3350 (PEG 3350)–0.2 M succinic acid (pH 7.0) buffer from hanging drops (1 μ l protein/1 μ l well solution) at 15°C. Crystals (approximate dimensions of 100 by 100 by 200 μ m) appeared after 4 to 5 days. Crystals were soaked for a few seconds in a stabilization solution containing 20% PEG 3350–0.2 M succinic acid (pH 7.0) buffer and 20% glycerol and flash frozen in liquid nitrogen. Diffraction data (obtained by using Beamline 8.3.1 of the Advanced Light Source, Berkeley, CA) were processed with the HKL2000 program suite (38). Molecular replacement was done by using Phaser (39) and a homology model from the Swiss-Model server generated by using the *E. coli* PBP5 structure (Protein Data Bank [PDB] accession number 1Z6F) as the template (40, 41). Initial attempts using the whole homology model failed. The two domains of the model were subsequently used separately to identify two copies of the larger N-terminal domains through molecular replacement. The C-terminal domain for each monomer was built manually. Refinement and model rebuilding were carried out with CCP4 (42) and Coot (43).

Computational methods. The X-ray structure of Pa sPBP5 provided the protein coordinates. To generate the substrate-bound model, the β -methyl NAM-L-Ala- γ -D-Glu-meso-DAP-D-Ala-D-Ala ligand (where DAP is diaminopimelate) was docked into the active site by using Gold (44, 45). Docking constraints ensured that the binding mode conformed

TABLE 1 Kinetic parameters for the carboxypeptidase activity of Pa sPBP5 at pH 9.5

Cell wall-based substrate	Mean K_m (mM) $\pm$ SD	Mean k_{cat} (s^{-1}) $\pm$ SD	Mean k_{cat}/K_m ($M^{-1} s^{-1}$) $\pm$ SD
$N_\alpha N_\epsilon$ -Diacetyl-Lys-D-Ala-D-Ala	8.7 ± 0.2	0.59 ± 0.04	65 ± 5
Methyl NAM-L-Ala- γ -D-Glu-meso-DAP-D-Ala-D-Ala	6.1 ± 1.4	0.31 ± 0.05	55 ± 20

to the experimentally determined binding mode of the same ligand in the crystal structure of *E. coli* PBP6 (PDB accession number 3ITB) (28). The best pose based on the docking score was used for a short molecular dynamics (MD) simulation of 1 ns to verify its stability. The Desmond module of the Schrödinger software suite was used to perform the MD simulation. The OPLS2005 force field provided simulation parameters. The averaged structure based on the last 500 ps of the simulation was subjected to conjugated gradient energy minimization. The acyl-enzyme species was generated by using this energy-minimized structure. The terminal D-Ala of the peptide was removed, and an ester linkage was formed between the remaining D-Ala and O- γ of Ser41. The structure was subjected to MD simulations as described above, and the averaged structure was energy minimized.

The imipenem-derived acyl-enzyme species of Pa sPBP5 and *E. coli* PBP5 were generated by using a combination of X-ray coordinates and modeling with Sybyl-X (2012; Tripos Inc., St. Louis, MO). The initial structures were solvated in a truncated octahedral box by using the TIP3P water model. Amber ff99 and gaff force fields provided simulation parameters, and the resp method was used to generate the partial atomic charges for the nonstandard species. Following a short equilibration phase, 20-ns-long trajectories were collected from the stable phase of the simulations.

Protein structure accession number. The structure of Pa sPBP5 has been deposited in the Protein Data Bank with accession number 4K91.

