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Enhancing Resistance to Cephalosporins in Class C β-Lactamases: Impact of Gly214Glu in CMY-2[†]

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ABSTRACT: The biochemical properties of CMY-32, a class C enzyme possessing a single-amino acid substitution in the Ω loop (Gly214Glu), were compared to those of the parent enzyme, CMY-2, a widespread class C β-lactamase. In parallel with our microbiological characterization, the Gly214Glu substitution in CMY-32 reduced catalytic efficiency (k_{cat}/K_m) by 50−70% against "good" substrates (i.e., cephalothin) while increasing k_{cat}/K_m against "poor" substrates (i.e., cefotaxime). Additionally, CMY-32 was more susceptible to inactivation by sulfone β-lactamase inhibitors (i.e., sulbactam and tazobactam) than CMY-2. Timed electrospray ionization mass spectrometry (ESI-MS) analysis of the reaction of CMY-2 and CMY-32 with different substrates and inhibitors suggested that both β-lactamases formed similar intermediates during catalysis and inactivation. We next showed that the carbapenems (imipenem, meropenem, and doripenem) form long-lived acyl−enzyme intermediates and present evidence that there is β-lactamase-catalyzed elimination of the C_6 hydroxyethyl substituent. Furthermore, we discovered that the monobactam aztreonam and BAL29880, a new β-lactamase inhibitor of the monobactam class, inactivate CMY-2 and CMY-32 by forming an acyl−enzyme intermediate that undergoes elimination of SO_3^{2-}. Molecular modeling and dynamics simulations suggest that the Ω loop is more constrained in CMY-32 than CMY-2. Our model also proposes that Gln120 adopts a novel conformation in the active site while new interactions form between Glu214 and Tyr221, thus explaining the increased level of cefotaxime hydrolysis. When it is docked in the active site, we observe that BAL29880 exploits contacts with highly conserved residues Lys67 and Asn152 in CMY-2 and CMY-32. These findings highlight (i) the impact of single-amino acid substitutions on protein evolution in clinically important AmpC enzymes and (ii) the novel insights into the mechanisms by which carbapenems and monobactams interact with CMY-2 and CMY-32 β-lactamases.

The most common mechanism responsible for β-lactam resistance in Gram-negative bacteria is the production of β-lactamases (EC 3.5.2.6) (*1*). At present, there are more than 850 different β-lactamases described in nature (http://www.lahey.org/Studies/). As a family of proteins, β-lactamases are grouped into four main classes (i.e., A−D) based upon amino acid sequence homology (*2*). Classes A, C, and D employ serine in the active site as the reactive nucleophile, while class B β-lactamases use a single metal ion or a pair of metal ions (Zn^{2+}) to catalyze the opening of the β-lactam ring. Both serine and metallo-β-lactamases make use of a strategically positioned water molecule to hydrolyze the lactam bond (*3*−*5*).

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In general, class A, C, and D β-lactamases follow a three-step reaction mechanism that is represented as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} E{-}S \xrightarrow[H_2O]{k_3} E + P \qquad (1)$$

where E is the β-lactamase enzyme, S is the β-lactam substrate, E·S is the Henri−Michaelis complex, E−S is the acyl enzyme, and P is the inactivated β-lactam. The rate constants for each step are designated by k_1, k_{-1}, k_2, and k_3.

Usually, class A and C β-lactamases are found in the Enterobacteriaceae. Acquired class A enzymes, often carried on mobile plasmids, are common in *Escherichia coli* and *Klebsiella pneumoniae*, are constitutively expressed, and confer resistance to penicillins and narrow-spectrum cephalosporins (*1*). Class C or AmpC type β-lactamases (AmpCs) are encoded by chromosomal genes in many Gram-negative pathogens (e.g., *Citrobacter freundii*, *Enterobacter* spp., and *Pseudomonas aeruginosa*), are inducible, and confer resistance to narrow- and extended-spectrum cephalosporins. Most recently, an increasing number of AmpC β-lactamase genes are being discovered on plasmids that also spread by horizontal transfer (*6*−*8*). Unlike class A enzymes,

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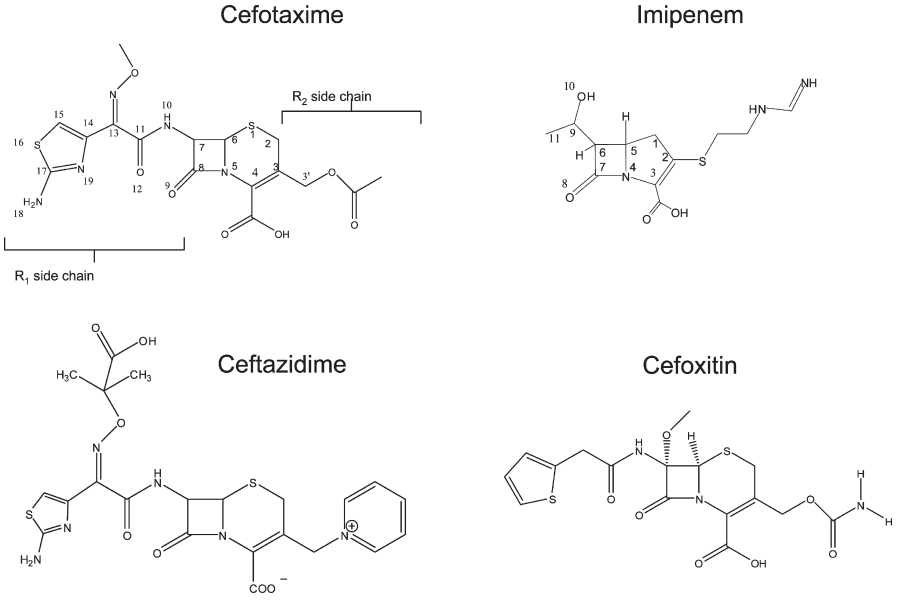

FIGURE 1: Chemical structures of several β-lactams.

AmpCs are poorly inhibited by the commercially available β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) (*7*).

Among class C enzymes, CMY-type[1] β-lactamases represent the most frequently detected plasmid-encoded AmpCs (pAmpCs) (*8*−*13*). Unfortunately, we know little regarding the ability of clinically important CMY-type enzymes to hydrolyze different classes of β-lactam antibiotics (*14*−*16*). Furthermore, the activity of novel β-lactamase inhibitors against the CMY-type pAmpCs has not yet been fully studied (*17*).

In class A β-lactamases, single-amino acid substitutions (primarily, but not exclusively, at Ambler positions Gly238 on the b3 β-strand and Arg164 or Asp179 in the Ω loop) remodel a "broad-spectrum" β-lactamase into an extended-spectrum β-lactamase (ESBL) (*18*−*20*). The bacterium possessing a class A ESBL becomes resistant to ceftazidime and/or cefotaxime. However, these changes come at a "price" (*18*, *21*). Among class A ESBLs, one usually detects a decrease in penicillin resistance (lower minimum inhibitory concentrations, MICs) and an increase in the susceptibility to β-lactamase inhibitors and carbapenems (*18*, *21*).

