Crystal Structures of KPC-2 β -Lactamase in Complex with 3-Nitrophenyl Boronic Acid and the Penam Sulfone PSR-3-226

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Class A carbapenemases are a major threat to the potency of carbapenem antibiotics. A widespread carbapenemase, KPC-2, is not easily inhibited by β -lactamase inhibitors (i.e., clavulanic acid, sulbactam, and tazobactam). To explore different mechanisms of inhibition of KPC-2, we determined the crystal structures of KPC-2 with two β -lactamase inhibitors that follow different inactivation pathways and kinetics. The first complex is that of a small boronic acid compound, 3-nitrophenyl boronic acid (3-NPBA), bound to KPC-2 with 1.62-Å resolution. 3-NPBA demonstrated a K_m value of $1.0 \pm 0.1 \mu\text{M}$ (mean $\pm$ standard error) for KPC-2 and blocks the active site by making a reversible covalent interaction with the catalytic S70 residue. The two boron hydroxyl atoms of 3-NPBA are positioned in the oxyanion hole and the deacylation water pocket, respectively. In addition, the aromatic ring of 3-NPBA provides an edge-to-face interaction with W105 in the active site. The structure of KPC-2 with the penam sulfone PSR-3-226 was determined at 1.26-Å resolution. PSR-3-226 displayed a K_m value of $3.8 \pm 0.4 \mu\text{M}$ for KPC-2, and the inactivation rate constant (k_{inact}) was $0.034 \pm 0.003 \text{ s}^{-1}$. When covalently bound to S70, PSR-3-226 forms a *trans*-enamine intermediate in the KPC-2 active site. The predominant active site interactions are generated via the carbonyl oxygen, which resides in the oxyanion hole, and the carboxyl moiety of PSR-3-226, which interacts with N132, N170, and E166. 3-NPBA and PSR-3-226 are the first β -lactamase inhibitors to be trapped as an acyl-enzyme complex with KPC-2. The structural and inhibitory insights gained here could aid in the design of potent KPC-2 inhibitors.

Carbapenemases pose a serious clinical threat to the “last-resort antibiotics,” the carbapenems (imipenem, meropenem, eripenem, and doripenem) (22, 33). In particular, the *Klebsiella pneumoniae* carbapenemases (KPCs) are troublesome, as they are often carried on a plasmid, leading to rapid dissemination (14, 22, 30, 33). The first isolated member of the KPC family, KPC-2 β -lactamase, was identified in 1996 in North Carolina as part of the Intensive Care Antimicrobial Resistance Epidemiology (ICARE) Project (36). To date, 11 KPC variants have been described (<http://www.lahey.org/studies/>), with KPC-2 and KPC-3 being the dominant variants; both KPC-2 and KPC-3 are becoming endemic in the United States, Greece, and Israel (35). KPCs have also emerged in China, South America, and many countries in Europe (35). Although *Klebsiella pneumoniae* is the predominant host species for KPC β -lactamases, other *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. have recently been reported to harbor *bla*_{KPC} genes (6, 23, 27, 32, 35). These reports represent a disturbing development in the spread of these carbapenemases.

Microbiological and biochemical studies have shown that KPCs are only weakly inhibited by clavulanic acid, sulbactam, and tazobactam (14, 18), spurring the need for development of inhibitors against this β -lactamase (7, 8, 25). A number of approaches exploiting different inhibitor scaffolds are being undertaken to find inhibitors of class A β -lactamases (3). In this report, we focus on two of these scaffolds: boronic acid compounds, which are used as probes to understand structural interactions in class A and C β -lactamases and in KPC detection assays (2, 4, 9, 21), and 6-(unsubstituted)penam sulfones, which have been shown to inhibit KPC-2 in the low micromolar range (18). We determined the structure of KPC-2 in complex with 3-nitrophenyl boronic acid (3-NPBA) and with PSR-3-226 (Fig. 1) and tested each compound's activity against KPC-2 (26). PSR-3-

226 is a derivative of SA2-13 (Fig. 1) with a different C-2 moiety. Previously, SA2-13 was rationally designed to stabilize the inhibitory *trans*-enamine intermediate (16). We present here the first structures of β -lactamase inhibitors in complex with KPC-2. The knowledge obtained from this structural analysis is a starting point for structure-based inhibitor optimization.

