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Understanding the Molecular Determinants of Substrate and Inhibitor Specificities in the Carbapenemase KPC-2: Exploring the Roles of Arg220 and Glu276

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β-Lactamases are important antibiotic resistance determinants expressed by bacteria. By studying the mechanistic properties of β-lactamases, we can identify opportunities to circumvent resistance through the design of novel inhibitors. Comparative amino acid sequence analysis of class A β-lactamases reveals that many enzymes possess a localized positively charged residue (e.g., R220, R244, or R276) that is critical for interactions with β-lactams and β-lactamase inhibitors. To better understand the contribution of these residues to the catalytic process, we explored the roles of R220 and E276 in KPC-2, a class A β-lactamase that inactivates carbapenems and β-lactamase inhibitors. Our study reveals that substitutions at R220 of KPC-2 selectively impact catalytic activity toward substrates (50% or greater reduction in k_{cat}/K_m). In addition, we find that residue 220 is central to the mechanism of β-lactamase inhibition/inactivation. Among the variants tested at Ambler position 220, the R220K enzyme is relatively "inhibitor susceptible" (K_i of 14 ± 1 μM for clavulanic acid versus K_i of 25 ± 2 μM for KPC-2). Specifically, the R220K enzyme is impaired in its ability to hydrolyze clavulanic acid compared to KPC-2. In contrast, the R220M substitution enzyme demonstrates increased K_m values for β-lactamase inhibitors (>100 μM for clavulanic acid versus 25 ± 3 μM for the wild type [WT]), which results in inhibitor resistance. Unlike other class A β-lactamases (i.e., SHV-1 and TEM-1), the amino acid present at residue 276 plays a structural rather than kinetic role with substrates or inhibitors. To rationalize these findings, we constructed molecular models of clavulanic acid docked into the active sites of KPC-2 and the "relatively" clavulanic acid-susceptible R220K variant. These models suggest that a major 3.5-Å shift occurs of residue E276 in the R220K variant toward the active S70 site. We anticipate that this shift alters the shape of the active site and the positions of two key water molecules. Modeling also suggests that residue 276 may assist with the positioning of the substrate and inhibitor in the active site. These biochemical and molecular modeling insights bring us one step closer to understanding important structure-activity relationships that define the catalytic and inhibitor-resistant profile of KPC-2 and can assist the design of novel compounds.

Resistance to antibiotics by bacteria is a major public health care problem throughout the world (2, 24). Bacteria have acquired multiple distinct resistance mechanisms to avoid the lethal effects of antibiotics. One major resistance mechanism possessed by Gram-negative pathogens is the expression of enzymes that hydrolyze β-lactams. These β-lactamases are of two major types: serine β-lactamases (classes A, B, and D) and metal-dependent β-lactamases (class B). Each serine β-lactamase possesses a distinct β-lactam or substrate profile, which can be altered due to single amino acid substitutions (6, 19, 20). To circumvent production of class A β-lactamases, mechanism-based inhibitors (i.e., clavulanic acid, sulbactam, and tazobactam) are given in combination with a β-lactam to treat patients infected with pathogens that produce these enzymes (6).

In recent years, the *Klebsiella pneumoniae* carbapenemase (KPC), the serine class A β-lactamase with the broadest profile with respect to substrates (including penicillins, cephalosporins, and carbapenems) and inhibitors, emerged and disseminated throughout the world (7, 16, 21, 23, 25). Given KPC-2's broad substrate spectrum, treatment strategies for patients infected with KPC-2-producing bacteria are limited, as the "last-resort" carbapenems are ineffective (4, 16, 18, 25). The biochemical characterization of the KPC β-lactamase is an essential endeavor to aid in the discovery of novel β-lactams and β-lactamase inhibitors.

In general, KPC-2 β-lactamase shares 39% and 41% sequence identity with TEM-1 and SHV-1, respectively. Unlike the TEM and SHV family β-lactamases, KPC-2 does not possess an Arg at position 244 (Ambler numbering system) (1). R244 in TEM and SHV β-lactamases has been shown to play a critical role in interacting with the C_3 carboxylate of carbapenems and β-lactamase inhibitors (9, 22, 26).

The goal of this work is to define the roles of 220 and 276 in the substrate profile of KPC-2. In previous investigations, detailed kinetic and molecular modeling analysis suggested that R220 forms important bridging hydrogen bonds between active site residues T237 and E276, amino acids suspected to be directly involved in substrate binding (13, 17). Comparative structural and biochemical analyses show that many class A serine β-lactamases

require a positively charged amino acid near the 220/276 region of the active site (10, 11, 15, 22, 26); an absolute conservation of a positively charged residue in this region of the active site is required (15). This positive charge is predicted to be necessary for the substrate specificity of each β-lactamase as well as the evolutionary potential of the β-lactamase to expand its hydrolytic profile based on single amino acid substitutions (15). As a result of comparative analyses of other important class A enzymes, we explored whether R220 is necessary for the binding and turnover of β-lactams and β-lactamase inhibitors by KPC-2. Based upon a knowledge of the crystal structure of KPC-2, we hypothesized that the high partition ratio (k_{cat}/k_{inact}) of KPC-2 with respect to clavulanic acid likely correlated with the absence of a key water molecule, which is anchored by R220 and E276, and is necessary for the inactivation of KPC-2 by clavulanic acid. By substituting other amino acids for R220 and E276, we found alterations in the kinetics of inactivation.

To further examine the roles of R220 and E276 in KPC-2, we also tested the impact of single amino acid substitutions at position 220 and 276 on the susceptibility of KPC-2-producing bacteria to β-lactams and β-lactamase inhibitors. We show here that R220 is "equivalent" to the R244 found in other class A β-lactamases (i.e., TEM-1 and SHV-1). Moreover, a positive charge at residue 220 is not required for the overall β-lactamase activity of KPC-2; many variants at 220 are "catalytically active" against tested substrates, except cefotaxime. Interestingly, residue 220 plays a critical role and impacts β-lactamase-inhibitor kinetics. In effect, we uncovered two "contrasting" β-lactamases, the R220K and R220M variants. The R220K variant is the first relatively "inhibitor-susceptible" variant of KPC-2 that maintains activity against β-lactams. On the other hand, the R220M variant demonstrates substantially increased K_m values for clavulanic acid, sulbactam, and tazobactam. Moreover, the positioning of 276 is also important to inhibitor kinetics. Finally, we propose that the location of water molecules in the active site of KPC-2 must be critical for inhibitor resistance and susceptibility. The synthesis of these findings is a "first step" toward intelligent β-lactamase-inhibitor design for this emerging threat to our β-lactam arsenal.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutagenesis. *K. pneumoniae* possessing bla_{KPC-2} and *Escherichia coli* with the pBR322-*catI*-bla_{KPC-2} plasmid and the cloning of bla_{KPC-2} from pBR322-*catI*-bla_{KPC-2} into pBC SK (+) phagemid (Stratagene, La Jolla, CA) and into pET24a(+) plasmid (Novagen, Darmstadt, Germany) were previously described (16, 17). The *K. pneumoniae* strain is the parent strain from which bla_{KPC-2} was identified and cloned (25). *E. coli* with pBR322-*catI*-bla_{KPC-2} is the original subclone of bla_{KPC-2} (25); *E. coli* with pBR322-*catI*-bla_{KPC-2} is predominantly used for carbapenem MICs, as it has been shown to express increased amounts of soluble KPC-2 β-lactamase compared to *E. coli* with pBC SK(+)bla_{KPC-2} (18). *E. coli* with pBC SK(+)bla_{KPC-2} was subcloned from pBR322-*catI*-bla_{KPC-2} (16). R220 and E276 variants were expressed from pBC SK(+)bla_{KPC-2} and/or pBR322-*catI*-bla_{KPC-2} carrying the R220 and E276 substitutions in *E. coli* DH10B cells (Invitrogen, Carlsbad, CA). A Quikchange XL site-directed mutagenesis kit (Agilent) was used to perform site saturation and site-directed mutagenesis using the manufacturer's protocol as previously described (17, 18).