RESULTS

A DD-carboxypeptidase and a β -lactamase. Soluble *P. aeruginosa* PBP5 (lacking the signal peptide and the membrane anchor and purified to homogeneity) demonstrated DD-carboxypeptidase activity using two synthetic cell wall-based substrates. The first substrate was $N_\alpha N_\epsilon$ -diacetyl-Lys-D-Ala-D-Ala (23), a minimal structure that has lysine in place of the diaminopimelate (DAP) residue that is found in the cell wall of *P. aeruginosa*. The second substrate was the DAP-containing methyl NAM-L-Ala- γ -D-Glu-meso-DAP-D-Ala-D-Ala (46). The pH profile for turnover of $N_\alpha N_\epsilon$ -diacetyl-Lys-D-Ala-D-Ala (see Fig. S1 in the supplemental material) has a pH optimum of 9.5 for both k_{cat} and k_{cat}/K_m . This pH optimum is close to that seen for *E. coli* PBP5. The turnover parameters for these substrates at pH 9.5 are given in Table 1. As is also the case for PBP5 and PBP6 of *E. coli*, the *in vitro* turnover of these substrates by Pa sPBP5 is modest. The pH profile for turnover is consistent with two active-site lysines undergoing titration.

The k_{cat}/K_m profile affords pK_a values of 8.8 ± 0.1 and 10.6 ± 0.2 for the two titrations, indicative of the state of the enzyme in the absence of the substrate. The corresponding profile for k_{cat} gives pK_a values of 9.1 ± 0.3 and 10.1 ± 0.2 for the enzyme-substrate complex. Optimal catalysis is consistent with one of the lysines being in its free-base form and the other in its protonated form. Comparison to our studies with PBP5 of *E. coli* (23, 29) indicates that Lys44 is in the free-base state and that Lys203 is protonated at the catalytically optimal pH. We then evaluated Pa sPBP5 for β -lactamase activity (Table 2). Here again, the activity was modest but quantifiable. Pa sPBP5 turned over penicillins (penicillin G and ampicillin), cephalosporins (nitrocefin and cephalothin), and carbapenems (imipenem, meropenem, and doripenem). A comparison to *E. coli* PBP5 showed Pa sPBP5 to be a less capable cephalosporinase but more capable against the penicillins and especially against the carbapenems evaluated. Indeed, turnover of carbapenems by *E. coli* PBP5 was undetectable (Table 2). Hence, Pa sPBP5 shows DD-carboxypeptidase activity and a broad spectrum of β -lactamase activity.

Structure of Pa sPBP5. Pa sPBP5 gave crystals at pH 7.0 that diffracted to a 2.05-Å resolution (Table 3). Each asymmetric unit in the C2 space group contained two monomers showing pseudo-2-fold symmetry (Fig. 1A). For each monomer, the densities for all residues (except at the C terminus) are well defined. The two monomers have nearly identical backbone conformations. Similar to *E. coli* PBP5 (25) and PBP6 (28), each monomer shows two domains. The larger N-terminal domain contains the active site (Fig. 1B), while the smaller C-terminal domain is β -sheet rich. The angle between the two domains is different between the two monomers and also compared to the other DD-carboxypeptidase structures, indicating that there is flexibility in the linker region connecting the two domains. This flexibility is not expected to be relevant to the catalytic mechanism of the enzyme. However, it (together with the relatively few intramolecular and intermolecular interactions involving the C-terminal domain) might explain the relatively lower quality of the electron densities for the C-terminal domain than for the N-terminal domain. The root mean square deviation (RMSD) between the N-terminal domain back-

TABLE 2 Kinetic parameters for the β -lactamase activities of *P. aeruginosa* sPBP5 and *E. coli* sPBP5 at pH 9.5^a

β -Lactam	<i>P. aeruginosa</i> sPBP5			<i>E. coli</i> sPBP5		
	Mean K_m (μ M) $\pm$ SD	Mean k_{cat} (s^{-1}) $\pm$ SD	Mean k_{cat}/K_m ($M^{-1} s^{-1}$) $\pm$ SD	Mean K_m (μ M) $\pm$ SD	Mean k_{cat} (s^{-1}) $\pm$ SD	Mean k_{cat}/K_m ($M^{-1} s^{-1}$) $\pm$ SD
Penicillin G	60 ± 17	$(5.0 \pm 0.7) \times 10^{-3}$	84 ± 4	279 ± 25	$(7.4 \pm 0.4) \times 10^{-3}$	27 ± 2
Ampicillin	129 ± 19	$(6.4 \pm 0.6) \times 10^{-3}$	50 ± 3	373 ± 54	$(7.9 \pm 0.8) \times 10^{-3}$	21 ± 2
Nitrocefin	226 ± 28	$(4.8 \pm 0.4) \times 10^{-3}$	21 ± 2	48 ± 6	$(5.1 \pm 0.3) \times 10^{-2}$	$1,047 \pm 44$
Cephalothin	353 ± 86	$(8.5 \pm 1.4) \times 10^{-4}$	2 ± 0.2	20 ± 2	$(9.6 \pm 0.2) \times 10^{-3}$	473 ± 117
Imipenem	701 ± 67	$(1.4 \pm 0.1) \times 10^{-3}$	2 ± 0.2	ND	ND	ND
Meropenem	489 ± 77	$(1.5 \pm 0.2) \times 10^{-2}$	30 ± 2	ND	ND	ND
Doripenem	82 ± 16	$(5.7 \pm 0.5) \times 10^{-3}$	69 ± 3	ND	ND	ND