MATERIALS AND METHODS

Cloning. A minor truncation of the C terminus of KPC-2 resulted in improved crystal growth (25). We therefore engineered a similar truncated KPC-2 construct by removing the last 4 KPC-2 amino acid residues by using the following primers: forward primer, 5'-GAATTCATATGTCAGTGTATCGCCGTCTAGTT-3'; reverse primer, 5'-CCGGAATTCCTTAGCCCAATCCCTCGAG-3'. The gene encoding the truncated KPC-2 was PCR amplified using Pfx polymerase (Invitrogen) and the pBR322-*catI*-*bla*_{KPC-2} vector template, gel purified using a QIAquick gel extraction kit (Qiagen), and ligated into the NdeI and EcoRI restriction sites of the pET30a vector (Novagen) prior to transformation into *Escherichia coli* DH5 α cells (36). After sequence confirmation, the plasmid was transformed into *E. coli* BL21 (DE3) competent cells (Invitrogen) for large-scale protein expression.

Expression and purification. Six liters of lysogeny broth (LB) containing 30 $\mu\text{g}/\text{ml}$ kanamycin was inoculated with 3% overnight culture,

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FIG 1 Chemical structures of the inhibitors 3-NPBA, PSR-3-226, and SA-2-13.

and cultures were grown at 37°C until the optical density at 600 nm reached 0.5 to 0.6. Subsequently, the temperature was lowered to 20°C, expression was induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma), and growth was allowed to continue overnight. Cells were subsequently pelleted by centrifugation and stored at -80°C until further use. Cell pellets were thawed and resuspended in 10 mM Tris buffer (pH 7.0) followed by lysis using sonication. After centrifugation, the filtered supernatant was passed through a preequilibrated phenylboronate affinity column as previously described (8). Briefly, a phenylboronate affinity column was preequilibrated with 10 mM Tris buffer (pH 7.0). The column was washed with 10 mM Tris buffer (pH 7.0)–0.5 M NaCl before eluting KPC-2 with 0.5 M boronate (pH 7.0)–0.5 M NaCl. KPC-2 was further purified using a Superdex 75 size exclusion column (GE Biosciences) equilibrated in 40 mM bis-Tris (pH 5.9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that the homogeneity of KPC-2 was over 95%. The protein was subsequently concentrated to 22 mg/ml in 40 mM bis-Tris (pH 5.9). About 60 mg of purified KPC-2 protein was obtained from a 6-liter culture.

Synthesis of PSR-3-226. PSR-3-226 was prepared as part of a large library of more than 50 2'-substituted 6-(unsubstituted)penam sulfones, which were synthesized and screened for activity against 8 different β -lactamases (17). These data, including the synthesis and structure-activity relationships (SARs), will be published separately. The synthetic scheme illustrating the methodology utilized in this library design is illustrated in Fig. 2.

Crystallization and soaking experiments. Initial crystallization conditions were obtained using sparse matrix crystallization screens and tested under the previously published KPC-2 crystallization conditions (8, 25). The best crystals were grown from 20% polyethylene glycol (M_w ,

6,000 Da) or polyethylene glycol 6000 (PEG 6000) in 100 mM potassium thiocyanate (KSCN) and 100 mM citrate (pH 4) at 20°C by using the vapor diffusion sitting drop method with a drop size of 1 μl protein and a 1 μl reservoir. To obtain an inhibitor-complexed KPC-2 structure, we did not include citrate buffer in the soaking and freezing solutions, since citrate might compete with the inhibitor in the active site (25). The crystals were therefore soaked overnight in a 3-NPBA soaking solution (containing 50 mM 3-NPBA in 25% PEG 6000 and 100 mM Tris-Cl [pH 7.2]) and were subsequently used for data collection. A similar soaking approach was attempted to obtain a PSR-3-226:KPC-2 structure, but this approach was not successful, as the crystals dissolved. To obtain the PSR-3-226-bound structure, KPC-2 crystals were soaked in a solution containing 50 mM PSR-3-226 with 25% PEG 6000 and 100 mM citrate (pH 4.0) for 2 h 45 min (longer soaking periods caused the crystal to visibly deteriorate). The soaked crystals were cryo-protected with 20% ethylene glycol in the corresponding mother liquor inhibitor soaking solution and flash-frozen in liquid nitrogen prior to data collection.

