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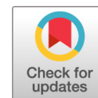
MECHANISMS OF RESISTANCE



Kinetics of Sulbactam Hydrolysis by β-Lactamases, and Kinetics of β-Lactamase Inhibition by Sulbactam

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ABSTRACT Sulbactam is one of four β-lactamase inhibitors in current clinical use to counteract drug resistance caused by degradation of β-lactam antibiotics by these bacterial enzymes. As a β-lactam itself, sulbactam is susceptible to degradation by β-lactamases. I investigated the Michaelis-Menten kinetics of sulbactam hydrolysis by 14 β-lactamases, representing clinically widespread groups within all four Ambler classes, i.e., CTX-M-15, KPC-2, SHV-5, and TEM-1 for class A; IMP-1, NDM-1, and VIM-1 for class B; *Acinetobacter baumannii* ADC-7, *Pseudomonas aeruginosa* AmpC, and *Enterobacter cloacae* P99 for class C; and OXA-10, OXA-23, OXA-24, and OXA-48 for class D. All of the β-lactamases were able to hydrolyze sulbactam, although they varied widely in their kinetic constants for the reaction, even within each class. I also investigated the inactivation kinetics of the inhibition of these enzymes by sulbactam. The class A β-lactamases varied widely in their susceptibility to inhibition, the class C and D enzymes were very weakly inhibited, and the class B enzymes were essentially or completely unaffected. In addition, we measured the sulbactam turnover number, the sulbactam/enzyme molar ratio required for complete inhibition of each enzyme. Class C enzymes had the lowest turnover numbers, class A enzymes varied widely, and class D enzymes had very high turnover numbers. These results are valuable for understanding which β-lactamases ought to be well inhibited by sulbactam. Moreover, since sulbactam has intrinsic antibacterial activity against *Acinetobacter* species pathogens, these results contribute to understanding β-lactamase-mediated sulbactam resistance in *Acinetobacter*, especially due to the action of the widespread class D enzymes.

KEYWORDS sulbactam, β-lactamase, kinetics, turnover number

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The β-lactam antibacterial drugs, including penicillins, cephalosporins, carbapenems, and monobactams, are widely used to treat bacterial infections. Unfortunately, this widespread use has led to the spread of resistance mediated by serine β-lactamase and metallo-β-lactamase enzymes that degrade the β-lactams. To counter resistance due to serine β-lactamases, β-lactams can be combined with β-lactamase inhibitors (1–4). Three of the four β-lactamase inhibitors in current clinical use, namely, sulbactam, tazobactam, and clavulanic acid (but not the diazabicyclooctanone avibactam), are themselves β-lactams and therefore are potentially subject to degradation by β-lactamases. Many clinical bacterial isolates contain multiple β-lactamase genes (5). Since the ability of β-lactamase inhibitors to inactivate β-lactamases is variable, these combinations of multiple enzymes may be capable of degrading both the β-lactam antibacterial drug and the β-lactamase inhibitor. Therefore, it is important to understand the susceptibilities of β-lactamase inhibitors to inactivation by β-lactamases.

As a β-lactamase inhibitor, sulbactam (Fig. 1) has been combined in clinical practice with ampicillin (6), cefoperazone (7), and ceftriaxone (8). In addition to its use as a β-lactamase inhibitor, sulbactam has intrinsic antibacterial activity against *Acinetobac-*

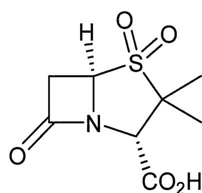

FIG 1 Structure of sulbactam.

ter species and a few other pathogens (9, 10). Since many clinical *Acinetobacter* strains express one or more β-lactamases, including class D enzymes that are not potently inhibited by sulbactam (3, 11), resistance to sulbactam is common (12).

This report supplies measurements of the steady-state kinetics of sulbactam hydrolysis by 14 purified β-lactamase enzymes, representing clinically important families within all four Ambler classes into which β-lactamases are grouped (13, 14), as well as the kinetics of inhibition of these enzymes by sulbactam. The class A serine β-lactamases were CTX-M-15, KPC-2, SHV-5, and TEM-1. The class B metallo-β-lactamases were IMP-1, NDM-1, and VIM-1. The class C serine β-lactamases were *Acinetobacter baumannii* ADC-7, *Pseudomonas aeruginosa* AmpC, and *Enterobacter cloacae* P99. The class D serine β-lactamases were OXA-10, OXA-23, OXA-24, and OXA-48.