Herein, we analyze the biochemical properties of a novel CMY-2-like pAmpC (i.e., CMY-32) cloned from an *E. coli* isolate found in the clinic. In comparison with CMY-2 [wild type (WT)], we determined that CMY-32 possesses a single-amino acid substitution in the Ω loop (i.e., Gly214Glu) resulting in significant changes in the resistance phenotype and hydrolytic profile (making this β-lactamase an "extended-spectrum type"). We also evaluated the mechanisms by which carbapenems, a monobactam antibiotic aztreonam, and the novel monobactam derivative, BAL29880, react and inhibit CMY-2 and CMY-32 enzymes. Our data reveal the impact of single-amino acid substitutions on protein evolution in this emerging family of resistance enzymes and highlight the manner in which carbapenems and monobactams inactivate class C enzymes.

[1]Abbreviations: CMY, active on cephamycins; TEM, class A β-lactamase named after the patient (Temoneira) providing the first sample; SHV, sulfhydryl reagent variable β-lactamase; CTX-M, active on cefotaxime, first isolated in Munich; ESBL, extended-spectrum β-lactamase; ESI-MS, electrospray ionization mass spectrometry; MIC, minimum inhibitory concentration; ATCC, American Type Culture Collection; CTX, cefotaxime; ATM, aztreonam; rmsd, root-mean-square deviation; LB, Luria-Bertani; pIEF, preparative isoelectric focusing; DS, discovery studio; MDS, molecular dynamic simulations.

MATERIALS AND METHODS

Detection and Cloning of bla_{CMY} Genes and Expression in E. coli DH10B Cells. We used two clinical *E. coli* isolates to obtain the *bla* genes employed in this study. The first strain carried the $bla_{\text{CMY-2}}$ gene and was detected in 2002 in Cleveland, whereas the second strain possessed the $bla_{\text{CMY-32}}$ gene and was discovered in 2006 in Pittsburgh (*22*, *23*).

Specific primers were used to amplify both bla_{CMY} genes (For, 5′-CCGGACACCTTTTTGCTTTT-3′; Rev, 5′-TATCCTGGGCCTCATCGTCAGTTA-3′). As previously reported, sequence analysis demonstrates that CMY-32 (GenBank accession number EU496815) possesses a single-amino acid substitution (i.e., Gly214Glu) compared to CMY-2 (GenBank accession number EF406116) (*23*). Each bla_{CMY} gene was cloned into the pBC SK(−) phagemid vector (Stratagene, La Jolla, CA) as previously described (*24*). Commercially purchased electrocompetent *E. coli* DH10B cells (Invitrogen Corp., Carlsbad, CA) were electroporated with the ligation mixture. We selected strains expressing the bla_{CMY} genes on Luria-Bertani (LB) agar plates containing chloramphenicol (20 μg/mL) and ampicillin (100 μg/mL).

Phenotypic Characterization. MICs for the following β-lactams were obtained using the Etest method (AB Biodisk, Solna, Sweden) on cation-adjusted Mueller-Hinton agar (BBL, Becton Dickinson, Sparks, MD): ampicillin, ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin, piperacillin-tazobactam, cefoxitin, cefotaxime (CTX), ceftazidime, cefepime, aztreonam (ATM), imipenem, and meropenem (see Figure 1 for the chemical structures of several of these compounds).

The inhibitory activity of BAL29880 (Basilea Pharmaceutica International Ltd.) was evaluated by microdilution in cation-adjusted Mueller-Hinton broth, according to the Clinical Laboratory Standard Institute criteria (*25*). This inhibitor was also combined with ampicillin and piperacillin (Sigma Chemical Co., St. Louis, MO) at a constant concentration of 4 μg/mL. The following American Type Culture Collection (ATCC) strains were used as controls: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603.

β-Lactamase Expression. Polyclonal anti-CMY-2 antibodies were produced by Genosys Biotech, Inc. (Woodlands, TX), and purified using a HiTrap Protein G HP column (GE

Healthcare Bio-Sciences AB, Uppsala, Sweden) as previously reported (*26*). *E. coli* DH10B isolates containing bla_{CMY-2} and bla_{CMY-32} were grown in LB broth to an OD_{600} of 0.8. Preparation of samples, immunoblotting, and assessment of β-lactamase expression were performed using a method previously described (*26*).

β-Lactamase Purification. Each *E. coli* DH10B strain containing the bla_{CMY} gene expressed in the pBC SK(−) vector was grown overnight at 37 °C in 4 L of SOB broth supplemented with ampicillin (100 μg/mL) and chloramphenicol (20 μg/mL). Cells were then pelleted and resuspended in 50 mL of 50 mM Tris buffer (pH 7.4). β-Lactamases were released using lysozyme, EDTA (Sigma Chemical Co.), and DNase (Promega, Madison, WI) according to a previously established protocol (*27*).

We performed preparative isoelectric focusing (pIEF) using a Sephadex G100 gel matrix and commercially prepared ampholines (pH 3.5−10.0) (Amersham Biosciences, Piscataway, NJ). The conditions used for pIEF were described previously (*28*). After pIEF had been run overnight, areas of the gel demonstrating β-lactamase activity by nitrocefin (NCF) overlay were cut from the gel, eluted with PBS (pH 7.4) on polyethylene glycol columns (Amersham Biosciences), and concentrated using an Amicon Ultra-4 concentrator (MW cutoff of 10000; Millipore, Carrigtwohill, County Cork, Ireland).

We further purified CMY-2 and CMY-32 β-lactamases to homogeneity using size exclusion chromatography with a Pharmacia AKTA purifier system (GE Healthcare Bio-Sciences AB). We employed a HiLoad 16/60 Superdex 75 column (GE Healthcare Bio-Sciences AB) and eluted with PBS (pH 7.4). We assessed the purity of β-lactamases by running the samples in sodium dodecyl sulfate−polyacrylamide gels. Gels were stained using Coomassie blue R-250 (Fisher, Pittsburgh, PA), and the protein molecular weight was estimated using prestained low-molecular weight standards (Bio-Rad Laboratories). Protein concentrations were determined by spectrophotometric assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

Kinetics Experiments. On the basis of the reaction scheme outlined in eq 1, we measured steady-state kinetic parameters (i.e., K_m, k_{cat}, and k_{cat}/K_m) by continuous assays at room temperature. The Michaelis constants were determined using nonlinear least-squares fit of the data (Henri−Michaelis equation, eq 2) employing the program Enzfitter (Biosoft Corp., Ferguson, MO) (*29*, *30*):

$$v = V_{max}[S]/(K_m + [S]) \quad (2)$$

We used an Agilent 8453 diode array spectrophotometer with a 1 cm path length and determined each initial velocity in triplicate in PBS buffer (pH 7.4). Initial velocity measurements were obtained for the following "good substrates" (as defined by $k_{cat} \geq 2\ s^{-1}$) (*31*): NCF ($\Delta\varepsilon_{482} = 17400\ M^{-1}\ cm^{-1}$), cephaloridine ($\Delta\varepsilon_{260} = -10200\ M^{-1}\ cm^{-1}$), cephalothin ($\Delta\varepsilon_{262} = -7660\ M^{-1}\ cm^{-1}$), and cefoxitin ($\Delta\varepsilon_{260} = -7700\ M^{-1}\ cm^{-1}$). For "poor substrates" ($k_{cat} < 2\ s^{-1}$ or nonmeasurable, i.e., cefotaxime, $\Delta\varepsilon_{260} = -7500\ M^{-1}\ cm^{-1}$), the apparent K_m values were obtained as competitive inhibition constants (K_i) in the presence of NCF.