Antibiotic susceptibility. Lysogeny broth (LB) agar dilution MICs, chosen according to the recommendations of the Clinical and Laboratory Standards Institute (5), were used to phenotypically characterize *K. pneumoniae* expressing bla_{KPC-2}, *E. coli* DH10B, and *E. coli* DH10B with bla_{KPC-2} and the bla_{KPC-2} R220 and E276 variants, as previously described (16). The chemical structures of the compounds tested are presented in Fig. 1.

β-Lactamase purification. The KPC-2 β-lactamase and the R220H, -K, -A, and -M and E276A and -D variants were purified from *E. coli* Origami 2 DE3 cells (Novagen) carrying wild-type (WT) or substituted pET24a(+)-bla_{KPC-2}, respectively. Cells were grown in super-optimal broth (SOB) to an optical density (OD) at 600 nm of 0.6, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction, and the cells were grown for an additional 3 h. All cells were pelleted and frozen for 18 h at −20°C. Pellets were resuspended in 50 mM Tris-chloride (Tris-Cl) at pH 7.4 with 1 mM magnesium sulfate. Pellets were lysed with lysozyme (Sigma) (40 mg/liter), and benzonase nuclease (Novagen) (1.0 U/ml) was added to digest nucleic acids, as some cells may burst during the extraction. EDTA (2.0 mM) was added to complete the periplasmic fractionation. The lysed cells were centrifuged at 12,000 rpm for 10 min to remove the cellular debris. The supernatant (e.g., crude extract) was further enriched for β-lactamase by the use of preparative isoelectric focusing (pIEF). A Sephadex G100 gel matrix and commercially prepared ampholines (Amersham Biosciences) (pH 3.5 to 10.0) were used in the pIEF gel. The pIEF gel was run overnight at 4°C at a constant power of 8 W on a Multiphor II isoelectric focusing apparatus (Amersham Pharmacia Biotech). After the pIEF gel was run overnight, areas of the gel demonstrating β-lactamase activity by nitrocefin (NCF) overlay were cut from the gel, eluted with 20 mM phosphate-buffered saline (PBS) (pH 7.4) on polyethylene glycol columns (Amersham Biosciences), and concentrated using an Amicon Ultra-4 concentrator (molecular weight [MW] cutoff of 10,000; Millipore). A HiTrap Q HP column (GE Healthcare Life Sciences) was used to perform fast protein liquid chromatography on an Äkta P-900 system (GE Healthcare Life Sciences). Proteins were eluted using a salt gradient (0 to 15%) and a mixture of 50 mM Tris-Cl at pH 8.8 and 50 mM Tris-Cl at pH 8.8 with 1.0 M sodium chloride. The purity of the fractions was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue R250. Protein concentrations were determined by measuring absorbance at $\lambda = 280$ nm and using the protein's extinction coefficient ($\Delta\varepsilon$; 39,545 M^{-1} cm^{-1} at 280 nm); the values were obtained using the ProtParam tool at http://us.expasy.org/tools.

Immunoblotting. *E. coli* strains expressing bla_{KPC-2} and all variants were grown in LB exponentially to an OD at 600 nm of between 0.7 and 0.8. Cells were pelleted and lysed to prepare crude extracts as described above. Positions 220 and 276 do not lie within the 3 major epitopes of the KPC-2 antibody, and immunoblotting was completed as previously described (17). Membranes were probed with a polyclonal anti-KPC-2 antibody (Sigma) and a loading control polyclonal anti-DNAK antibody (Stressgene). For detection, protein G-horseradish peroxidase (protein G-HRP) conjugate (Bio-Rad) and anti-mouse secondary-HRP conjugate antibody were used. Blots were developed using an ECL-Plus developing kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. A Fotodyne Luminary/FX workstation was used to capture images.

Kinetics. Steady-state kinetic parameters were determined using an Agilent 8453 diode array spectrophotometer (Santa Clara, CA) as previously described (16, 18). Briefly, each assay was completed using 10 mM PBS at pH 7.4 at room temperature (25°C) with the ratio of the concentration of the enzyme (E) to that of the substrate (S) chosen to establish pseudo-first-order kinetics. To measure hydrolysis rates, we used the following extinction coefficients ($\Delta\varepsilon$): nitrocefin (NCF) $\Delta\varepsilon$, 17,400 M^{-1} cm^{-1} at 482 nm; ampicillin $\Delta\varepsilon$, −900 M^{-1} cm^{-1} at 235 nm; piperacillin $\Delta\varepsilon$, −820 M^{-1} cm^{-1} at 235 nm; cefotaxime $\Delta\varepsilon$, −7,250 M^{-1} cm^{-1} at 262 nm; imipenem $\Delta\varepsilon$, −9,000 M^{-1} cm^{-1} at 299 nm, and clavulanic acid $\Delta\varepsilon$, −1,630 M^{-1} cm^{-1} at 235 nm. Using Enzfitter (Biosoft Corporation, Ferguson, MO), a nonlinear least-square fit of the data (Henri Michaelis-