RESULTS

Sulbactam hydrolysis by β-lactamases. For the class A β-lactamases tested, there was a wide range of kinetic constants for sulbactam hydrolysis (Table 1). The K_ms ranged from ≤2 μM for SHV-5 and TEM-1 to >6.25 mM for CTX-M-15, and the k_{cat}s ranged from 0.07 s^{-1} for SHV-5 to 14 s^{-1} for CTX-M-15. The k_{cat}/K_m values ranged from 470 M^{-1} s^{-1} for CTX-M-15 to ~1,000,000 M^{-1} s^{-1} for TEM-1.

In contrast to the highly varied class A β-lactamases, the class B, C, and D β-lactamases had more similar kinetic constants within each class, with only a 10-fold range of k_{cat}/K_m values. The class C enzymes had lower K_ms than the class B and D enzymes

TABLE 1 Steady-state kinetic constants for hydrolysis of sulbactam by β-lactamases, determined by initial rate and progress curve analyses

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})	
Enzyme[a]	Initial rate	Progress curve	Initial rate	Progress curve	Initial rate	Progress curve
Class A						
CTX-M-15	>6.25	30	ND	14	ND	470
KPC-2	1.4	1.1	7.4	14	5,400	13,000
SHV-5	<0.0025	NT	0.072	NT	>29,000	NT
TEM-1	~0.002	NT	1.8	NT	~1,000,000	NT
Class B						
IMP-1	10	12	170	160	17,000	13,000
NDM-1	3	ND	430	ND	140,000	ND
VIM-1	0.96	0.89	13	13	13,000	15,000
Class C						
ADC-7	0.26	1.1	0.22	0.55	830	500
AmpC	0.42	0.82	0.34	0.52	800	630
P99	0.55	0.76	0.52	0.55	950	720
Class D						
OXA-10	1.5	2.0	2.0	2.2	1,300	1,100
OXA-23	1.7	0.97	16	14	9,400	14,000
OXA-24	9.7	8.4	55	42	5,700	5,000
OXA-48	3.9	2.5	43	48	14,000	19,000

[a]AmpC, *Pseudomonas aeruginosa* class C chromosomal AmpC; P99, *Enterobacter cloacae* class C chromosomal β-lactamase; ADC-7, *Acinetobacter baumannii* class C chromosomal β-lactamase; NT, not tested; ND, could not be determined.

TABLE 2 Inhibition constants for inhibition of β-lactamases by sulbactam[a]

Enzyme[b]	k_{inact}/K_i (M^{-1} s^{-1})	k_{off} (s^{-1})	K_i (μM)
Class A			
CTX-M-15			0.018
KPC-2	26	0.0007	
SHV-5	129,000	0.002	
TEM-1	5,300	0.001	
Class B			
VIM-1			2,400
Class C			
ADC-7	44	0	
AmpC	32	0.00003	
P99	19	0	
Class D			
OXA-10	22	0	
OXA-23			130
OXA-24	0.4	0.0008	
OXA-48	4	0.0002	

[a]Examples of the global fit of the data to the kinetic model are shown in Fig. S3 for SHV-5, CTX-M-15, and P99.
[b]AmpC, *Pseudomonas aeruginosa* class C chromosomal AmpC; P99, *Enterobacter cloacae* class C chromosomal β-lactamase; ADC-7, *Acinetobacter baumannii* class C chromosomal β-lactamase.

but also had lower k_{cat}s, resulting in lower k_{cat}/K_ms than for the class B and D enzymes. TEM-1 had the highest k_{cat}/K_m, by far, of the 14 enzymes tested.

Two methods were employed here to measure the kinetics of sulbactam hydrolysis by β-lactamases, i.e., traditional initial rate measurements and progress curve analysis by numerical integration. The two methods gave similar results (Table 1). Individual kinetic constants for CTX-M-15 could not be measured using initial rates because the Michaelis plot was linear, due to the very high K_m, but the constants could be measured by progress curve analysis (see Fig. S2 in the supplemental material).