To characterize the β-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam, and BAL29880), we measured the first-order rate constants for enzyme and inhibitor complex inactivation (k_{inact}) by monitoring the reaction time courses in the presence of inhibitor and NCF under steady-state conditions. A fixed concentration of enzyme (i.e., $E = 1.6$ nM for CMY-2, or $E = 3.1$ nM for CMY-32), 100 μM NCF, and increasing concentrations of inhibitor (I) were used in each assay. The k_{obs} values were determined using a nonlinear least-squares fit of the data employing Origin 7.5 (OriginLab Co., Northampton, MA):

$$A = A_0 + v_f t + (v_0 - v_f)[1 - \exp(-k_{obs} t)]/k_{obs} \quad (3)$$

where A is the absorbance, v_0 (expressed in variation of absorbance per unit time) is the initial steady-state velocity, v_f is the final steady-state velocity, and t is the time (*32*). Each k_{obs} was plotted versus I and fit to determine k_{inact} according to eq 4

$$k_{obs} = (k_{inact}[I])/(K_i + [I]) \quad (4)$$

In a similar manner to our determination of K_i for poor substrates, we determined the K_i of each inhibitor from the initial steady-state velocities. We corrected the K_i values for the presence of NCF using eq 5.

$$K_i(\text{corrected}) = K_i(\text{observed})/(1 + [S]/K_{m,NCF}) \quad (5)$$

We used eq 6 to calculate the percentage of the standard error (SE):

$$\text{SE of } k_{cat}/K_m \text{ or } k_{inact}/K_i = [(\text{SE of } k_{cat} \text{ or } k_{inact}/k_{cat} \text{ or } k_{inact})^2 + (\text{SE of } K_m \text{ or } K_i/K_m \text{ or } K_i)^2]^{1/2} \quad (6)$$

Ultraviolet Difference (UVD) Spectroscopy. UVD absorption spectra were obtained for CMY-2 and CMY-32 (both at 8 nM) reacted with CTX (8 μM) in 10 mM PBS (pH 7.4). Wavelengths from 200 to 500 nm were analyzed using the Agilent 8453 spectrophotometer for 1 h at room temperature.

Electrospray Ionization Mass Spectrometry (ESI-MS). We performed timed ESI-MS of each CMY β-lactamase alone or in combination with β-lactam antibiotics or β-lactamase inhibitors on a Q-STAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nanospray source as described previously (*33*). Two incubation times (i.e., 1 and 15 min) were analyzed for each β-lactamase inhibitor tested, whereas for the carbapenems, only a 15 min incubation was performed. We desalted the reaction mixtures using C_{18} ZipTips (Millipore, Billerica, MA). The CMY-2 or CMY-32 β-lactamase sample was then diluted with 50% acetonitrile and 0.1% trifluoroacetic acid to a concentration of 10 μM. We next infused this protein solution at a rate of 0.5 μL/min, and spectral data were collected for 2 min. Spectra were deconvoluted using Analyst (Applied Biosystems). Notably, all data reported in Results should be considered with a ±3 amu standard error.

Molecular Modeling. To generate a model of the CMY-32 enzyme, we used the crystal structure of CMY-2 (Protein Data Bank entry 1ZC2) and Discovery Studio (DS) version 2.1 (Accelrys, San Diego, CA). We prepared the protein for molecular dynamics simulations (MDS) by adding hydrogen atoms and setting the pH at 7.4. The CMY-32 model was created using the Build Module of DS by inserting a substitution at position 214 (i.e., Gly to Glu).

First, the crystallographic waters were removed, and the two β-lactamase models were immersed in a water box (i.e., 7 Å from any face of the box) using explicit periodic boundary conditions (PBCs). Then, the complexes were minimized in several steps,

using Steepest Descendent and Conjugate Gradient algorithms to reach the minimum convergence (i.e., 0.02 kcal mol^{-1} $Å^{-1}$) after 10000 iterations. All energy minimizations and MDSs were performed using CHARMm force field parameters. We employed the particle mesh Ewald (PME) method to treat long-range electrostatic interactions. Bonds that involved hydrogen atoms were constrained with the SHAKE algorithm. Following equilibration, two separate (2 fs) MDS runs (heating/cooling and production), at constant pressure and temperature (300 K), were conducted for the CMY-2 and CMY-32 models. The trajectories collected after the production steps were analyzed, and the conformations with the minimum energies were chosen. The CMY-2 and CMY-32 models were superimposed.

The model of the preacylation complex of CMY-32 and CTX was developed using the Flexible Docking Module of DS version 2.1. The side chains of the active site amino acids (i.e., Ser64, Glu120, Asn152, Tyr221, Tyr150, Lys67, and Ser318) were allowed to move during docking. The low-energy CTX conformations were docked into the active site of the CMY-32 model using LibDock (*34*). In the presence of CTX, the active site side chain residues were refined using the ChiRotor algorithm (*35*). A final simulated annealing and energy minimization of each CTX conformation was performed using CDOCKER (*34*). The 10 resulting conformations of CTX were manually analyzed for hydrogen bonds, steric clashes, and distances between atoms. The energies were evaluated, and the most favorable conformation was chosen. The complex that was created was further energy minimized.

A model of the acyl−enzyme complex of the CMY-2 enzyme with BAL29880 was also constructed. The chemical structure of BAL29880, built using the Fragment Builder tools of DS version 2.1, was minimized using a Standard Dynamic Cascade protocol. The CDOCKER algorithm was employed to dock BAL29880 in the active site of CMY-2 β-lactamase (*34*). The BAL29880 placement in the active site was specified using a binding site sphere. Random ligand conformations were generated from the initial structure through high-temperature MDS. Grid-based simulated annealing followed by force field minimization refined the generated conformations. Starting with the most energetically favorable conformation, we created and energy minimized the acyl−enzyme complex.

RESULTS

Phenotype Characterization. To measure the impact of a single-amino acid substitution on the hydrolytic activity of the CMY-2 and CMY-32 β-lactamases in *E. coli*, we performed MICs in a uniform genetic background. As shown in Table 1, both CMY β-lactamases expressed in *E. coli* DH10B confer high-level resistance to ampicillin, amoxicillin-clavulanate, piperacillin, and ceftazidime (MICs of >256 μg/mL), whereas piperacillin-tazobactam, cefepime, and carbapenems demonstrated MICs in the susceptible ranges (≤6 μg/mL) (*36*). Noticeably, the strain expressing CMY-32 was more susceptible to ampicillin-sulbactam and cefoxitin than the strain harboring CMY-2. In contrast, the MIC for CTX was significantly higher for CMY-32 (64 μg/mL) than for CMY-2 when expressed in *E. coli* (16 μg/mL). We observed the same trend when testing ATM (MICs of 12 and 6 μg/mL, respectively). Although a slight increase in MIC was observed for both *E. coli* clones containing CMY-2 or CMY-32 when compared to *E. coli* DH10B, full resistance to cefepime was not established (Table 1).