^a ND, not detectable.

TABLE 3 Crystallographic statistics

Parameter	Value(s) ^a
Data collection statistics	
Space group	C2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	232.1, 39.9, 85.5
α, β, γ (°)	90.0, 111.0, 90.0
Resolution (Å)	50.0–2.05 (2.09–2.05)
<i>R</i> _{merge} (%) ^b	5.2 (53.2)
<i>I</i> /σ <i>I</i>	18.4 (2.1)
Completeness (%)	99.8 (99.6)
Refinement statistics	
<i>R</i> _{work} / <i>R</i> _{free} (%)	21.2/26.3
No. of atoms	
Protein/ligand/water	5,285/24/155
RMSD ^c	
Bond length (Å)	0.009
Bond angle (°)	1.287
B factor (Å ²)	
Protein/ligand/water	46.3/49.1/44.1
Ramachandran plot	
Most favored (%)	89.5
Additionally allowed (%)	10.0
Generally allowed (%)	0.5

^a Values in parentheses represent highest-resolution shells.

^b Calculated by Scalepack (38).

^c Refined by Refmac in CCP4 (42).

bone atoms of Pa sPBP5 and its *E. coli* counterpart is only 1.06 Å (aligning 869 atoms), and the RMSD between the C-terminal domains is 1.35 Å (aligning 325 atoms). The RMSD similarity underscores the structural conservation in these two enzymes. The active sites of both Pa sPBP5 monomers in the asymmetric unit are located at the dimer interface (Fig. 1A). Each active site is partially blocked by residues from the neighboring monomer, due to extensive interactions involving a small active-site loop consisting of residues 76 to 79. Although these contacts allow access to the active site by small compounds such as succinate (Fig. 1C), larger substrates such as β-lactam antibiotics or peptidoglycan substrate analogs are excluded. This observation likely accounts for our inability to obtain complexes by crystal soaking. It further suggests that the crystallographic dimer interface is unlikely to be biologically relevant, notwithstanding reports that some DD-carboxypeptidases may exist as homooligomeric species *in vivo* (47).

Active-site residues. The entire N-terminal domain of Pa sPBP5, including the active site, resembles not only those of *E. coli* PBP5 and PBP6 but also the active-site domain structure of the class A β-lactamases. We closely compared the active-site configurations of the two monomers of Pa sPBP5. The configurations are similar but not identical. The points of difference reflect the different crystal packing and likely an inherent structural flexibility to the protein as well. Ser41 and Lys44 have nearly identical conformations in both monomers (Fig. 1C and D), with a hydrogen bond between Ser41O-γ and Lys44N-ζ (at a distance of 3.0 Å in monomer 1), consistent with the role of Lys44 as a general base to deprotonate the catalytic serine during acylation. Lys44N-ζ has two additional hydrogen bonds, with Asn103O-δ1 (2.6 Å) and Thr142O (3.1 Å). Other residues, including Ser78, Ser101, Asn103, and Lys203, have different conformations in monomers 1 and 2 (Fig. 1C and D). The serine corresponding to Ser78 of Pa

sPBP5 has been proposed to stabilize the leaving group (by interaction with D-Ala nitrogen) during the acylation reaction on the basis of the structure of *E. coli* PBP6 complexed with a substrate analog (28). Serine 78 likely has the same function in Pa sPBP5. In monomer 1, Ser78 points its hydroxyl group in the direction of Ser41, a conformation similar to that observed in the *E. coli* PBP6 complex structure. In monomer 2, the Ser78 side chain is directed outward toward the solvent, a conformation likely to be catalytically unimportant. In monomer 1, Lys203 hydrogen bonds with the backbone oxygen atoms of Ile97 (2.7 Å) and Ile98 (2.6 Å) and