Interesting features of the reaction of sulbactam with β-lactamases were revealed by considering the entire reaction progress curves rather than just the initial rates. Whereas the simple 2-step steady-state kinetic mechanism ($E + S \leftrightarrow ES \rightarrow E + P$) was sufficient for numerical integration in some cases (CTX-M-15, IMP-1, and VIM-1), other enzymes required more complex models that included formation of a covalent complex or inhibited state, dissociation of the covalent complex, and substrate inhibition due to binding of sulbactam to that complex or inhibited state (see Materials and Methods and Fig. S3 to S5). The possibility that kinetic mechanisms other than the selected ones could perform equally well in the progress curve analysis cannot be excluded, however.

A particularly interesting observation from the progress curve analysis is the crossing of the progress curves at different sulbactam concentrations that was seen with class C β-lactamases, an example of which is shown in Fig. S5 for *E. cloacae* P99. This was interpreted as being due to substrate inhibition by sulbactam binding to an inhibited state of the enzyme that accumulates gradually. No significant substrate inhibition was observed in the Michaelis plots, based on initial rates (Fig. S1). The equilibrium dissociation constants for this interaction with *A. baumannii* ADC-7, *P. aeruginosa* AmpC, and *E. cloacae* P99 were 84 nM, 340 nM, and 180 nM, respectively.

Inhibition of β-lactamases by sulbactam. The values of the second-order rate constant (k_{inact}/K_i) for inhibition of the serine β-lactamases varied widely, from 0.4 M^{-1} s^{-1} for OXA-24 to 129,000 M^{-1} s^{-1} for SHV-5 (Table 2). For CTX-M-15 and OXA-23, the inhibition appeared not to be time dependent on the time scale of the measurements; therefore, a value for the equilibrium inhibition constant K_i (the equilibrium dissociation constant of the inhibitor) was determined. It is likely that the inhibition of these enzymes was, in fact, time dependent but the value of the off-rate constant k_{off} was so

TABLE 3 Turnover numbers for sulbactam with β-lactamases, measured after 1 h or 5 h of incubation[a]

Enzyme[b]	Turnover number	
	1–2 h	5–6 h
Class A		
CTX-M-15	70	280
KPC-2	2,300	10,000
SHV-5	440	660
TEM-1	14,000	9,400
Class C		
ADC-7	9	9
AmpC	16	18
P99	30	33
Class D		
OXA-10	6,400	2,900
OXA-23	18,000	23,000
OXA-24	≫34,000	≫34,000
OXA-48	43,000	42,000

[a]Experimental data are shown in Fig. S3.
[b]AmpC, *Pseudomonas aeruginosa* class C chromosomal AmpC; P99, *Enterobacter cloacae* class C chromosomal β-lactamase; ADC-7, *Acinetobacter baumannii* class C chromosomal β-lactamase.

high that the time dependence was not observable. CTX-M-15 was potently inhibited by sulbactam, with a K_i of 0.018 μM. In contrast, OXA-23 was weakly inhibited, with a K_i of 130 μM.

Estimates of k_{off}, the rate constant for dissociation/hydrolysis of sulbactam from the enzyme, were obtained from the same global progress curve-fitting used to obtain the k_{inact}/K_i measurements. In some cases (*A. baumannii* ADC-7, *E. cloacae* P99, and OXA-10), k_{off} was too low to measure by this method and therefore was given as zero. Measured values ranged from 3×10^{-5} s^{-1} for *P. aeruginosa* AmpC to 0.002 s^{-1} for SHV-5. Examples of nitrocefin hydrolysis progress curves for β-lactamases with weakly time-dependent inhibition (SHV-5), apparent time-independent inhibition (CTX-M-15), and strongly time-dependent inhibition (*E. cloacae* P99) by sulbactam are shown in Fig. S6.

The metallo-β-lactamases IMP-1 and NDM-1 were not significantly inhibited by sulbactam concentrations up to at least 5 mM. VIM-1, in contrast, was inhibited by millimolar concentrations of sulbactam, and the K_i was calculated to be 2.4 mM. Inhibition was not time dependent.

Sulbactam turnover numbers for inhibition of β-lactamases. The turnover number may be described as the average number of molecules of an inhibitor per enzyme molecule required to achieve 100% inhibition, given sufficient time. Since sulbactam is both a substrate (Table 1) and an inhibitor (Table 2) of serine β-lactamases, turnover numbers greater than 1 are expected, as reflected in the literature (see Discussion).