Table 1: Antimicrobial Susceptibility Tests for the *E. coli* DH10B Clones Expressing the CMY-2 or CMY-32 β-Lactamase

	MIC (μg/mL)[a]		
antibiotic	*E. coli* DH10B pBC SK(−)	*E. coli* DH10B pBC SK(−)/ CMY-2	*E. coli* DH10B pBC SK(−)/ CMY-32
ampicillin	4	>256	>256
ampicillin-sulbactam	4	128	24
ampicillin-BAL29880	2	2	2
amoxicillin-clavulanate	4	>256	>256
piperacillin	2	>256	>256
piperacillin-tazobactam	2	6	4
piperacillin-BAL29880	1	4	2
cefoxitin	4	>256	32
cefotaxime (CTX)	<0.032	16	64
ceftazidime	<0.032	>256	>256
cefepime	<0.032	0.25	0.19
aztreonam (ATM)	<0.032	6	12
BAL29880	>32	>32	>32
imipenem	<0.032	0.5	0.38
meropenem	<0.032	0.047	<0.032

[a]MICs were obtained using the Etest method. MICs for BAL29880 alone or in combination with ampicillin or piperacillin were obtained by microdilution according to CLSI criteria (*25*).

To further characterize the phenotype of CMY-32 in *E. coli* DH10B, we also tested the activity of the novel monobactam inhibitor and compared it to that of ATM. BAL29880 alone did not exhibit activity against *E. coli* DH10B, including those expressing the bla_{CMY} genes (MICs of >32 μg/mL). However, in combination with ampicillin or piperacillin, BAL29880 lowered MICs considerably (i.e., ≤4 μg/mL) for strains expressing CMY-2 or CMY-32 (Table 1).

β-Lactamase Expression. To ensure that the differences in MICs between the *E. coli* DH10B producing bla_{CMY-2} and bla_{CMY-32} were not due to changes in expression levels of the β-lactamases, we performed immunoblots using a specific anti-CMY-2 polyclonal antibody. Steady-state expression levels of CMY-2 and CMY-32 in *E. coli* DH10B showed that the strain expressing CMY-2 produced similar quantities of β-lactamase as the strain harboring CMY-32 pAmpC (data not shown).

Kinetics and UVD Spectroscopy. Steady-state kinetic parameters revealed that CMY-32 possessed significantly less catalytic efficiency (k_{cat}/K_m) for hydrolyzing the narrow-spectrum cephalosporins (NCF, cephaloridine, and cephalothin) compared to CMY-2 (Table 2). These results were mainly due to reductions in k_{cat} rather than K_m. A decrease in k_{cat}/K_m was also seen with cefoxitin, a 7-α-methoxy cephalosporin (*14*, *37*) (Table 2).

The turnover of CTX, an oxyimino amino-thiazole cephalosporin that is regarded as a poor substrate, is often difficult to measure because of the low k_{cat} (*14*, *31*). Here, we followed the hydrolysis of CTX by CMY-2 and CMY-32 using UVD absorption spectra. In the Supporting Information (Figure 1), we show the increase in the hydrolysis of CTX by CMY-32 versus CMY-2 (see λ_{max} = 228 nm). Consistent with MICs, cefepime, an extended-spectrum cephalosporin that is also a poor substrate for class C enzymes, was poorly hydrolyzed by CMY-2 and CMY-32 [K_m > 100 μM, and k_{cat} not measurable (Table 2)] (*14*, *37*).

For ATM and imipenem, we did not observe hydrolysis by either CMY-2 or CMY-32 under saturating conditions (Table 2).

Table 2: Steady-State Kinetic Parameters of CMY-2 and CMY-32[a]

	K_m (μM) [or K_i(corrected)]		k_{cat} (s^{-1})		k_{cat}/K_m (μM^{-1} s^{-1})	
β-lactam	CMY-2	CMY-32	CMY-2	CMY-32	CMY-2	CMY-32
			Good Substrates			
nitrocefin (NCF)	11.2 ± 1.2	10.7 ± 0.7	534.8 ± 16.0	225.9 ± 3.2	47.6 ± 5.23	21.1 ± 1.4
cephaloridine	42.7 ± 7.2	53.8 ± 6.6	408.5 ± 24.1	139.6 ± 6.7	9.6 ± 1.7	2.6 ± 0.3
cephalothin	7.8 ± 0.6	9.8 ± 1.2	140.0 ± 22.4	74.5 ± 2.2	17.9 ± 3.2	7.6 ± 1.0
cefoxitin	17.9 ± 5.5	22.9 ± 4.3	6.8 ± 0.7	4.7 ± 0.3	0.38 ± 0.04	0.21 ± 0.04
			Poor Substrates			
cefotaxime (CTX)	1.8 ± 0.2	4.05 ± 0.26	NM[d]	0.90 ± 0.06	NM[d]	0.22 ± 0.02
cefepime [b,c]	108.1 ± 7.0	988.9 ± 0.3	NM[d]	NM[d]	NM[d]	NM[d]
aztreonam (ATM) [b,c]	0.12 ± 0.008	0.10 ± 0.003	NM[d]	NM[d]	NM[d]	NM[d]
imipenem [b,c]	0.57 ± 0.059	0.40 ± 0.036	NM[d]	NM[d]	NM[d]	NM[d]

[a]Standard errors are reported. [b]k_{cat} not measurable. [c]K_i measured. [d]Not measurable.

Table 3: Determination of K_i Values, First-Order Rate Constants (k_{inact}), and k_{inact}/K_i Values[a]

	K_i(corrected) (μM)		k_{inact} (s^{-1})		k_{inact}/K_i (μM^{-1} s^{-1})	
β-lactam	CMY-2	CMY-32	CMY-2	CMY-32	CMY-2	CMY-32
tazobactam	50.0 ± 9.5	13.9 ± 1.2	0.045 ± 0.0018	0.129 ± 0.0024	0.0009 ± 0.00005	0.009 ± 0.0002
sulbactam	101.3 ± 7.6	87.9 ± 3.1	0.025 ± 0.0007	0.038 ± 0.001	0.0002 ± 0.00001	0.0004 ± 0.00002
clavulanate	4365 ± 471	500 ± 24.5	NM[b]	0.031 ± 0.0007	NM[b]	0.00006 ± 0.000004
BAL29880	4.1 ± 0.40	5.5 ± 0.60	0.04 ± 0.001	0.06 ± 0.005	0.0098 ± 0.0025	0.011 ± 0.0023

[a]Standard errors are reported. [b]NM = not measurable.

To understand why this occurred, we employed ESI-MS (see below) and observed that ATM and imipenem formed long-lived covalent complexes with both β-lactamases. In competition experiments with NCF, we observed that the K_i for CMY-2 and CMY-32 was greater than that for any substrate tested ($K_i \leq 600$ nM).