Data collection and structure determinations. Data for the KPC-2: 3-NPBA and the KPC-2:PSR-3-226 complexes were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline BL11-1 and Advanced Photon Source (APS) beamline 23-ID, respectively. Both data sets were processed using HKL2000 (15). The KPC-2 complex structures were determined using molecular replacement with the program Phaser (11) with chain A of the truncated KPC-2 structure (PDB 3C5A) (25) as the search model. Crystallographic refinement was performed using REFMAC (12), and model building was done using COOT (5). The PRODRG2 server (29) was used to obtain the parameter and topology files for the chemical structures built in the active site, including the 3-NPBA

FIG 2 Synthesis of PSR-3-226. Reagents and conditions were as follows: (a) H_2SO_4 , NaNO_2 , KBr, ethanol (EtOH), 6 to 8°C , 3.5 h; (b) peracetic acid, benzophenone hydrazone, KI, DCM/ H_2O , 0°C , 1 h; (c) Zn dust, $\text{AcOH}/\text{CH}_3\text{CN}$, 0°C , 1 h; (d) MBT, toluene, 114°C , 2 h; (e) $\text{ClCH}_2\text{CO}_2\text{H}$, AgOAc , DCM, room temperature (RT), 4 h; (f) KMnO_4 , $\text{AcOH}-\text{H}_2\text{O}$ -acetone (5:1:1), RT, 1 h; (g) thiourea, EtOH, 70°C , 1 h; (h) DMP, DCM, RT, 1 h; (i) NaClO_2 , NaH_2PO_4 , 3-methyl-2-butene, $\text{tBuOH}-\text{THF}$ (3:1), RT, 1 h; (j) oxalyl chloride, pyr, DCM, RT, 1 h; (k) $\text{HOCH}_2\text{CONH}_2$, pyr, DMAP; (l) (i) H_2 , cat. Pd and (ii) NaHCO_3 or (i) TFA, anisole, -10°C and (ii) NaHCO_3 .

TABLE 1 Data collection and refinement statistics^a

Study phase and parameter	3-NPBA:KPC-2	PSR-3-226:KPC-2
Data collection		
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
a, b, c (Å); α , β , γ (°)	50.4, 66.6, 73.6; 90, 90, 90	49.1, 66.3, 71.8; 90, 90, 90
Wavelength (Å)	0.97945	0.98400
Resolution (Å)	50.00–1.62 (1.68–1.62)	50.00–1.26 (1.31–1.26)
R _{sym}	5.9 (36.1)	7.3 (27.2)
I/ σ I	21.3 (3.3)	23.1 (3.4)
Completeness (%)	91.9 (75.7)	97.9 (82.5)
Redundancy	4.4 (4.4)	6.7 (2.7)
Refinement		
Resolution range (Å)	27.8–1.62 (1.661–1.619)	33.2–1.26 (1.293–1.260)
No. of reflections	28,074	59,322
R(work)/R(free)	16.0/19.2 (25.8/29.2)	15.4/16.9 (22.5/24.6)
No. of atoms		
Protein, inhibitor, water, others	2,010, 12, 246, 0	2,013, 21, 316, 26
RMSD ^b		
Bond length (Å)	0.010	0.010
Bond angles (°)	1.226	1.369
Avg B factors (Å ²)		
Protein, inhibitor, water, others	15.1, 22.5, 30.6, 0	13.4, 53.0, 28.3, 73.8
Ramachandran plot statistics (%)		
Core regions	94.7	94.7
Additional allowed regions	5.3	5.3
Generously allowed regions	0.0	0.0
Disallowed regions	0.0	0.0

^a Values in parentheses refer to the highest-resolution shell.^b RMSD, root mean square deviation.

and the *trans*-enamine intermediate of PSR-3-226. Crystallographic refinement was monitored using the program DDQ (31), and the final model quality was assessed using PROCHECK (10). Data collection and refinement statistics are shown in Table 1.