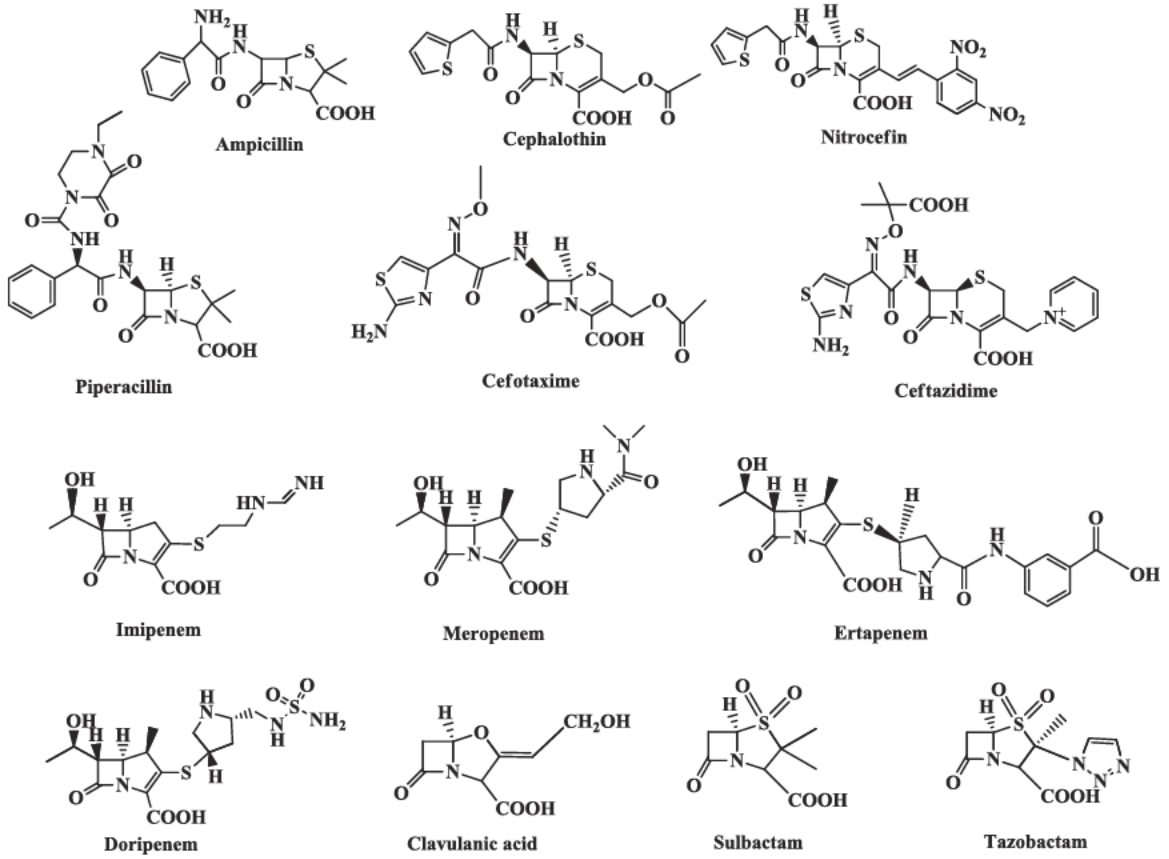

FIG 1 Compounds used in this study.

Menten equation) determined the kinetic parameters V_{max} and K_m as follows:

$$v = (V_{max} \times [S])/(K_m + [S]) \quad (1)$$

Based on our previous analyses, Fig. 2 is a representation of the KPC-2 β-lactamase (E; see below) reaction pathway with the β-lactamase inhibitor (I) clavulanic acid to form products (P_1 and P_2, the latter of which represents multiple potential products) (16–18) (the sulfones may follow a different scheme and have different microscopic rate constants). For the purposes of these experiments, formation of the noncovalent Michaelis complex, E:I, is represented by the dissociation constant, K_i, which is equivalent to k_{-1}/k_1. k_2 is the first-order rate constant for the acylation step, or formation of E-I. k_3 is the rate constant for the hydrolysis of the E-I acyl-enzyme.

Our experience to date leads us to maintain that the reaction of KPC-2 and variants with clavulanic acid is more complex than a three-step process (16–18). The suspected intermediates also likely include an

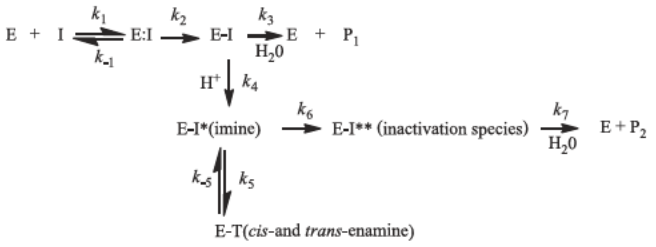

FIG 2 A representation of the KPC-2 β-lactamase (E) reaction pathway with a β-lactamase inhibitor (I) (i.e., clavulanic acid).

imine and an enamine species. In Fig. 2, we show that the rearrangement of E-I to the imine form of E-I* is governed by the rate constant k_4 (which, in the case of clavulanic acid, requires protonation of an oxonium ion intermediate [9]). The rate constants for the tautomerization of E-I* to E-T or the *cis*- and *trans*-enamine are k_5 and k_{-5}; this may be a reversible process. The E-I* imine can also undergo molecular rearrangements to form E-I** inactivation products, as indicated by the rate constant k_6. The hydrolysis of the E-I** is represented by rate constant k_7. We note that additional rate constants apply for formation of *cis*- versus *trans*-enamine E-T as well as formation of the different inactivation species and products.

In the set of experiments performed here, we determined an approximate K_m of the inhibitors for the enzyme by the use of a direct competition assay under steady-state conditions (16, 18). Nitrocefin (NCF) was used as the reporter substrate at a final concentration of 100 μM. Data were linearized by plotting inverse initial steady-state velocities ($1/v_0$) against inhibitor concentration (I). The velocity (v_0) obtained after mixing corresponds to Eq. 2. K_m (observed) was determined by dividing the value for the *y* intercept by the slope of the line. The data were corrected to account for the affinity of NCF for the β-lactamase as previously defined (16, 18).

$$v_0 = (V_{max} \times [S])/\{K_{m(ncf)} \times (1 + I/K_m) + [S]\} \quad (2)$$

The partition ratios (k_{cat}/k_{inact}, where k_{cat} is k_3 and k_{inact} corresponds to k_4) for the inhibitors were determined with KPC-2 and variant β-lactamases by the titration method, as described by others (8, 9, 14). Various ratios of increasing concentrations of inhibitor (from 10 μM to 10 mM) to enzyme (350 nM) (I:E) were mixed and incubated for 15 min at room temperature in 10 mM PBS (pH 7.4). The enzyme activity remaining was assayed by monitoring the initial rate of hy-

TABLE 1 MICs of β-lactams[a]

Strain	MIC (mg/liter)								
	AMP	PIP	THIN	TAZ	TAX	IMI[b]	ERTA[b]	MERO[b]	DORI[b]
K. pneumoniae	8,192	1,024	1,024	32	16	8	8	4	4
E. coli DH10B pBR322-*catI*-bla_{KPC-2}	4,096	1,024	512	32	8	4	2	2	2
E. coli DH10B pBC SK(+) bla_{KPC-2}	256	128	256	2	2	0.5	0.25	0.25	0.25
R220A	512	32	32	0.5	0.125	1	0.06	0.125	0.06
R220G	512	16	32	0.5	0.06	0.5	0.06	0.06	0.125
R220I	32	2	4	0.5	0.06	0.25	0.06	0.06	0.06
R220L	512	32	64	0.5	0.06	1	0.06	0.06	0.125
R220M	1,024	32	32	0.5	0.06	2	0.125	0.125	0.125
R220V	256	16	32	0.5	0.25	1	0.06	0.06	0.06
R220F	512	32	16	0.25	0.125	2	0.06	0.06	0.125
R220W	256	4	8	0.25	0.125	0.5	0.06	0.06	0.125
R220Y	512	32	16	0.5	0.06	1	0.06	0.06	0.125
R220C	128	8	16	0.25	0.125	0.5	0.06	0.06	0.06
R220N	64	4	8	0.25	0.06	0.5	0.06	0.06	0.06
R220P	64	4	4	0.25	0.06	N/A[c]	N/A	N/A	N/A
R220Q	512	32	64	0.5	0.125	2	0.125	0.125	0.25
R220S	512	32	64	0.5	0.125	2	0.125	0.06	0.125
R220T	256	16	32	0.5	0.125	1	0.06	0.06	0.125
R220D	64	4	16	0.25	0.06	0.5	0.06	0.06	0.06
R220E	512	32	64	0.5	0.125	2	0.125	0.06	0.125
R220H	1,024	64	128	1.0	0.125	2	0.25	0.125	0.25
R220K	256	16	64	0.5	0.25	2	0.5	0.5	0.5
E276A[d]	512	64	128	N/D[e]	2	0.25	0.25	0.25	0.125
E276D[d]	2,048	256	512	N/D	4	0.5	0.25	0.5	0.5
E276N[d]	512	128	128	N/D	2	1	0.25	0.125	0.25
E276R[d]	32	4	16	N/D	0.25	0.25	0.06	0.06	0.06
E. coli DH10B	1	2	4	0.25	0.06	0.25	0.06	0.06	0.06
E. coli DH10B pBC SK (+)	1	2	4	0.25	0.06	0.25	0.06	0.06	0.06

[a] The *K. pneumoniae* strain is the primary strain from which bla_{KPC-2} was first identified and cloned (25). *E. coli* with pBR322-*catI*-bla_{KPC-2} is the original subclone of bla_{KPC-2} and has been shown to express increased amounts of KPC-2 relative to *E. coli* with pBC SK(+)bla_{KPC-2}. β-Lactam abbreviations: ampicillin, AMP; piperacillin, PIP; cephalothin, THIN; cefotazidime, TAZ; cefotaxime, TAX; imipenem, IMI; ertapenem, ERTA; meropenem, MERO; doripenem, DORI. The data shown are from four independent determinations.
[b] *E. coli* with pBR322-*catI*-bla_{KPC-2} is predominantly used for determination of carbapenem MICs (as indicated by shading) (18, 25).
[c] N/A, variant R220P was not obtained during the initial screen for mutants.
[d] All E276 variants were expressed from pBC SK (+) bla_{KPC-2} in *E. coli* DH10B.
[e] N/D, values were not determined.

drolysis of 100 μM NCF. The I:E ratio necessary to inhibit the initial rate of hydrolysis of NCF by greater than 90% compared to the control (enzyme without inhibitor) was determined.