The commercially available β-lactamase inhibitors (tazobactam, sulbactam, and clavulanate) were not very effective at inhibiting the two CMY enzymes (Table 3). Consistent with our MIC determinations showing an increase in susceptibility to ampicillin-sulbactam and piperacillin-tazobactam, these inhibitors possessed lower K_i values (by at least 40%) for CMY-32 β-lactamase than for CMY-2 (Table 3). The K_is of BAL29880, the novel monobactam, for CMY-2 and CMY-32 was lower than those of the three commercially available inhibitors. Furthermore, our MIC data indicated that BAL29880 requires a β-lactam partner for full efficacy (MIC ≤ 4 μg/mL).

Additionally, we observed a notable finding regarding the kinetics of inactivation of CMY-2 and CMY-32 with tazobactam. Compared to the other inhibitors, tazobactam demonstrates a k_{inact} value at least 3-fold greater than those of sulbactam, clavulanate, and BAL29880. This more rapid inactivation constant coupled with a lower K_i is also consistent with the MIC results.

ESI-MS. We employed ESI-MS to gain insight into the nature of the intermediates formed in the inactivation of CMY-2 and CMY-32 β-lactamases. To begin, we established that the molecular weight (MW) of CMY-2 is 39854 amu, whereas that of CMY-32 is 39926 amu, consistent with the predicted molecular weights of the proteins (Figure 2).

Since carbapenems are known to be poorly hydrolyzed by both class A and C β-lactamases, we first sought to determine if imipenem, meropenem, and doripenem would form a stable acyl−enzyme adduct with CMY-2 and CMY-32 β-lactamases (*38*). At 15 min, the inactivation of CMY-2 and CMY-32 with imipenem in a ratio of 1:1000 produced adducts of 299, corresponding to the molecular weight of imipenem (Figure 2). However, when the E:I ratio was reduced to 1:20, an additional minor adduct with a mass defect of 44 amu was seen (data not shown). We observed the same phenomenon with meropenem and doripenem (Figure 2).

We next inactivated CMY-2 and CMY-32 with tazobactam, sulbactam, ATM, and BAL29880. In the course of our analysis, it became evident that the ESI-MS determinations obtained after 1 and 15 min incubations were equivalent. Therefore, in Figure 3, we present only one of the timed reactions.

As shown in Figure 3, inactivation of both β-lactamases with the sulfones yielded products with MWs of +72 amu and +87 amu. The fragmentation of sulbactam to yield adducts similar to tazobactam supports a common mechanism of inactivation by sulfone inhibitors. As stated above, we also demonstrated that both ATM and BAL29880 form acyl−enzyme species by ESI-MS. As with the carbapenems, we detected two species (Figure 3), one with a MW of 80 amu lower than the other. This suggested to us that there is fragmentation of ATM and BAL29880 as is seen with the carbapenems.

DISCUSSION

In this work, we studied the biochemical characteristics of CMY-2 and its variant, CMY-32. Our analysis points to the distinctive properties that define the "extended-spectrum" phenotype of CMY-32. To emphasize the singular features of this "class C ESBL", we draw comparisons to the well-studied class A ESBLs. Our work highlights the consequence of "natural protein

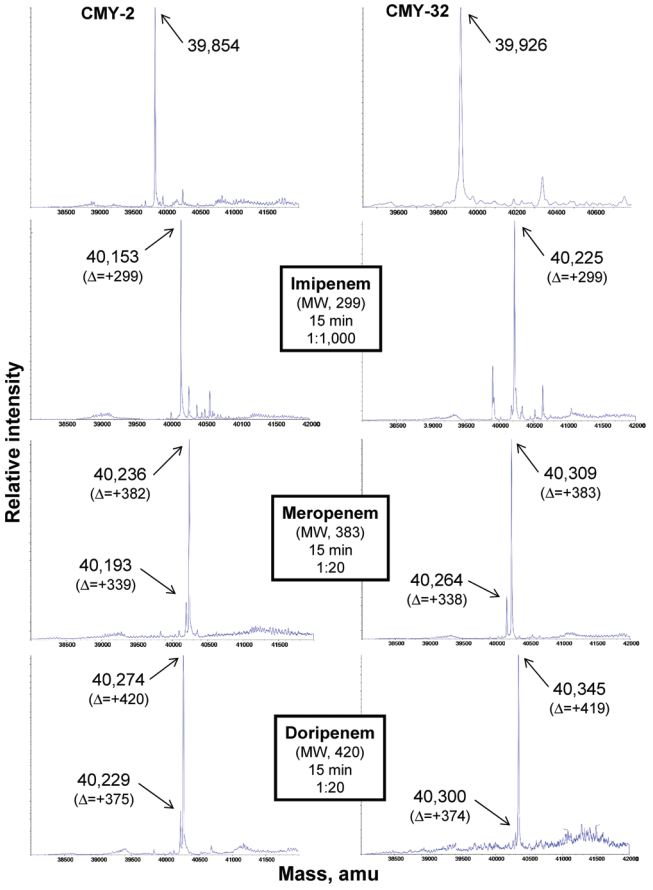

FIGURE 2: Mass spectrometry of the two purified CMY pAmpC β-lactamases alone and in combination with different carbapenems: (left) CMY-2 β-lactamase and (right) CMY-32 β-lactamase. Molecular weights (MW), times of incubation, and ratios of CMY protein to carbapenem are indicated within the squares. All results should be considered with a ± 3 standard error.

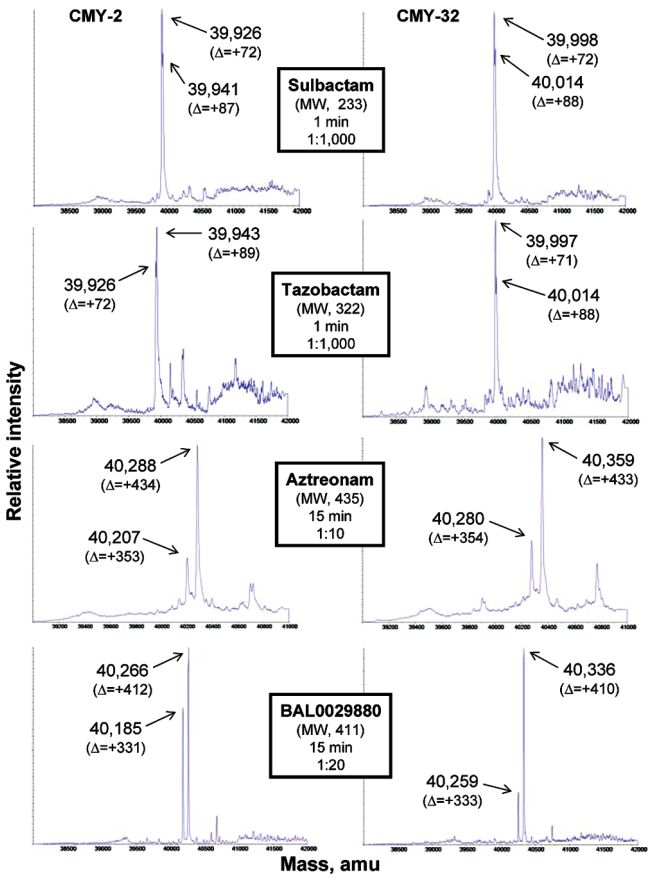

FIGURE 3: Mass spectrometry of the two purified CMY pAmpC β-lactamases in combination with different β-lactamase inhibitors and ATM: (left) CMY-2 β-lactamase and (right) CMY-32 β-lactamase. Molecular weights (MW), times of incubation, and ratios of CMY protein to inhibitor are indicated within the squares. All results should be considered with a ± 3 standard error.

engineering" (i.e., previous antibiotic selection pressure) on substrate specificity (i.e., emergence of the CTX-resistant phenotype). Next, we describe the reaction intermediates observed when these carbapenems, sulfones, and monobactams react with CMY-2 and CMY-32 β-lactamases.