β -Lactamase inhibition assays. Steady-state kinetic parameters were determined with an Agilent 8453 diode array spectrophotometer at 25°C in 10 mM PBS buffer using purified KPC-2. The kinetic parameters $V_{\max}$ and K_m , calculated from initial steady-state velocities (v), were obtained by using an iterative nonlinear least squares fit of the data to the Henri-Michaelis equation and utilizing Enzfitter (Biosoft Corporation), according to the following equation: $v = (V_{\max}[S])/(K_m + [S])$, where v is the observed velocity, $V_{\max}$ is the maximum velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis constant determined for nitrocefin.

For the purposes of these analyses, 3-NPBA and PSR-3226 were regarded as mechanism-based inhibitors. As a result, K_m values of the inhibitors were determined in competition assays using 100 μ M nitrocefin ($\Delta\epsilon_{482}$, 17,400 M⁻¹ cm⁻¹). Plots of $1/[S]$ versus activity values were linear and provided the y intercept/slope values used for K_m determinations. K_m values were corrected by taking into account the nitrocefin affinity, according to the following equation: $K_m(\text{corrected}) = K_m(\text{observed})/\{1 + [S]/K_m(\text{nitrocefin})\}$, where $[S]$ is the concentration of nitrocefin (100 μ M) and $K_m(\text{nitrocefin})$ is the Michaelis constant determined for nitrocefin (for SHV-1, 20 μ M; for KPC-2, 5 μ M). The kinetic parameters were calculated using data from experiments. The k_{inact} values were determined using a fixed concentration of enzyme and nitrocefin and increasing in-

TABLE 2 Kinetics of inhibition^a

Complex	K_m (μ M)	k_{inact} (s ⁻¹)
KPC-2:3-NPBA	1.0 $\pm$ 0.1	ND
SHV-1:PSR-3-226	4.43 $\pm$ 0.22	0.10 $\pm$ 0.01
KPC-2:PSR-3-226	3.8 $\pm$ 0.4	0.034 $\pm$ 0.003

^a Values are means $\pm$ standard errors. ND, not determined.

hibitor concentrations. The k_{obs} values were determined using a nonlinear least squares fit of the data employing Origin 7.5 software and the following equation: $A = A_0 + v_f t + (v_0 - v_f)[1 - \exp(-k_{\text{obs}} t)]/k_{\text{obs}}$, where A is the absorbance, v_0 (expressed as the variation of absorbance per unit of time) is the initial velocity, v_f is the final velocity, and t is time.

Next, k_{obs} was plotted versus the inhibitor concentrations of the experiment and fit to determine k_{inact} according to the following equation: $k_{\text{obs}} = (k_{\text{inact}}[I])/(K_I + [I])$.

MIC determinations and disk diffusion assays. The susceptibilities of *Klebsiella pneumoniae* possessing *bla*_{KPC-2} and *E. coli* DH10B pBR322-*catI* expressing *bla*_{KPC-2} were assessed using Mueller-Hinton (MH) agar dilution MICs according to a method described by the Clinical and Laboratory Standards Institute (CLSI) (1). MICs were determined using a Steers replicator, which delivered 10 μ l of culture containing 10⁴ CFU per spot. The cefotaxime concentration was varied from 0.06 mg/liter to 128 mg/liter, while 3-NPBA was maintained at 4 mg/liter. 3-NPBA and cefotaxime were purchased from Sigma (St. Louis, MO).

Disk diffusion assays were performed following CLSI guidelines (13). Disks containing 30 μ g of cefotaxime were used alone (Becton Dickinson). Ten micrograms of 3-NPBA, 10 μ g of PSR-3-226, and 30 μ g of PSR-3-226 were resuspended in dimethyl sulfoxide (DMSO) and pipetted onto disks containing 30 μ g of cefotaxime or blank (no inhibitor) disks and allowed to dry for 1 h; in addition, DMSO alone was used as a control. Colonies of *E. coli* DH10B that expressed pBR322-*catI*-*bla*_{KPC-2} were directly resuspended into MH broth in an amount producing the equivalent of a 0.5 McFarland standard and were used to inoculate MH agar plates. The disks were carefully placed on each plate. The bacteria were grown at 37°C for 18 h, and zone diameters were measured.