To measure overall activity of KPC-2 and the R220K and -M variants upon incubation with clavulanic acid, progress curves were taken at a fixed concentration of enzyme (10 nM), 50 μM NCF, and increasing concentrations of clavulanic acid (400 to 3,200 μM).

ESI-MS. Electrospray ionization mass spectrometry (ESI-MS) of the intact KPC-2 and R220K variant β-lactamases was performed using an Applied Biosystems (Foster City, CA) QStar Elite quadrupole-time-of-flight mass spectrometer equipped with a TurboIon spray source. β-Lactamases were incubated with clavulanic acid for 1, 2.5, 5, 10, and 20 min at room temperature in 10 mM PBS (pH 7.4). The ratio of inhibitor to β-lactamase (I:E) was 100:1 for all reactions. Then, reactions were terminated by addition of 0.2% formic acid. All samples were desalted and concentrated using a C_{18} ZipTip (Millipore, Billerica, MA) pipette tip according to the manufacturer's protocol. Eluted protein samples were diluted with 50% acetonitrile and 0.2% formic acid to a concentration of 10 mM and infused at a rate of 0.5 μl per min, and data were collected for 2 min. Spectra were deconvoluted using the Applied Biosystems Analyst program.

Molecular modeling. The crystal coordinates of KPC-2 (Protein Data Bank [PDB] accession code 2OV5 [13]) were used to construct representations of the R220A, R220M, R220K, E276D, and E276A variants of KPC-2, as well as to represent the Michaelis complexes of clavulanic acid, with the KPC-2 β-lactamase and the R220K variant by the use of Discovery Studio 2.5 (DS 2.5; Accelrys, Inc., San Diego, CA) molecular modeling software as previously described (18). The R220A, R220M, R220K, E276D, and E276A variants were designed by substitution of the residues at positions 220 and 276, respectively. Clavulanic acid was constructed using Fragment Builder tools and was minimized using a Standard Dynamics Cascade protocol of DS 2.5. The unhydrolyzed clavulanic acid was automatically docked into the active site of KPC-2 β-lactamase by the use of the Flexible Docking module of DS 2.5. The protocol allowed placement of a "flexible" β-lactamase inhibitor in the "flexible" active site of KPC-2. After docking, the most favorable pose of clavulanic acid was chosen (demonstrating a short distance [2 to 3 Å] between Ser70:O and C_7 of clavulanic acid as well as anticipated active site contacts) and the complex was created. The Michaelis complex of the R220K variant with clavulanic acid was created in a similar fashion. To check the stability and look for possible conformational changes of the complex, Molecular Dynamics Simulation (MDS) was conducted on apo-KPC-2, KPC-2–clavulanic acid, and the R220K:clavulanic acid complexes, as previously described (18).

RESULTS

Susceptibility testing of R220 and E276 variants. To assess the impact of amino acid substitutions at positions 220 and 276 on the

TABLE 2 MICs of β-lactam–β-lactamase-inhibitor combinations[a]

Strain or variant	MIC(s) (mg/liter)		
	AMP/ clavulanic acid	AMP/ sulbactam	PIP/ tazobactam
K. pneumoniae	32	512	512/64
E. coli DH10B pBC SK(+)bla_{KPC-2}[b]	4	128	64/8
R220A	16	32	32/4
R220G	8	32	16/2
R220I	≤1	≤1	4/0.5
R220L	8	32	32/4
R220M	8	64	64/8
R220V	2	16	16/2
R220F	16	32	32/4
R220W	8	16	16/2
R220Y	16	32	32/4
R220C	8	16	16/2
R220N	2	8	8/1
R220P	2	8	4/0.5
R220Q	16	32	32/4
R220S	16	32	32/4
R220T	8	32	32/4
R220D	≤1	4	8/1
R220E	8	32	32/4
R220H	8	32	32/4
R220K	≤1	8	8/1
E276A	8	32	64/8
E276D	32	256	256/32
E276N	8	64	128/16
E276R	≤1	≤1	4/0.5
DH10B	≤1	≤1	4/0.5
DH10B pBC SK(+)	≤1	≤1	4/0.5

[a] Ampicillin (AMP) was maintained at a constant concentration of 50 mg/liter, and clavulanic acid and sulbactam concentrations ranged from 1 mg/liter to 32 mg/liter and 1 mg/liter to 512 mg/liter, respectively. Piperacillin (PIP)/tazobactam ratios ranged 8:1 from 1/0.125 mg/liter to 512/64 mg/liter. The data shown are from a single experiment representative of four independent experiments.
[b] All variants were expressed from pBC SK (+) bla_{KPC-2} in *E. coli* DH10B; see Table 1 footnote for additional information on strains.

TABLE 3 KPC-2 R220 variant substrate kinetics

β-Lactam	KPC-2 variant	K_m (μM) (± SD)	k_{cat} (s^{-1}) (± SD)	k_{cat}/K_m (μM^{-1} s^{-1}) (± SD)
Piperacillin	WT	12 ± 1	65 ± 7	5.4 ± 0.5
	R220H	13 ± 1	68 ± 7	5.2 ± 0.5
	R220A	164 ± 16	193 ± 20	1.2 ± 0.1
	R220M	35 ± 4	85 ± 9	2.4 ± 0.2
	R220K	23 ± 2	40 ± 4	1.7 ± 0.2
	E276A	12 ± 1	59 ± 6	4.8 ± 0.5
	E276D	5.2 ± 0.5	38 ± 4	7.2 ± 0.7
Cephalothin	WT	38 ± 4	223 ± 20	5.9 ± 0.6
	R220H	108 ± 11	206 ± 20	1.9 ± 0.2
	R220A	207 ± 21	379 ± 38	1.8 ± 0.2
	R220M	98 ± 10	156 ± 16	1.6 ± 0.2
	R220K	45 ± 5	88 ± 9	2.0 ± 0.2
	E276A	24 ± 2	157 ± 16	6.5 ± 0.6
	E276D	12 ± 1	139 ± 14	11 ± 1
Nitrocefin	WT	8 ± 1	130 ± 13	16 ± 2
	R220H	16 ± 2	260 ± 26	16 ± 2
	R220A	54 ± 5	327 ± 33	6.1 ± 0.6
	R220M	94 ± 9	724 ± 72	7.7 ± 0.8
	R220K	10 ± 1	120 ± 12	12 ± 1
	E276A	4.9 ± 0.4	97 ± 10	19 ± 2
	E276D	4.1 ± 0.4	77 ± 8	19 ± 2
Cefotaxime	WT	190 ± 19	140 ± 14	0.7 ± 0.1
	R220H	N/D[a]	N/D	N/D
	R220A	N/D	N/D	N/D
	R220M	N/D	N/D	N/D
	R220K	N/D	N/D	N/D
	E276A	278 ± 28	118 ± 12	0.4 ± 0.1
	E276D	253 ± 25	100 ± 10	0.5 ± 0.1
Imipenem	WT	21 ± 2	21 ± 2	1.0 ± 0.1
	R220H	11 ± 1	9 ± 1	0.8 ± 0.1
	R220A	18 ± 2	4 ± 1	0.2 ± 0.1
	R220M	28 ± 3	10 ± 1	0.4 ± 0.1
	R220K	5 ± 1	7 ± 1	1.4 ± 0.1
	E276A	12 ± 1	11 ± 1	0.9 ± 0.1
	E276D	10 ± 1	13 ± 1	1.3 ± 0.1