Impact of the Gly214Glu Substitution on Phenotype. As a result of the Gly214Glu substitution, the phenotype we observed is very reminiscent of class A ESBLs in *E. coli* and illustrates that in class C β-lactamases, a rather specific substitution located in the Ω loop alters susceptibility and resistance to many β-lactams. Most strikingly, resistance to CTX and ATM is increased for *E. coli* bearing CMY-32 compared to CMY-2. In contrast, the class C CMY-32 β-lactamase expressed in *E. coli* DH10B resulted in a lower level of resistance to cefoxitin, as compared to CMY-2. Moreover, there is increased susceptibility to β-lactam$-\beta$-lactamase inhibitor combinations and carbapenems for CMY-32. These properties are the "clinical hallmarks" that alert microbiology laboratories to the presence of ESBLs in *E. coli*, *K. pneumoniae*, and *Proteus* spp. Interestingly, there are many class A ESBLs of the TEM and SHV families that possess substitutions in the Ω loop that behave similarly (*18*, *21*).

Impact of the Gly214Glu Substitution on the Turnover of Good and Poor Substrates. The steady-state kinetic parameters of both CMY-2 and CMY-32 pAmpCs were consistent with the antimicrobial susceptibility profiles. First, we saw an overall reduction in k_{cat}/K_m for first-generation cephalosporins (i.e., cephaloridine and cephalothin), substrates that are generally "preferred" by class C enzymes (*7*). Likewise, cefoxitin, a 7-α-methoxy cephalosporin, which is usually hydrolyzed by AmpCs, shows a 45% reduction in k_{cat}/K_m when hydrolyzed by CMY-32. Most strikingly, CMY-32 exhibits the ability to hydrolyze CTX [$k_{cat}/K_m = 0.22\ \mu M^{-1}\ s^{-1}$ for CMY-32 compared to not measurable for CMY-2 (see Table 2 and the Supporting Information, Figure 1)].

Consistent with the kinetic behavior of other class C enzymes, CMY-2 and CMY-32 hydrolyze imipenem poorly (k_{cat} not measurable). Following the reaction with timed ESI-MS, we see that imipenem accumulates in the unfragmented acyl$-$enzyme form for both CMY pAmpCs (Figure 2). We observed this phenomenon only when the ratio of imipenem to CMYs was 1000:1. Reducing the ratio to 20:1, we noted identical patterns with all three carbapenems. After the covalent adduct is formed, there is fragmentation to an acyl$-$enzyme species that is 44 amu smaller (Figure 2). Similar results were observed with meropenem and doripenem. As previously observed for a chromosomal class A β-lactamase expressed by *Mycobacterium tuberculosis* and the ADC-7 β-lactamase (a class C enzyme) of *Acinetobacter baumannii*, these results suggest that, after β-lactam ring opening, the covalently bound carbapenem inactivation pathway splits between (i) direct hydrolysis with formation of an unfragmented acyl$-$enzyme intermediate and (ii) elimination of the hydroxyethyl substituent (*39*, *40*). Investigations to define the impact of this finding and identify the residues that may facilitate this process are underway.

Impact of the Gly214Glu Substitution on β-Lactamase Inhibitors. For CMY-32, the Gly214Glu substitution lowers the K_i for all clinically used inhibitors (the most dramatic change was for tazobactam and sulbactam) and significantly increases the k_{inact}, a property that is also very common among class A ESBLs (*1*). Timed ESI-MS showed that both sulfone inhibitors follow a pattern of inhibition that generates the same postulated intermediates for both AmpCs (i.e., Δ = +88 amu for the hydrated aldehyde, and Δ = +72 amu for the aldehyde). Originally, this was described for class A β-lactamases and CMY-2 (*17*, *41*). As sulbactam is regarded as a very poor inhibitor of class C enzymes (K_i > 100 μM), we were surprised to see adducts similar to those formed by tazobactam, a more effective inhibitor. Therefore, we maintain that even these class C serine β-lactamases pass through the imine and enamine transitional stages and that CMY-32 may form certain intermediates more readily than CMY-2. We stress that these findings also shed significant insight into why tazobactam serves as a good inhibitor of CMY β-lactamases, albeit unrecognized as such. Our data show that tazobactam possesses a first-order rate constant of inactivation (k_{inact}) for CMY-32 that is 3-fold greater than those of commercially available inhibitors. Together with the lower K_i, the more rapid inactivation adds to the potency of this β-lactamases inhibitor combinations.

Aztreonam, a Monobactam Substrate, and BAL29880, a Monobactam Inactivator. The most intriguing observations were made with ATM and BAL29880. We generally regard ATM as a poor substrate, and BAL29880 is an inhibitor. As a result of the Gly214Glu substitution, CMY-32 expressed in *E. coli* has increased MICs versus aztreonam. In contrast, BAL29880 does not appear to possess antibacterial activity (MICs of > 32 μg/mL for *E. coli* with CMY-2 and CMY-32 β-lactamases). However, when combined with ampicillin or piperacillin, BAL29880 lowers MICs at least 6-twofold (from ≥256 to ≤4 μg/mL), a range similar to that of ATM alone against CMY-2 and CMY-32 (Table 1).

To understand why two monobactams behave so differently, we performed direct competition experiments using NCF as an indicator substrate and studied the reactions using timed ESI-MS. Here, ATM showed 30−55-fold lower K_i for CMY-2 and CMY-32 when compared to BAL29880 (Tables 2 and 3). This compelled us to explore possible mechanisms by which ATM and BAL29880 interact with CMY-2 and CMY-32. Timed ESI-MS analysis demonstrated that BAL29880 and ATM follow similar pathways of inhibition against both CMY-2 and CMY-32 pAmpCs (Figure 3). Both monobactams acylate the CMY β-lactamases (i.e., Δ = +411 amu for BAL29880, or Δ = +435 amu for ATM) and form long-lived acyl−enzyme intermediates. These findings were reminiscent of those of Oefner et al., who demonstrated that ATM, a poor substrate, acylates the active site Ser64 of the *C. freundii* AmpC β-lactamase (Protein Data Bank entry 1FR1), an enzyme that is 97% homologous in amino acid content to CMY-2 and CMY-32 (*42*). Therefore, we anticipated the presence of the full adduct added to CMY-2 and CMY-32 when ATM and BAL29880 were reacted with these class C cephalosporinases (*42*, *43*). Unexpectedly, we discovered that a second adduct was also formed. The MW of the second adduct is approximately 80 amu lower than the first, suggesting that both monobactams undergo fragmentation after acylation of the CMY β-lactamase (Figure 3). Mechanistically, the addition of water to the acyl−enzyme species would allow hydrolysis of the sulfonamide to yield the free sulfate (Figure 4a,b).