Protein Data Bank accession numbers. The coordinates and structure factors for the KPC-2 complexes have been deposited with the Protein Data Bank (PDBID for 3-NPBA:KPC-2, 3RXX; PDBID for PSR-3-226:KPC-2, 3RXW).

RESULTS AND DISCUSSION

MICs, kinetics, and the KPC-2:3-NPBA complex. In susceptibility testing, 3-NPBA at 4 mg/liter decreased cefotaxime MICs from 32 mg/liter to 4 mg/liter for *K. pneumoniae* and *E. coli* DH10B strains expressing *bla*_{KPC-2}. For *E. coli* DH10B carrying pBR322-*catI*-*bla*_{KPC-2}, zone diameters during disk diffusion assays increased from 20 mm for cefotaxime alone to 37 mm for cefotaxime combined with 3-NPBA.

3-NPBA inhibited KPC-2 β -lactamase with a K_m value of 1.0 $\pm$ 0.1 μ M (mean $\pm$ standard error) (Table 2).

The X-ray crystal structure of KPC-2:3-NPBA was determined to 1.62-Å resolution. During the initial model-fitting stages, an unbiased omit *Fo*-*Fc* map contoured at 2.5 σ clearly revealed the 3-NPBA ligand covalently attached to the O γ atom of the catalytic S70 residues (Fig. 3A). Density for the nitro moiety of 3-NPBA is somewhat weaker than that for the rest of 3-NPBA, which is perhaps explained by the distance of this moiety from the boron-carbon bond of 3-NPBA. A relatively small rotational heterogeneity around this bond would displace the more distant nitro group, yielding a weaker density for this moiety than for the rest of 3-NPBA. In addition to 3-NPBA, 246 water molecules were in-

FIG 3 Unbiased omit F_o-F_c electron density maps contoured at 2.5σ , depicting 3-NPBA in the active site of KPC-2 β -lactamase (A) or the *trans*-enamine intermediate of PSR-3-226 in the active site of KPC-2 β -lactamase (B). Citrate molecules in panel B were omitted for clarity. (C) Partially occupied citrate molecules in the PSR-3-226-bound KPC-2 structure.

cluded in the model, and the final model refined to an $R(\text{work})$ value of 16.0% and an $R(\text{free})$ value of 19.2% (additional data and refinement statistics are listed in Table 1).

The overall structure of KPC undergoes little change upon ligand binding, as the 3-NPBA-bound KPC-2 structure was similar to that of the full-length KPC-2 and the C-terminally truncated structures, yielding a root mean square deviation (RMSD) for $C\alpha$ atoms of 0.574 and 0.226 Å, respectively.

In Fig. 4, the interactions of 3-NPBA with the KPC-2 active site are illustrated. First, 3-NPBA forms a dative bond, via its boron atom, with the O_γ atom of S70. Second, both boronate oxygens form extensive interactions; one boronate oxygen hydrogen bonds with the backbone nitrogens of S70 and T237 and the carbonyl oxygen of T237, whereas the other boronate oxygen interacts with the N170 and E166 side chains. Third, the CP5 carbon atom of the phenyl ring of 3-NPBA undergoes a 3.8-Å van der Waals interaction with W105 via an edge-to-face interaction, a common interaction for aromatic moieties (1). Furthermore, the π ring electrons of the phenyl ring of 3-NPBA interact via cation- π interactions with K73 (4.0 Å) and N132 (3.6 Å). Both types of interactions with phenyl rings have been observed previously (24, 37). Finally, 3-NPBA allows an interaction with a water molecule (Fig. 4A). The positions of the boronic acid oxygens of 3-NPBA bound in the active site indicate that the inhibitor adopts a conformation that is reminiscent of a deacylation transition state inhibitor. Such a boronic acid transition state analog conforma-