The lowest sulbactam turnover numbers measured here were for the class C enzymes, with ratios ranging from 9 to 33 (Table 3 and Fig. S7). Turnover numbers for the class A enzymes varied widely, from 70 for CTX-M-15 to 14,000 for TEM-1. High turnover numbers were measured for all four of the class D enzymes tested. The turnover number for OXA-24 was unmeasurably high, with no inhibition observed under the conditions of the experiment.

In most cases, the turnover numbers measured after a 1- to 2-hour preincubation of sulbactam with the β-lactamase were approximately the same as those measured after a 5- to 6-hour preincubation. For CTX-M-15, KPC-2, SHV-5, and OXA-23, however, the turnover numbers were higher after the longer preincubation. This indicates that complete inhibition of these enzymes was not achieved within the time frame of the shorter preincubation. This result is consistent with the relatively high sulbactam k_{off} values of these enzymes, which prevents the enzymes from being completely inhibited

by the sulbactam concentrations used, allowing a substantial amount of the sulbactam to be degraded during the course of the long preincubation. In contrast, the turnover number for OXA-10 decreased with incubation time. This indicates that the shorter incubation time was insufficient to achieve full inhibition of this enzyme. Owing to the negligible off-rate constant of the sulbactam-OXA-10 complex, however, more complete inhibition was achieved after the longer incubation. Turnover numbers were not measured for the metallo-β-lactamases, because IMP-1 and NDM-1 were not inhibited in the range of concentrations tested and the inhibition of NDM-1 was purely competitive and not time dependent.

DISCUSSION

Sulbactam hydrolysis by β-lactamases. The K_m of 1.4 mM measured for KPC-2 was higher than the value of 135 μM reported previously by Papp-Wallace et al. (15). The reason for this is not known but could be related to differences in the buffer conditions used for testing (10 mM phosphate-buffered saline [PBS] [pH 7.4] in the study by Papp-Wallace et al. [15] versus 0.1 M sodium phosphate, 10 mM $NaHCO_3$, 0.005% Triton X-100 in this study). The closely related enzyme KPC-3 (differing from KPC-2 by a single amino acid residue) was reported by Alba et al. (16) to have a sulbactam K_m of 30 μM, a k_{cat} of 4 s^{-1}, and a k_{cat}/K_m of 1×10^5 M^{-1} s^{-1}.

Results reported here for TEM-1 are consistent with previously published measurements. Brenner and Knowles (17) reported a sulbactam K_m of 0.8 μM and a k_{cat} of 2 s^{-1} for RTEM (TEM-1), such that the k_{cat}/K_m was 2.5×10^6 M^{-1} s^{-1}. Imtiaz et al. (18) and Meroueh (19) also reported a sulbactam k_{cat} of 2 s^{-1} for TEM-1, and Delaire et al. (20) reported a k_{cat} of 0.84 s^{-1}.

SHV-5 differs from SHV-1 at only two adjacent residues but is considered to be hypersusceptible to mechanism-based inhibition as a result (21). Consistent with this conception, Thomson et al. (22) reported a sulbactam k_{cat} of 730 s^{-1} for SHV-1, compared with the measurement reported here of 0.072 s^{-1} for SHV-5. Since the two residues in question, i.e., Gly-234 and Glu-235 in SHV-1 versus Ser-234 and Lys-235 in SHV-5 (when numbering from the initiator Met; residues 238 and 239 when numbering the active site nucleophile as Ser-70 or residues 238 and 240 when using the canonical Ambler class A β-lactamase alignment, which omits residue 239) are adjacent to the active site, it is not surprising that the substitution results in an enormous change in k_{cat}, rendering SHV-5 far slower than SHV-1 at sulbactam hydrolysis.

Measurements reported here of the kinetic constants for hydrolysis of sulbactam by CTX-M-15 appear to be the first reported for a member of the clinically important CTX-M family of β-lactamases. Interestingly, CTX-M-15 had by far the lowest catalytic efficiency for sulbactam hydrolysis among the four class A enzymes tested, due to its comparatively high K_m.