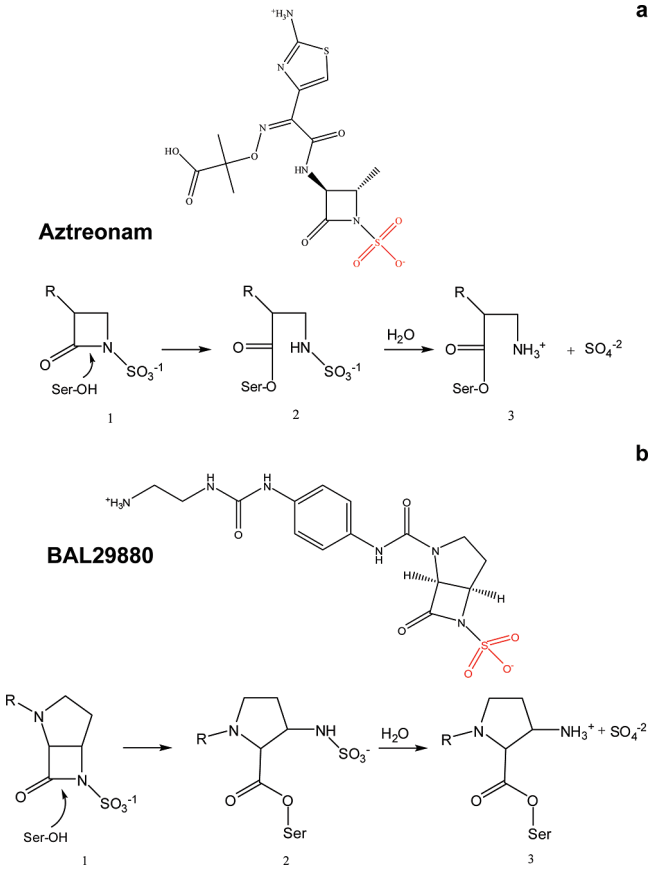

FIGURE 4: (a) Chemical structure of ATM and its mechanism of inactivation by CMY β-lactamases responsible for the second adduct of 353 amu seen with ESI-MS (See Figure 3). (b) Chemical structure of BAL29880 and its mechanism of inactivation by CMY β-lactamases responsible for the second adduct of 333 amu seen with ESI-MS (See Figure 3). In the case of ATM and BAL29880, the *N*-sulfonate monobactam binds in the oxyanion hole and the Ser64 hydroxyl attacks the activated carbonyl (C═O), forming the acyl−enzyme intermediate and a sulfonamide (NH-SO_3^-). The sulfonamide is subsequently hydrolyzed (by H_2O in the active site) to generate the amine (NH_3^+) and sulfate (SO_4^{2-}).

Molecular Models. To explain the basis of the affinity and specificity of the Gly214Glu substitution in the Ω loop of CMY-2, we constructed three molecular models.

In the first model, we superimposed the energy-minimized structures of CMY-32 and CMY-2 (rmsd = 1.0 Å) using backbone atoms and compared the conformations of the active sites, paying particular attention to the Ω loop region (Val211−Val223) (Figure 5a,b). The analysis of the trajectories generated after MDS showed an average potential movement of 1−3 Å in the region of the Gly214Glu substitution. Our model shows that there is also significant change in the position of the Tyr221 side chain in the CMY-32 β-lactamase (Figure 5b). As a result of the Gly to Glu substitution, Tyr221 likely moves within hydrogen bonding distance of Glu214 [1.9 Å (Figure 5b)]. Our interpretation is that this newly formed hydrogen bond limits the overall flexibility and mobility of the Ω loop. Additionally, we observed that because of the Glu214−Tyr221 hydrogen bond, Gln120 adopts a new conformation in which the side chain is flipped more than 3.5 Å (see Figure 6).

In our second model, we present an explanation of why CTX is hydrolyzed by CMY-32 β-lactamase. Using the energy-minimized structure of CMY-32, we docked the carbonyl of the four-member ring of CTX in the oxyanion hole (Figure 6). Because of

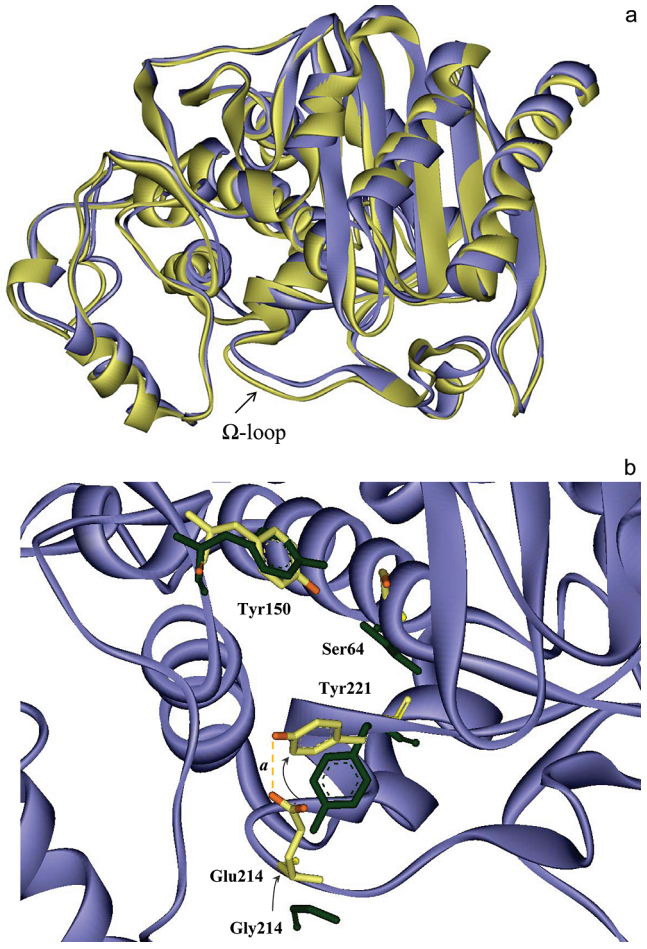

FIGURE 5: (a) Molecular modeling of CMY-32 (purple) superimposed onto CMY-2 (yellow) (rmsd = 1 Å) showing the conformational changes introduced by the substitution at position 214. The most significant differences between the conformations of the two enzymes were observed in the Ω loop region (Val211−Val223) with a displacement of up to 3 Å between the backbone of the amino acids at position 214. (b) Change in the position of the Tyr221 side chain (more than 90° rotation of the aromatic ring) and the Gly214Glu substitution (~3 Å change in the position of the backbone) that allow the formation of the hydrogen bond between Tyr221:H and Glu214:OE2 (a = 1.93 Å). The new conformation in the CMY-32 enzyme limits the mobility of the Ω loop. CMY-2 residues are colored green, and CMY-32 is colored yellow, with the oxygens colored red. The hydrogen atoms are not shown.

the repositioning of Gln120 in CMY-32, we see a new hydrogen bond contact between Glu120:O and the terminal amide of the R1 side chain of CTX. In this model, the C4 carboxylate is within hydrogen bonding distance of Arg349 and the carbonyl oxygen of the R2 side chain makes favorable interactions with Asn346 (Figures 1 and 6).