FIG 1 Overall structure and active site of Pa sPBP5. (A) Two monomers are drawn in a ribbon representation in the asymmetric unit, with the catalytic Ser41 shown as spheres. (B) Stereo view of the ribbon representation of Pa sPBP5 (green) superimposed on that of *E. coli* PBP5 (salmon). Each monomer consists of a large N-terminal domain (bottom) and a smaller C-terminal domain (top). (C) Stereo view of 2F_o-F_c electron density (1.5 σ) of active-site residues in monomer 1. The carbon atoms in succinate are shown in yellow. Hydrogen bonds are shown as dashed lines. (D) Active site of monomer 2, with both Ser101 and Lys203 adopting different conformations.

FIG 2 Comparison of active sites. (A) Conformational similarities between active-site residues in *E. coli* PBP5 (light gray) and Pa sPBP5 monomer 1 (green). Residue numbers are based on the Pa sPBP5 sequence. (B) Conformational changes between sPBP5 monomer 1 and monomer 2. (C) Conformational similarities between the active-site residues in PBP5 monomer 2 (green) and CTX-M-9 class A β -lactamase (light gray). (D) Changes in two active-site loops in *E. coli* PBP5, Pa sPBP5, and CTX-M-9 class A β -lactamase. For the two PBP5s, only the N-terminal domains are shown. The loops of residues 77 to 87 in *E. coli* PBP5, 74 to 78 in Pa sPBP5, and 101 to 105 in β -lactamase are shown in red. Residues 147 to 158, 138 to 149, and 162 to 179 in each respective enzyme are shown in yellow.

to Ser101O- γ (2.8 Å) (Fig. 1C). In monomer 2, the interactions between Lys203 and the two Ile residues are lost as a different hydrogen bond to Thr204O (3.3 Å) is formed while maintaining a weaker contact with Ser101 (the distance between Ser101O- γ and Lys203N- ζ is 3.6 Å) (Fig. 1D). Serine 101 moves its O- γ atom to form a long hydrogen bond (3.1 Å) with Ser41O- γ , in contrast to a distance of 4.6 Å in monomer 1. Although different conformations for Ser101, Asn103, and Lys203 have been observed in other PBPs (30), Pa sPBP5 represents a unique example where multiple residues simultaneously switch their rotameric states to give different active-site configurations of the same protein. Even more interestingly, whereas the conformations of these residues in monomer 1 resemble those of the equivalent residues in *E. coli* PBP5 and PBP6 (Fig. 2A), in monomer 2, the conformations are similar to those observed in class A β -lactamases such as CTX-M (Fig. 2C). Comparing the two Pa PBP5 monomers, conformational changes that are more significant than side chain rotameric states are seen for residues 76 and 77 and residues 188 to 191. The C- α atoms of these residues differ by 0.9 to 1.6 Å between the two monomers. In particular, Arg189 in monomer 2 appears to show two separate conformations. The density for the second conformation is too ambiguous to model, and the density for the first conformation is less well defined than the Arg189 density in monomer 1.

Active-site loops. Despite the structural similarities among Pa sPBP5, *E. coli* PBP5, and class A β -lactamases, the two loops in the active sites are distinctly different among these enzymes (Fig. 2D). In *E. coli* PBP5, these loops are residues 77 to 87 (loop 1; 11 amino acids) and residues 147 to 158 (loop 2; 12 amino acids). In CTX-M class A β -lactamase, loop 1 is shortened to 5 amino acids (residues 101 to 105), while loop 2 is longer by 6 residues (residues 162 to 179). In Pa sPBP5, loop 1 is also shortened (residues 74 to 78), similar to CTX-M β -lactamase, whereas loop 2 (residues 138 to 149) resembles that of *E. coli* PBP5.