[a] N/D, values could not be determined; variants did not hydrolyze cefotaxime at detectable levels. In addition, attempts to obtain a K_i for cefotaxime were not successful even when using excess (up to 10 mM) cefotaxime, revealing that binding of cefotaxime was also compromised in these variants.

KPC-2 β-lactamase in bacteria, we tested the susceptibility profile of *E. coli* containing variants of KPC-2. Our analysis shows that variants at R220 demonstrated decreased MICs for all tested β-lactams, with the exception of ampicillin, in comparison to the WT enzyme expressed in *E. coli* DH10B (Table 1). Only the R220H variant expressed in *E. coli* DH10B exhibited MICs nearest to WT levels.

Increased ampicillin-clavulanic acid MICs were detected for 12 of the 19 variant strains (i.e., R220A, -G, -L, -M, -F, -W, -Y, -Q, -S, -T, -E, and -H; Table 2). In stark contrast, none of the 19 variant strains demonstrated increased sulbactam or tazobactam MICs. The R220M variant displayed MICs closest to the KPC-2 levels for all the tested β-lactam–β-lactamase-inhibitor combinations. Surprising, the R220K variant exhibited significantly decreased MICs for the β-lactam–β-lactamase-inhibitor combinations in comparison to KPC-2. Other variants (R220D, -I, -N, and -P) also demonstrated decreased MICs for β-lactam–β-lactamase-inhibitor combinations, but unlike the R220K variant, they were also more susceptible to the partner β-lactam. Replacing E276 with uncharged amino acids, such as alanine or asparagine, β-lactam and β-lactam–β-lactamase-inhibitor resistance was maintained (Tables 1 and 2).

Immunoblotting of the R220 and E276 variants reveals only slight differences in protein expression among the variants. Immunoblotting using a polyclonal anti-KPC-2 antibody revealed minimal changes in expression among the variants (see Fig. S1 in the supplemental material). We note that the R220I, -K, and -P mutants did not express as well as KPC-2.

Kinetic analysis of selected variants of KPC-2 indicates that a positive charge at position 220 is not an absolute requirement for hydrolysis of β-lactams, with the exception of cefotaxime. KPC-2 and the R220K, -H, -M, and -A and E276A and -D variants were purified to ≥95% homogeneity. Steady-state kinetics analyses were performed using piperacillin, cephalothin, NCF, cefotaxime, and imipenem (Table 3).

TABLE 4 KPC-2 R220 variant inhibitor kinetics[a]

Parameter	KPC-2 variant	Value (±SD) for: Clavulanic acid	Sulbactam	Tazobactam
K_m (μM)	WT	25 ± 3	184 ± 18	66 ± 7
	R220M	485 ± 49	3,600 ± 400	257 ± 26
	R220K	14 ± 1	146 ± 15	36 ± 4
	E276A	15 ± 1	219 ± 22	82 ± 8
	E276D	11 ± 1	319 ± 32	192 ± 20
k_{cat} (s^{-1})	WT	18 ± 2	N/A[b]	N/A
k_{cat}/k_{inact} (15 min)	WT	9,000	3,000	500
	R220M	30,000	>100,000	250
	R220K	250	250	25
	E276A	4,500	1,000	250
	E276D	9,000	2,000	500

[a] NCF at 100 μM was used as the substrate.
[b] N/A, values were not determined.

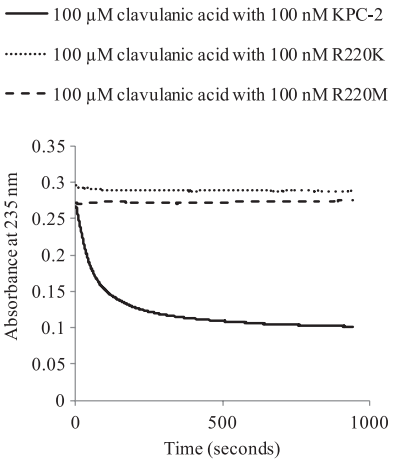

FIG 4 Monitoring hydrolysis using clavulanic acid with KPC-2 and the R220K and -M variants. Clavulanic acid was incubated with KPC-2 or the R220M or -K variant, and hydrolysis at 235 nm was monitored over time.

For the variants tested against the penicillins, cephalosporins, and carbapenems studied, there was an overall decrease in catalytic efficiency (k_{cat}/K_m reduced by 50% or greater). For piperacillin, nitrocefin, and imipenem, the R220H variant behaved similarly to the WT enzyme. The R220K variant displayed decreased k_{cat}/K_m values (i.e., ≤3.2-fold) for most substrates. Interestingly, the K_m value for imipenem for the R220K variant decreased from 21 ± 2 μM for KPC-2 to 5 ± 1 μM for the R220K variant, but overall, the k_{cat}/K_m ratio remained the same.

Measuring the kinetics for the R220M variant against various substrates revealed some decreases (i.e., ≤3.7-fold) in overall catalytic efficiency, mostly the result of increased K_m values for substrates (Table 3). The R220A variant showed significantly increased K_m values for piperacillin, cephalothin, and NCF from 5.4-fold to upwards of 13.7-fold. In contrast, slight increases in the k_{cat} value of ≤3-fold were also detected. Cefotaxime hydrolysis was not measurable with any R220 variant and thus appeared to be the process most affected by loss of arginine at position 220. The kinetic analysis of the E276A and -D variants revealed kinetic parameters similar to those of KPC-2 (Tables 3 and 4).

R220K cannot hydrolyze clavulanic acid, while R220M demonstrates increased K_m values for all β-lactamase inhibitors. Inhibition/inactivation by clavulanic acid is a complex multistep process involving the formation of many intermediates (Fig. 2) (3, 6). To further dissect the differences between KPC-2 and the R220K and -M variants, we performed detailed kinetics.

The K_m values for the purified R220K β-lactamase were lower than those seen with KPC-2; however, the partition ratios (k_{cat}/k_{inact}) after 15 min for all inhibitors were significantly decreased (i.e., up to a 36-fold decrease for clavulanic acid) (Table 4).

Assessing the activity of the R220K variant, we found that with 400 μM clavulanic acid, the R220K variant was mostly inactivated (Fig. 3C), while at 3.2 mM clavulanic acid, KPC-2 still retained β-lactamase activity (Fig. 3A). Measuring hydrolysis of clavulanic acid revealed that KPC-2 had a k_{cat} value of 18 ± 2 s^{-1} for clavulanic acid, while the R220K variant's levels of clavulanic acid hydrolysis were undetectable (Table 4 and Fig. 4).