tion was described previously for larger boronic acid transition state inhibitor (BATSI) compounds when bound to SHV-1 β -lactamase (9) (Fig. 4B). In this conformation, one of the boron hydroxyl groups is positioned in the oxyanion hole and the other one displaces the deacylation water positioned normally between E166 and N170. A previous structure analysis of 3-NPBA bound to the class C β -lactamase AmpC (26) revealed a different orientation for the ligand compared to the KPC-2:3-NPBA structure. The 3-nitro moiety of 3-NPBA in the AmpC complex is in a position that would not be compatible within the KPC-2 structure, as it would sterically clash with the KPC-2 N170 side chain. One common feature, however, between the two 3-NPBA structures is the phenyl ring-Asn interaction, as Asn152 of AmpC is in a similar position as Asn132 in KPC-2.

MICs, kinetics, and the KPC-2:PSR-3-226 complex. In disk diffusion assays, 10 μg of PSR-3-226 combined with cefotaxime did not change zone sizes of *E. coli* DH10B pBR322-*catI*-*bla*_{KPC-2}. When 30 μg of PSR-3-226 was combined with cefotaxime, an increase in zone size from 16 mm to 19 mm was observed.

The measured affinities of PSR-3-226 for KPC-2 and SHV-1 were similar, although it had a slightly lower K_m for KPC-2 (Table 2). The inactivation rate constant k_{inact} is, however, about 3-fold lower for KPC-2 compared to SHV-1, likely pointing to KPC-2's inherent reduced susceptibility to inhibition.

The X-ray crystal structure of the KPC-2:PSR-3-226 complex was determined to 1.26-Å resolution. The unbiased omit *Fo-Fc*

FIG 4 3-NPBA bound to KPC-2. (A) Stereo view of interactions of 3-NPBA in the active site of KPC-2 β -lactamase. (B) Active site superposition of the KPC2:3-NPBA structure with the SHV-1:cefoperazone BATSI structure (PDB ID 3MKF). SHV-1:cefoperazone BATSI is shown in white, and the KPC-2:3-NPBA structure is in black. Residues used for superpositioning include 70, 73, 105, 130, 132, 166, 170, and 234 to 237.

FIG 5 PSR-3-226 bound to KPC-2. (A) Stereo view of interactions of PSR-3-226 in the active site of KPC-2. Citrate molecules were omitted for simplicity. Dashed black lines indicated hydrogen bonds. (B) Active site superposition of the KPC-2:PSR-3-226 structure with the SHV-1:SA2-13 structure (PDB ID 2H5S). KPC-2:PSR-3-226 is shown in black, while SHV-1:SA2-13 is white, with the protein shown in a transparent cartoon representation. Residues used for superposition are the same as those used in Fig. 4.

map contoured at 2.5σ revealed density for a ligand covalently attached to the O γ atom of the catalytic S70 residue (Fig. 3B). A *trans*-enamine intermediate was modeled into this density, and the dihedral angle for this *trans*-enamine was refined to 179.9° . Clear density for the atoms of this intermediate was observed up to its CB atom, whereas the electron density for the rest of the tail of the compound was poor. This suggests that the tail region is flexible, in particular around the CA-CB bond, and these poorly resolved atoms include the sulfone moiety, methyl group, and amide tail R2 linker. The apparent flexibility of this part of the compound was also evident from the refined higher B factors of these tail atoms, which is consistent with their poorly resolved density. Occupancy for the PSR-3-226 ligand was varied and showed that the best refinement results occurred with an occupancy of 0.7. Adjacent to PSR-3-226, a citrate molecule with two partial occupancies was also modeled, of which 5 citrate atoms had strong density (Fig. 3C). After careful examination and adjustment of the structure, we placed a water molecule with occupancy of 0.3 in the oxyanion hole and a deacylation water molecule with 0.5 occupancy, bridging E166 and N170, to account for the extra density at these corresponding positions. In total, 316 water molecules were included in refinement, and the final model had an R(work) of 15.4% and an R(free) of 16.9% (additional refinement statistics are listed in Table 1).