Loop 1 plays an important role in *E. coli* PBP5 catalysis. It ends with Ser87, the equivalent of Ser78 in Pa sPBP5. The O- γ atom of Ser87 is only 3.6 Å away from the boronate oxygen of a transition-state analog for the acylation event, which is suggested to mimic the leaving-group nitrogen in carboxypeptidase catalysis (26). The equivalent serine residue in *E. coli* PBP6 places its O- γ atom 3.8 Å away from this leaving group (the backbone nitrogen of the terminal D-Ala) in the Michaelis complex structure with a substrate analog (28). These observations suggest that this serine may stabilize the leaving group (backbone nitrogen of the terminal D-Ala) during acylation. Loop 1 may thus play a role in placing Ser87 in the correct position to carry out its function. Additionally, residues from this loop contribute to substrate binding through hydrogen bonds and van der Waals contacts with the substrate peptide, as shown by both the *E. coli* PBP5 and PBP6 complex structures. The importance of loop 1 and its adjacent residues for *E. coli* PBP5 catalysis is underscored by previous mutagenesis and biochemical studies, where DD-carboxypeptidase activity was abolished by deletion of residues 74 to 90 or as a result of its disordering by a G105D mutation (24).

Compared to *E. coli* PBP5, a short helix is eliminated in loop 1 of CTX-M β -lactamase (Fig. 2D). Interestingly, this reduction may open space for the introduction of a new helix in loop 2 in CTX-M. Loop 2 is the so-called Ω -loop of the class A β -lactamases. A key catalytic residue of these β -lactamases, Glu166, is situated at the end of this short helix. Glu166 functions as the general base in promoting the hydrolytic water molecule used in class A β -lactamase deacylation and is responsible for the dramatic increase of the deacylation efficiency of class A β -lactamases compared to PBPs.

Computational analysis of ligand binding. Although the narrow dimer interface in the crystal prohibits binding of substrate (we tried NAM-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala-D-Ala and ampicillin), a succinate molecule from the crystallization solution

accessed the active site. Its positioning revealed valuable information. Like many of the active-site residues, this succinate adopts slightly different conformations within the two monomers (Fig. 1C and D). In both monomers, each carboxylate of the succinate engaged hydrogen bonds. One bond is to the oxyanion hole formed by the backbone amide groups of Ser41 and His206, and the other is to the pocket normally occupied by the terminal carboxylate of the peptidoglycan substrate (or the carboxylate of the β -lactam antibiotics). When the structure of the peptidoglycan substrate in complex with *E. coli* PBP6 is superimposed onto monomer 1 of Pa sPBP5 using conserved active-site motifs as points of reference, the carboxylate of the terminal D-Ala is nearly identical to that of one of the succinate carboxylates (Fig. 3A). The terminal D-Ala carboxylate of the substrate used in the hybrid quantum mechanical and molecular mechanical study of the DD-carboxypeptidase reaction of *E. coli* PBP5 (29) and in the Pa sPBP5 computational model (described below) and the bound succinate all show interactions with Thr204 and Ser101. These interactions are stable throughout our dynamics simulations. The C- α atom of the terminal D-Ala and the carbonyl group of the penultimate D-Ala are positioned close to other atoms from the succinate molecule (Fig. 3A).

This structural information and our experimental coordinates of the Michaelis complex of the peptidoglycan bound to *E. coli* PBP6 were used in modeling the Michaelis complex of β -methyl NAM-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala-D-Ala with Pa sPBP5. The energy-minimized complex (Fig. 3B and C) shows Ser41 poised for addition to the carbonyl of the penultimate D-Ala, with the carbonyl ensconced in the oxyanion hole. Figure 3D shows the energy-minimized model of the acyl-enzyme species, with the ester carbonyl maintaining the two requisite hydrogen bonds to the oxyanion hole. The terminal D-Ala has departed the active site. The side chain of the diaminopimelate is fully solvated at the active-site opening.