The R220M variant displayed a nearly 20-fold increase in K_m values for clavulanic acid and sulbactam and partition ratios of 30,000 and >100,000, respectively (Table 4). The K_m value of the R220M variant for tazobactam also increased from 66 μM to 257 μM, with a minor reduction (i.e., 2-fold) in the partition ratio. The overall activity of the R220M variant measured with time in the presence of increasing concentrations of clavulanic acid revealed that it was more susceptible to inactivation than KPC-2 (Fig. 3A and B). Like those of the R220K variant, the R220M vari-

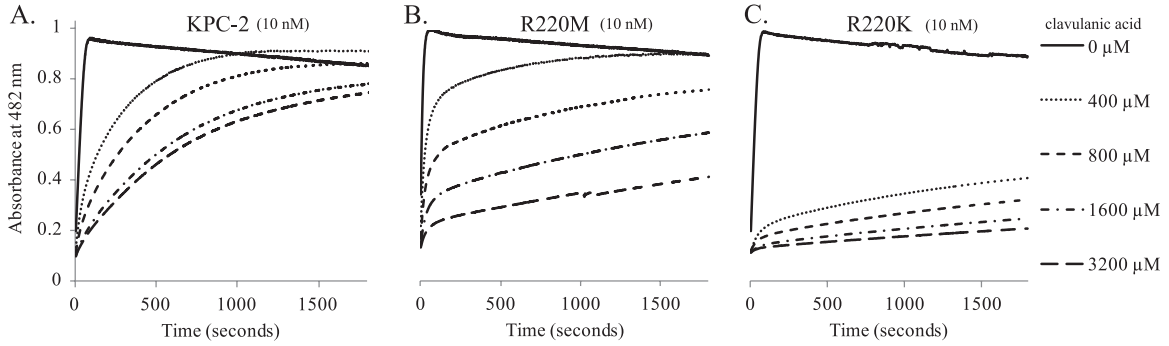

FIG 3 Activity curves of the kinetic interactions of clavulanic acid with KPC-2 and the R220M and -K variants. The reaction of 10 nM KPC-2 (A) or the R220M (B) or -K (C) variant with increasing concentrations of clavulanic acid over time was monitored at an absorbance of 482 nm using 50 μM nitrocefin as a reporter substrate for β-lactamase activity. Compared to KPC-2 and the R220M variant, the R220K variant is readily inactivated by clavulanic acid.

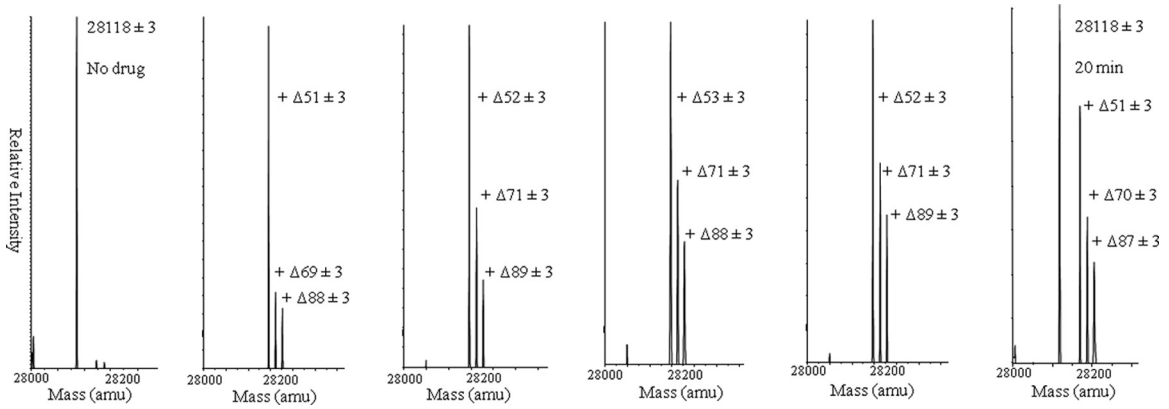

FIG 5 Timed mass spectrometry of the R220K variant with clavulanic acid. In a timed mass spectrometry experiment, clavulanic acid was reacted with the R220K variant. Samples were collected at 1 min, 2.5 min, 5 min, 10 min, and 20 min. An enzyme-alone control was also run (i.e., the R220K variant; 28,118 ± 3 atomic mass units [amu]). Mass adducts of +51 ± 3 amu (propynyl enzyme or enol ether), +69 ± 3 amu (aldehyde), and +88 ± 3 amu (hydrated aldehyde) were observed with the R220K variant.

ant's levels of hydrolysis of clavulanic acid were also undetectable (Table 4 and Fig. 4).

Mass spectrometry reveals adducts with the R220K variant but not KPC-2. To further discern the nature of the intermediates of inactivation by clavulanic acid in the reaction pathway of KPC-2 and the R220K variant, we performed electrospray ionization mass spectrometry (ESI-MS). Timed mass spectrometry data showed that R220K reacts with clavulanic acid to form various inactivation products (+52, +70, and +88) (Fig. 5) (3, 6). These inactivation products formed within 1 min and increased in intensity during a 10-min time course; free β-lactamase reappeared by 20 min. Adducts were not observed with KPC-2 even at high ratios of inhibitor to enzyme (data not shown).

Modeling of KPC-2 and the R220K variant in apo-enzyme and complexed forms. To gain insight into the structural changes that might have been occurring, we performed molecular modeling and MDS. Modeling of the KPC-2 and R220A, -M, and -K apo-enzymes predicted a significant 3.5-Å shift of the E276 side chain in toward the active site in the R220K variant (Fig. 6A). All other movements of active-site residues (S70, K73, W105, N132, E166, N170, T216, K234, T235, T237, and H274) were less than 1.7 Å. The overall topologies of the active sites of the two proteins were different; the active site of the R220K variant appeared larger and more electropositive (Fig. 6B and C).

Unhydrolyzed clavulanic acid was docked into the active sites of KPC-2 and the R220K variant, and MDS was run. KPC-2 required 100 ps to reach equilibration with the Michaelis complex (data not shown). The KPC-2 apo-enzyme took only 10 ps to reach equilibration (data not shown). The R220K variant reached equilibration rapidly, i.e., within 5 ps, with the Michaelis complex (data not shown).

Our simulations indicated that the inhibition of β-lactamases by clavulanic acid was largely dependent on the position of two key water molecules. One water molecule (Wat1) is critical for deacylation, while the other water molecule (Wat2) is necessary for protonation of the clavulanic acid oxonium intermediate, thus driving inhibition/inactivation. In the KPC-2–clavulanic acid complex, Wat1 was clearly anchored via hydrogen bonds to E166, a residue shown to be essential for deacylation (Fig. 6B). Wat2 formed interactions with E276 but not with clavulanic acid. In the R220K–clavulanic acid model, the movement of E276 resulted in the displacement of Wat1 away from E166 in most conformations (Fig. 6C). In addition, Wat2 made hydrogen bonds with E276 and clavulanic acid and thus was available to accelerate secondary ring opening and promote inhibition (Fig. 6C).

Consistent with our MIC and kinetic data, molecular modeling studies demonstrated that major structural changes in the apo-enzymes of E276A and -D were not present (data not shown).

DISCUSSION

KPC-2 represents one of the most clinically important serine carbapenemases found in Gram-negative bacteria (7, 16, 21, 23, 25). Our analysis of the amino acid sequence requirements for catalysis reveals that the positive charge at position 220 modulates the catalytic activity of KPC-2 β-lactamase against most substrates; substitution at position 220 by other amino acids results in active enzymes that possess a wide range of catalytic efficiencies. This observation is in sharp contrast to the role of R220 in *Streptomyces albus* G β-lactamase, where the substitution of R220 by leucine completely abrogates activity of this enzyme (11). Similar observations were noted with R244 variants of TEM-1 and SHV-1 (10, 11, 15, 22, 26). Thus, R220 is the "R244" equivalent in KPC-2.