The overall protein conformation of KPC-2 when complexed to PSR-3-226 is similar to the uncomplexed full-length KPC-2 and the C-terminally truncated KPC-2 structures, with an RMSD of 0.591 and 0.158 Å, respectively. The active site undergoes little change upon ligand binding, except for residues W105 and S130, which have two alternative conformations that appeared to correlate with the 0.7 occupancy of PSR-3-226 and alternate conformations of citrate, respectively. The alternate W105 conformations, with 30 and 70% occupancies, are likely a result of avoiding steric clashes with the inhibitor, which has 70% occupancy. PSR-3-226 forms a linear *trans*-enamine intermediate in the active site, with the carbonyl oxygen being positioned in the oxyanion hole, as was seen before for SA2-13 in SHV-1 (Fig. 5) and also in the R164 mutants of SHV-1 (16, 28). Additional interactions are formed by the C-3 carboxylate group of PSR-3-226, which is within hydrogen bonding distance of N132, E166, and N170 (Fig. 5A). Such

interactions were also observed for the SHV-1:SA2-13 complex. Furthermore, a superpositioning of these two structures revealed that substantial parts of SA2-13 and PSR-3-226 are in a similar conformation (Fig. 5B) (16). The interactions of the mostly disordered tail region of PSR-3-226 are not further discussed here, due to the large uncertainty of their atomic positions and because of their poor electron densities. In addition to PSR-3-226, there was also a partially ordered citrate molecule observed in the active site. The position of the carboxylate of citrate interacting with the KPC-2 active site was localized in the general carboxylate binding site of serine β -lactamases, similar to where one of the other carboxylates of citrate, bicine, and even BLIP residue D49 were shown to interact in earlier-described KPC-2 structures (7, 8, 25). The presence of a carboxyl moiety, (i.e., that of citrate), situated in the carboxyl binding pocket in close proximity to the covalent O γ -carbonyl carbon bond, is reminiscent of SA2-13 bound to SHV-1, in which SA2-13's own carboxyl linker is situated in this position. Although we assigned this density as a partially ordered citrate, we did consider the possibility that this density represented the amide linker of PSR-3-226 when in the *trans*-enamine intermediate conformation; this did not yield a satisfactory fit with the electron density, likely due to the linker being two atoms shorter than SA2-13. Therefore, this density was modeled as citrate. Note that citrate was also present in the previous KPC-2 crystal structure with one of its carboxyl moieties in a similar position as in our structure (25). Interactions of the carboxyl moiety of citrate in the active site involved KPC-2 residues S130, adopting two different conformations, T235, T237, and K234.

We did attempt to obtain a KPC-2:SA2-13 complex structure via soaking SA2-13 with KPC-2 crystals, but we were unsuccessful. This could have been due to steric repulsion, as superpositioning of the SA2-13:wtSHV-1 structure with that of the PSR-3-226-bound KPC-2 structure indicated that SA2-13 binding to KPC-2 would be sterically hindered due to W105 in either of the two observed conformations, as extrapolated van der Waals distances would be less than 2.5 Å. We note that the region of the active site, encompassing residues W105 and P104, has previously been observed to have a key role in affecting substrate catalysis and inhibition efficacy in KPC-2 (19, 34).

In conclusion, 3-NPBA and PSR-3-226 are the first β -lacta-

mase inhibitors to be trapped as acyl-enzyme complexes with KPC-2. The KPC-2:PSR-3-226 structure presented here may serve as a good lead for further structure-based inhibitor optimization based on the penam sulfone inhibitor scaffold. The observation of a *trans*-enamine conformation suggests that this mode of inhibition has significant potential against KPC-2 β -lactamases, and the structure provides insights into how to further stabilize this deacylation-resistant intermediate.

The KPC-2:3-NPBA structure is also a potential starting point for future drug design efforts to optimize boronic acid transition state inhibitors. Such small boronic acid compounds have clear potential as inhibitors, because similar compounds, such as 3-aminophenylboronic acid, have a broad specificity for class A carbapenemases (20). Further studies are under way to optimize these types of inhibitors.

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