Computational analysis of Pa sPBP5 carbapenemase activity. Comparison of the apo-enzyme structures and of the substrate kinetics for PBP5 of *P. aeruginosa* and for PBP5 of *E. coli* shows remarkably similar enzymes. However, the significant difference is the ability of Pa sPBP5 to turn over carbapenems. We evaluated the basis for this new catalytic ability using 20-ns MD simulations of computationally generated, carbapenem-derived acyl-enzymes for the two PBP5s. The initiation of acyl-enzyme hydrolysis requires a conformation wherein the catalytic Lys44 hydrogen bonds the hydrolytic water molecule when this water molecule occupies an “attack” angle and distance with respect to the carbonyl of the acyl-enzyme. The relative population of such conformations was assessed in the MD simulation using the hydrogen bond distance between the amine of Lys44 and the water molecule (distance d_1) and the distance between the oxygen of the water molecule and the carbon of the carbonyl (distance d_2) as coordinates (Fig. 4A). The *E. coli* sPBP5 MD simulation shows a relatively rigid ensemble of conformations (black data points in Fig. 4B) characterized by a catalytically close distance between the water and the carbon of the acyl-enzyme (d_2 is clustered at a distance of approximately 4 Å) but having a noncatalytic distance between the lysine and the water molecule (d_1 is clustered at a distance of approximately 6 Å). The oxygen of the carbonyl rests in the oxyanion hole, and the water molecule is hydrogen bonded to the NH of the dihydropyrrole segment. Neither the oxygen nor the methyl group of the hydroxyethyl side chain of the carbapenem

FIG 3 Substrate binding to Pa sPBP5. (A) Pa sPBP5 binding of succinate, compared to that of the peptidoglycan substrate. The substrate fragment (yellow) from the X-ray structure of the complex of a peptidoglycan substrate with *E. coli* PBP6 is superimposed onto the Pa sPBP5 structure using residues 41 and 204 to 206, which constitute the oxyanion hole and are involved in ligand binding. The two D-Ala residues of the substrate peptide are shown in full, together with some atoms of the preceding two residues. Hydrogen bonds between the succinate and active-site residues are shown as dashed lines. (B) Stereo view of the computational model for binding of the peptidoglycan substrate analog β -methyl NAM-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala-D-Ala to Pa sPBP5 in the Michaelis complex. Peptide and NAM carbon atoms are shown in gray, oxygens are in red, and nitrogens are in blue. The model shows the NAM-pentapeptide, ensconced in the active site based on the X-ray data of the binding of the same ligand to PBP6 of *E. coli*. (C) Close-up view of panel B within the active site, showing hydrogen bonds as yellow dashed lines. (D) Stereo view of the close-up of the acyl-enzyme species. The ester carbonyl is housed in the oxyanion hole, with Lys44 shown nearby.

contacts the water molecule. The computational structure of the Pa sPBP5 carbapenem-derived acyl-enzyme is virtually identical to the computational structure of the same acyl-enzyme with *E. coli* sPBP5 (Fig. 4C and 5A). Notwithstanding their near identity, a key contrast between these two enzymes is revealed by computational MD analysis. There is substantially greater motion avail-

FIG 4 Interactions of the hydrolytic water molecule during MD simulation. (A) Schematic representation of the distances between the closest water molecule to both Lys44 and the ester carbonyl of an imipenem-derived acyl-enzyme. (B) Distance distribution for these interactions extracted from the 20-ns MD study. Each data point corresponds to a recorded snapshot for the Pa sPBP5 acyl-enzyme (red dots) and for the *E. coli* sPBP5 acyl-enzyme (black dots). (C) Representative snapshot of the computational structure of an imipenem-derived acyl-enzyme of Pa PBP5. This structure is obtained by MD simulation and shows the position of the hydrolytic water molecule (red sphere) in a conformation permissive for initiating acyl-enzyme hydrolysis ($d_1 = 2.7$ Å; $d_2 = 3.7$ Å). The carbapenem acyl-enzyme is the Δ^2 tautomer of the dihydropyrrrole, as has been seen in experimental studies with other PBPs. The protein is represented in green ribbons, while the imipenem acyl-enzyme and Lys44 are represented as capped sticks (gray, C; red, O; blue, N; yellow, S).