For susceptibility testing with β-lactam–β-lactamase-inhibitor combinations, 12 of 19 variants displayed increased MICs for ampicillin-clavulanic acid. However, the variants do not have increased MICs for ampicillin-sulbactam or piperacillin-tazobactam, revealing a critical difference between the inhibition mechanisms of oxapenams versus sulfones. We previously reported the "clavulanic acid-resistant sulfone-susceptible" phenotype with T237 and W105 variants of KPC-2 (17, 18). These results led us to hypothesize that this phenotype was due to differences in the inactivation chemistry of the sulfones versus clavulanic acid with KPC-2 (18). Now, we also predict that a critically positioned water molecule, Wat2, may additionally be further displaced in the KPC-2 variants that result in this phenotype (Fig. 6). Without water present, we postulate that the enolate becomes a poor leaving group and that clavulanic acid is rapidly deacylated in these variants (higher partition ratios) (Fig. 6 and 7). The sulfones do not require Wat2 for secondary ring opening and inactivation and thus are unaffected by movement of Wat2 in variant β-lactamases.

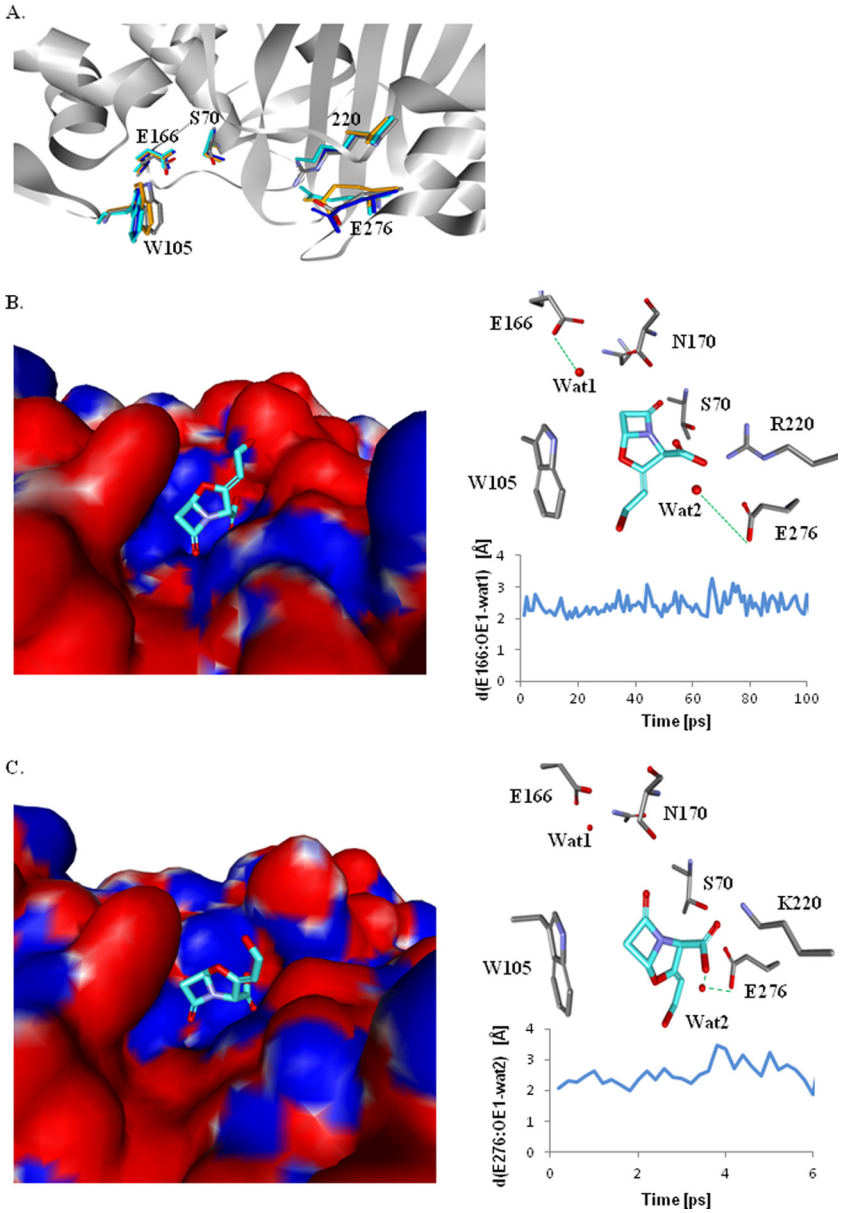

FIG 6 Molecular modeling of KPC-2 and the R220A, -M, and -K variants. (A) The apo-enzyme of the KPC-2 (gray) was superimposed on the apo-enzymes of the R220A (orange), -M (dark blue), and -K (cyan) variants, revealing a 3.5-Å shift in E276 for the R220K variant. (B) Electrostatic surface of the active site of KPC-2–clavulanic acid (left). The molecular representation of KPC-2 (gray) with clavulanic acid (blue) reveals that Wat1 (red) forms hydrogen bonds (green dashed line) with E166 (top right). The distance (d) in angstroms between E166 and Wat1 throughout the 100-ps simulation (bottom right) is indicated. (C) Electrostatic surface of the active site of the R220K variant-clavulanic acid (left). The molecular representation of the interaction of the R220K variant (gray) with clavulanic acid (blue) indicates that Wat2 (red) is hydrogen bonded (green dashed line) to clavulanic acid and E276 (top right). The distance (d) in angstroms between C7 of clavulanic acid and Wat2 throughout the 5-ps simulation (bottom right) is indicated.

Two "different" β-lactamases, R220M and R220K, were further studied. The R220M variant demonstrates MICs similar to or higher than WT levels and displays increased K_m values for the commercially available β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). The mechanism of resistance suggests that there is decreased binding affinity ($K_i = k_{-1}/k_1$); when high concentrations of inhibitor (>320,000:1 I:E ratio) were added, inactivation was measured. This mechanism of resistance is different from that of WT KPC-2, as the R220M variant has the ability to confer inhibitor resistance based on hydrolysis as opposed to loss of binding.

The MICs for the R220K variant were lower than those seen with the WT for all tested β-lactam–β-lactamase combinations. Interestingly, by substituting the side chain of arginine for lysine, binding of clavulanate, sulbactam, and tazobactam is improved, and k_{cat}/k_{inact} becomes much lower. Unlike the results seen with the WT, hydrolysis of clavulanic acid by the R220K variant cannot be detected and the variant is readily inactivated by clavulanic acid over time. Taken together, the combination of these factors produces a relatively "inhibitor-susceptible" variant of KPC-2.