Lastly, we modeled the acyl−enzyme complex of BAL29880 without the SO_3^{2-} group (Figure 7). In this energy-minimized model, we docked the carbonyl of BAL29880 into the oxyanion hole of CMY-2 (see Figure 4b). In this conformation, the oxygen is advantageously positioned to form hydrogen bonds with the protonated Lys67. Rotating the acyl−amide bond, we observe a stabilizing hydrogen bond interaction with Asn152 (Figure 7) (*44*). Thus, the inhibitor BAL29880, like ATM, adopts an arrangement in the acyl−enzyme intermediate that primarily exploits contacts with conserved residues (Lys67 and Asn152) of the active site. Despite these similarities, ATM and BAL29880 behave very differently (one a "poor substrate" and the other an inhibitor).

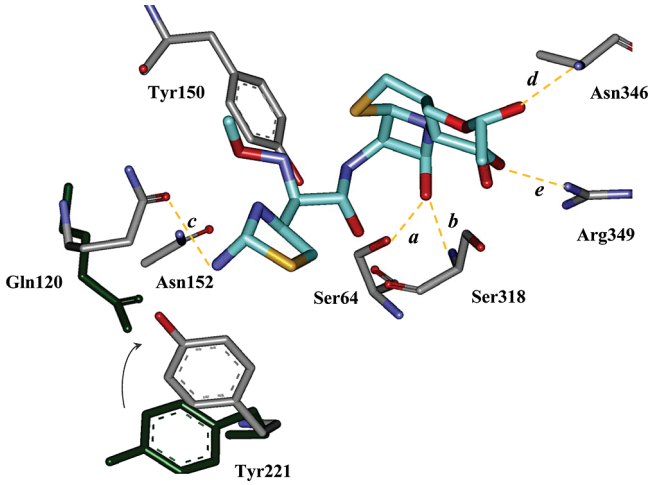

FIGURE 6: CTX modeled in the active site of CMY-32 β-lactamase as a preacylation complex. CTX (carbon atoms colored blue) was docked with the carbonyl of the β-lactam ring in the oxyanion hole of CMY-32 in alignment with the backbone amides of Ser64 and Ser318 (a = ~2.1 Å; b = ~2.2 Å). The hydrogen bonds of CTX with CMY-32 (colored by element) are represented as dashed lines (c = ~2.0 Å; d = ~2.1 Å; e = ~2.3 Å). In CMY-32, Gln120 and Tyr221 (gray) adapt a different conformation than in CMY-2 (green).

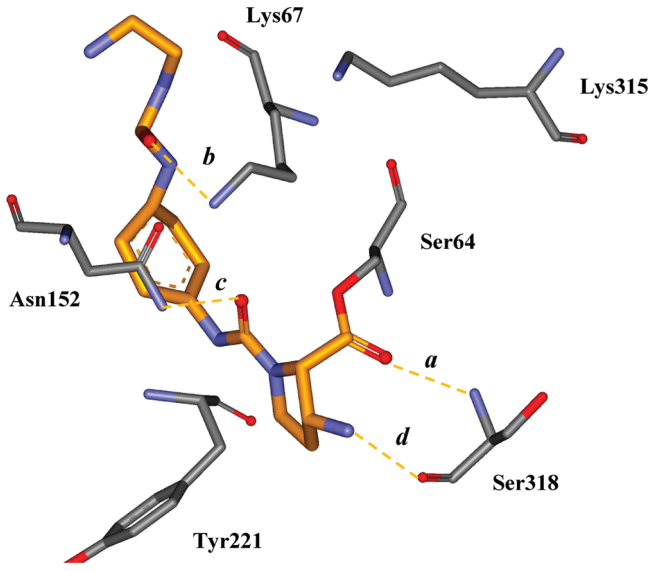

FIGURE 7: BAL29880 (carbon atoms colored orange) modeled in the active site of CMY-2 β-lactamase as an acyl−enzyme complex with the carbonyl group in the oxyanion hole formed by Ser318 and Ser64 (a = ~1.9 Å). Dashed lines represent the hydrogen bonds between BAL29880 and CMY-2 (colored by element; b = ~1.8 Å; c = ~2.1 Å; d = ~2 Å). BAL29880 was modeled without the SO_3^- group.

Conclusions. We emphasize four important findings arising from this work. First, we demonstrate how a single-amino acid change in the Ω loop of CMY-2 can considerably alter MICs, modify the spectrum of substrate hydrolysis, and impact the efficacy of β-lactamase inhibitors. In this context, we argue that in class C ESBLs, movement of the Ω loop and repositioning of key amino acids may be the explanation for the phenotypic and kinetic properties of this class C. In contrast to the findings in class A ESBLs, we propose that the major impact of this substitution is to constrain rather than expand or disorder the Ω loop and active site cavity (*20*, *45*, *46*). Here, the inherently larger space in the WT CMY-2 enzyme active site, which is optimally configured to hydrolyze narrow-spectrum

cephalosporin substrates, appears to be tailored in CMY-32 to also accommodate CTX and ATM.

Second, comparing our analysis to other class C β-lactamases that are also considered "extended-spectrum" types, we see that amino acid substitutions at multiple positions, additions, and deletions all give rise to this ESBL phenotype in class C β-lactamases (*15*, *47*, *48*). Among class A enzymes, the number and types of changes able to confer ESBL phenotype are limited (*18*). Hence, we predict that there will be even more opportunities in class C enzymes to evolve novel catalytic abilities by natural protein engineering.

Third, we propose a novel mechanism for the interaction of class C β-lactamases with monobactam substrates and inhibitors. One wonders if the elimination of the SO_3^{2-} group from both ATM and the BAL29880 facilitates hydrolysis or serves to further stabilize the acyl−enzyme complex and results in a long-lived terminal species. We also do not know if this phenomenon is unique to CMY type β-lactamases (*49*). In NMC-A, a similar pattern was seen by Mourey et al. studying monobactam inhibitors (*49*).

Lastly, we observed in this class C serine β-lactamase that the formation of the acyl−enzyme intermediates of carbapenems is accompanied by fragmentation of the substrate with loss of the hydroxyethyl substituent at the C6 position. This seems to be a property of CMY- and ADC-type class C β-lactamases (*40*).

As we contemplate these major conclusions, we are certain that the complexity of β-lactamases and the mechanisms responsible for β-lactamase-mediated resistance will continue to pose significant challenges to our understanding of the nature and the evolution of protein structure−function relationships in the years ahead. Our study of the mechanism of inactivation by monobactams and carbapenems forms a template for the future rational design of new inhibitors of this class of enzymes.

SUPPORTING INFORMATION AVAILABLE

UV difference spectra of CTX with CMY-2 and CMY-32. This material is available free of charge via the Internet at http://pubs.acs.org.

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