able to the Pa sPBP5 acyl-enzyme, as assessed by the distribution of d_1 distances (red points in Fig. 4B) extracted from conformational snapshots. In particular, Pa sPBP5 shows a new conformational cluster centered at a d_1 distance of approximately 2.8 Å and a d_2 distance of approximately 4.0 Å. The location of this new cluster coincides with expectations for an ability to initiate acyl-enzyme hydrolysis. Pa PBP5 is able to access the conformations within this new cluster on the basis of the approximately 1-Å-greater width of its active-site cleft, as represented by the distances between the C- α atoms of Ser41 and Gly102 (distance d_4) and Ser41 and Ser78 (distance d_5 in Fig. 5B), compared to that of *E. coli* sPBP5 (Fig. 5C).

DISCUSSION

The pathogenic strains of *P. aeruginosa* that are encountered clinically today are vastly different from the *P. aeruginosa* strains used initially to demonstrate a role for its PBP5 in intrinsic β -lactam resistance. The modern *P. aeruginosa* pathogen—such as occurs in cystic fibrosis-associated infection (48)—perseveres by acquisition of an array of resistance mechanisms against virtually all chemotherapeutic agents (49, 50). While the resistance role of its PBP5 has receded, this PBP5 presents a new variation on the catalytic domain found in both the serine β -lactamases and the PBP family. This catalytic domain is versatile with respect to both mechanism (DD-carboxypeptidation, transpeptidation, and β -lactam hydrolysis) and substrate breadth (51), and the structural relationship between this adaptability and resistance development continues to compel its mechanistic study. We undertook this study of PBP5 of *P. aeruginosa* as a result of its historical contribution to intrinsic β -lactam resistance and for comparison to *E. coli* PBP5. We confirm *in vitro* DD-carboxypeptidase activity for Pa sPBP5, with a pH profile and kinetic parameters very similar to those of *E. coli* PBP5 (a bona fide DD-carboxypeptidase). In addition, we observed modest but broad (penicillin, cephalosporin, and carbapenem) β -lactamase activity for Pa sPBP5. Its ability to slowly turn over penicillins and cephalosporins parallels the known behavior of *E. coli* PBP5. The ability of Pa sPBP5 to catalyze carbapenem hydrolysis, however, is a new activity compared to *E. coli* PBP5. The structural basis for this activity is suggested by our MD simulations.

Although the highest k_{cat} values seen for carbapenems for class A β -lactamases (i.e., class A carbapenemases) are significantly lower (typical values of 0.1 to 5 s⁻¹) than the k_{cat} values for penicillins and cephalosporins (10² to 10³ s⁻¹) (52, 53), these values are sufficient for the β -lactamase to impart clinical resistance to carbapenem chemotherapy (54, 55). The poorer turnover for the carbapenems results from interference with the approach of the hydrolytic water to the acyl-enzyme by the 6 α -hydroxyethyl group of the carbapenem (56–58). Binding of carbapenems also causes conformational changes within the active site of the β -lactamase and particularly affects the residues in the Ser-X-Asn motif (e.g., Ser130 and Asn132 in TEM and SHV enzymes) and the residues of loop 1 (e.g., Tyr105). For enzymes that have evolved efficient carbapenemase activity, such as the KPC-2 β -lactamase, the active-site cavity is enlarged, and the residues spanning positions 96 to 105 have become flexible (59). The carbapenem turnover ability found for Pa sPBP5, but not for *E. coli* PBP5, reflects this same change. The flexibility and enlargement of the active site (by approximately 1 Å) are most evident with respect to the Gly76-Gly77 motif found in loop 1 of Pa PBP5 (loop 1 is equivalent to residues 101 to 105 in class A β -lactamases). This enlargement (which is accompanied by conformational changes within the Ser-X-Asn motif) underscores the correlation of carbapenemase activity to flexibility within the Pa sPBP5 active site. Increased active-site flexibility is also a key aspect of substrate expansion in the extended-spectrum β -lactamases (60). Loop 1 of *E. coli* PBP5 (residues 77 to 87) is 6 amino acids longer and includes hydrophobic residues (Val82 and Phe83) that have no counterpart in Pa sPBP5. The numerous hydrophobic contacts that Val82 and Phe83 establish with other hydrophobic residues (including Leu88, Val116, and Leu153) may reduce movement among both themselves and neighboring residues. In a recently reported acyl-enzyme complex