Molecular modeling studies suggested that the shift of E276 toward the active site drives inhibitor susceptibility in this en-

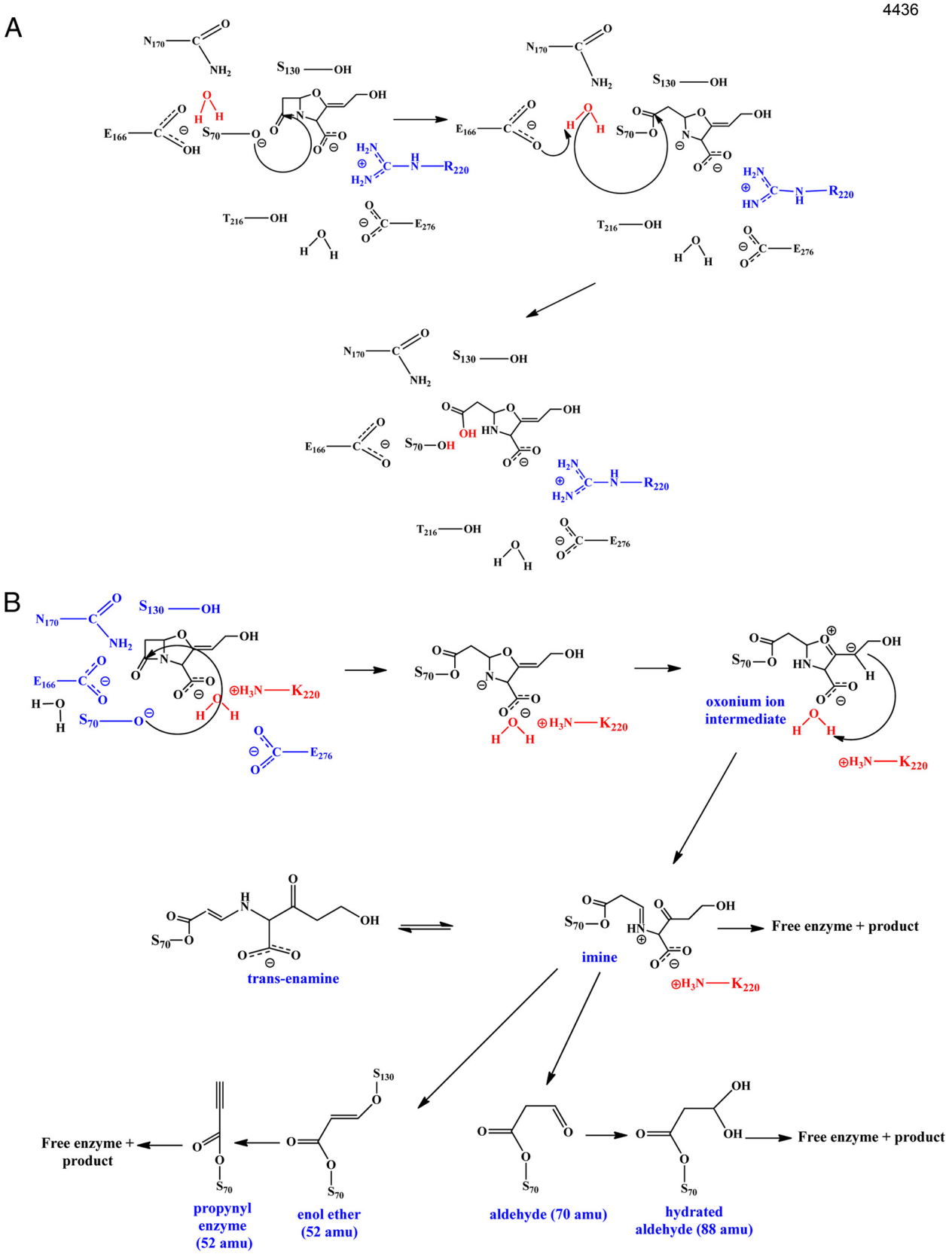

FIG 7 Mechanisms of clavulanic acid interactions with KPC-2 and the R220K variant. (A) Mechanism of clavulanic acid resistance by WT KPC-2. The major pathway is illustrated; only minor amounts of enamine may form and thus are not depicted in this scheme. The S70 nucleophile attacks the β-lactam ring. The deacylation water is positioned close to E166, promoting deacylation of the acyl-enzyme complex. (B) Mechanism of clavulanic acid inhibition of the R220K variant. The S70 nucleophile attacks the β-lactam ring. The protonation water molecule is closely anchored by E276 and clavulanic acid, thus promoting secondary ring opening and protonation of the oxonium ion intermediate to form the imine. The imine is mostly converted to the inactivation products: an enoyl ether which decomposes to the propynyl enzyme and an aldehyde which gets hydrated to generate a hydrated aldehyde. A minor fraction may tautomerize to the enamine form; thus, it is not represented.

zyme through repositioning of two key water molecules (i.e., the deacylation water and the protonation water) in the active site.

In agreement with the contrasting substrate profiles of WT KPC-2 and the R220K variant, during MDS, KPC-2 required 100 ps to reach equilibration with the Michaelis complex. However, the R220K variant reached equilibration rapidly (within 5 ps) with the Michaelis complex, suggesting that it quickly forms a stable complex with clavulanic acid, thus further supporting the inhibition of R220K by clavulanic acid.

Our analysis of WT KPC-2 in comparison to the R220K variant with respect to clavulanic acid is indicative of two very different reaction pathways with clavulanic acid (Fig. 7). For WT KPC-2, the rate constant for deacylation, k_3, is much larger than k_4, the rate constant for ring opening and tautomerization, and we predict that only a very small portion is converted to either enamine intermediate, as these species as well as other inactivation products are not trapped by mass spectrometry. Thus, after the S70 nucleophile attacks the β-lactam ring forming the acyl-enzyme, the majority of the acyl-enzyme complex is hydrolyzed (Fig. 7A). Our modeling studies suggest that Wat1 is positioned close to E166, promoting deacylation of the acyl-enzyme complex rather than protonation of the oxonium ion intermediate from Wat2.

In the case of the R220K variant, k_3, the rate constant for deacylation is still larger than k_4 but is much lower than k_3 for WT KPC-2, as evidenced by a partition ratio for clavulanic acid that is lower by 36-fold and the lack of hydrolysis of clavulanic acid. In addition, k_6, the rate constant for the formation of the inactivation products, is larger than k_5, the rate constant for formation of the enamine species; we predict that little of the enamine intermediate is formed. The rate of the reaction of the R220K variant with clavulanic acid is slower than that of the WT, as mass spectrometry revealed that inactivation products formed in a 20-min timed study.

How does one explain this in a mechanism? The S70 nucleophile attacks the β-lactam ring forming the acyl-enzyme, but the position of E276 stimulates inhibition in the R220K variant due to the placement of Wat1 and Wat2 (Fig. 7B). The Wat2 molecule is closely anchored by E276, thus promoting secondary ring opening and protonation of the oxonium ion intermediate to form the imine. The imine is mostly converted to the inactivation products: enoyl ether which deacylates to the propynyl enzyme and an aldehyde which gets hydrated to generate a hydrated aldehyde. A minor fraction tautomerizes to the *trans*-enamine form. Eventually, inactivation products decompose, since mass spectrometry showed that, by 20 min, the β-lactamase began to deacylate and the presence of free enzyme was observed.

In summary, we report here for the first time the identification of an "inhibitor-susceptible" variant of KPC-2 that maintains catalytic activity against β-lactams. The predicted shift of one residue in the active site of KPC-2 makes this notoriously inhibitor-resistant β-lactamase susceptible to clavulanic acid. Thus, R220 appears to function as a "switch" between inhibitor resistance and susceptibility in the KPC-2 β-lactamase. These insights allow us to consider the future design of novel inhibitors of this carbapenemase. The challenge for the medicinal chemist is to alter the R_2 side chain of clavulanic acid so that Wat2 approximates E276 and R220 to promote inactivation, thus generating a potent inhibitor of the KPC-2 β-lactamase. We also hasten to add that the mechanistic considerations that apply to sulfone inhibitors may offer an important alternative to inhibit KPC-2 β-lactamases (12).

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