FIG 5 The larger active-site-cleft size for Pa sPBP5 observed by MD simulation. (A) Distances measured between the C- α atoms of Ser41 and Thr142 (d_3), Ser41 and Gly102 (d_4), and Ser41 and Ser78 (d_5). (B) Distances throughout a 20-ns MD simulation of Pa PBP5. Each change represents a 50-snapshot running average of the respective distances: d_3 , in brown; d_4 , in red; and d_5 , in blue. (C) Same distances as those in panel B measured for *E. coli* PBP5. Note the approximately 1-Å-longer distances for d_4 and d_5 of Pa PBP5 than of *E. coli* PBP5.

structure of *E. coli* PBP5 and imipenem, Leu153 forms favorable van der Waals contacts with the 6 α -hydroxyethyl group of imipenem, locking in a conformation that prevents access of the hydrolytic water to the acyl-enzyme (61). In Pa sPBP5, the equivalent leucine is more distant and does not have interactions with the acyl-enzyme (see Fig. S2A in the supplemental material). The increased volume and flexibility of the Pa PBP5 active site allow water molecules to access both Lys44, a potential general base, and the acyl-enzyme linkage, leading to the formation of a transient deacylation-competent complex, as revealed by our MD simulations (see Fig. S2B in the supplemental material). The formation of such a complex would be difficult for *E. coli* PBP5 due to the narrow and rigid features of its active site.

Our comparison of Pa sPBP5 with its *E. coli* PBP5 counterpart is particularly relevant, as it suggests that the ability to hydrolyze a wide range of β -lactam structures is intrinsically encoded in the protein fold. The molecular details of the β -lactam hydrolysis reaction mediated by Pa sPBP5 await future investigation into the particular roles of specific active-site residues, such as Ser101, and a clearer sense of the identity and position of the residues involved in deacylation. As an enzyme with dual functions, the DD-carboxypeptidase and β -lactamase activities of Pa sPBP5 are likely to share the same catalytic apparatus and similar mechanisms. Our new crystal structure and the modeled substrate complexes suggest that Pa sPBP5 may catalyze the DD-carboxypeptidase reaction following the catalytic pathway of *E. coli* PBP5, during which a neutral Lys44 functions as the general base for both acylation and

deacylation. This lysine may assume a similar role during the hydrolysis of β -lactam compounds. The catalytic role of Ser101 in the conserved Ser-X-Asn motif is less clear, as it is proposed to play different roles in DD-carboxypeptidase and β -lactamase reactions. The side chain of this serine facilitates the DD-carboxypeptidase reaction in *E. coli* PBP5 by hydrogen bonding with the substrate. On the other hand, the equivalent serine in class A β -lactamase might serve as a proton relay during catalysis (62). This serine has different conformations in *E. coli* PBP5 compared to those in the CTX-M class A β -lactamase. Importantly, both conformations are observed in the current Pa sPBP5 structure, in each of the two monomers in the asymmetric unit. In certain DD-carboxypeptidases, such as the *Actinomadura* sp. strain R39 DD-peptidase, the equivalent serine assumes the conformation more common to β -lactamases and is hypothesized to have a similar role in proton relay (63).

The PBP enzymes are a significant component of Gram-positive β -lactam resistance and are emerging as a source of Gram-negative resistance in bacteria such as *Acinetobacter baumannii* (64). Our studies of Pa sPBP5 provide a detailed analysis of its dual DD-carboxypeptidase and β -lactamase activities and suggest the structural basis for its remarkable ability to hydrolyze carbapenems. By shedding light on the similarities and differences between the *E. coli* and *P. aeruginosa* PBP5 enzymes, these data provide valuable information for further investigation into the role of Pa sPBP5 in carbapenem resistance as well as on the evolution of β -lactamase activity from the PBP enzymes.

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