The reported kinetic constants for sulbactam hydrolysis by metallo-β-lactamases range widely. The kinetic constants measured here for IMP-1, NDM-1, and VIM-1 are within this range. Marcoccia et al. (23) reported K_m, k_{cat}, and k_{cat}/K_m values for NDM-1 of 1.4 mM, 50 s^{-1}, and 36,000 M^{-1} s^{-1}, respectively. Franceschini et al. (24) reported K_m, k_{cat}, and k_{cat}/K_m values for VIM-1 of 194 μM, 10 s^{-1}, and 52,000 M^{-1} s^{-1}, respectively. The BlaB metallo-β-lactamase of *Chryseobacterium meningosepticum* had a K_m of 1.4 mM, a k_{cat} of 470 s^{-1}, and a k_{cat}/K_m of 3.4×10^5 M^{-1} s^{-1} (25). The IND-5 metallo-β-lactamase of *Chryseobacterium indologenes* had a sulbactam K_m of 1.3 mM, a k_{cat} of 3.3 s^{-1}, and a k_{cat}/K_m of 2,500 M^{-1} s^{-1} (26). Metallo-β-lactamases from *Aeromonas hydrophila*, *P. aeruginosa*, and *Bacteroides fragilis* had k_{cat}/K_ms of 10, 13,700, and 5,900 M^{-1} s^{-1}, respectively (27). The *A. hydrophila* A2 metallo-β-lactamase, *Pseudomonas maltophilia* L-1 metallo-β-lactamase, and *Bacillus cereus* metallo-β-lactamase II were reported (28) to have sulbactam K_ms of 37 μM, 76 μM, and 5.2 mM, respectively, k_{cat}s of 0.12 s^{-1}, 210 s^{-1}, and 10 s^{-1}, respectively, and k_{cat}/K_ms of 3,240 M^{-1} s^{-1}, 2.8×10^6 M^{-1} s^{-1}, and 1,900 M^{-1} s^{-1}, respectively. The kinetic constants shown here for the hydrolysis of sulbactam by class C and class D β-lactamases appear to be the first such measurements reported.

Inhibition of β-lactamases by sulbactam. I measured the second-order rate constants (k_{inact}/K_i) for sulbactam inhibition of each β-lactamase or the K_i, as appropriate (Table 2). These results are consistent with the generally held view that sulbactam has utility as an inhibitor of class A enzymes only (3). Potent inhibition was seen with CTX-M-15, SHV-5, and TEM-1. In comparison, Faheem et al. (29) reported a k_{inact}/K_i value of 29,000 M^{-1} s^{-1} for CTX-M-15 and a K_i of 62 nM, similar to our K_i result of 18 nM. Imtiaz et al. (18) and Merouehet al. (19) reported a k_{inact}/K_i of 125 M^{-1} s^{-1} for TEM-1. In contrast, Labia et al. (30) and Bret et al. (31) reported k_{inact}/K_i values of 1,300 and 2,200 M^{-1} s^{-1}, respectively. Therefore, the k_{inact}/K_i measurement of 5,300 M^{-1} s^{-1} for TEM-1 reported here is higher than the earlier reports.

Published reports include other examples of potent inhibition of class A β-lactamases. Mariotte-Boyer et al. (32) reported a k_{inact}/K_i of 1,325 M^{-1} s^{-1} for the class A NMC-A carbapenemase from *E. cloacae*. Thomson et al. (22) reported a k_{inact}/K_i of 6,500 M^{-1} s^{-1} for SHV-1. Data reported by Yamaguchi et al. (33) and Sawai and Tsukamoto (34) were used to calculate k_{inact}/K_i values of 1,100 M^{-1} s^{-1} and 2,300 M^{-1} s^{-1}, respectively, for type 1b (TEM-2-type) penicillinase, and Labia et al. (30) similarly reported 1,000 M^{-1} s^{-1}. Therrien et al. (35) reported a k_{inact}/K_i of 3,300 M^{-1} s^{-1} for the class A PSE-4 (also called CARB-1) β-lactamase.

Sulbactam had much lower potency against the class A β-lactamase KPC-2, however. This enzyme differs from the other class A enzymes in having potent carbapenemase activity. Similarly low inhibitory activities of sulbactam were observed with the class B, C, and D enzymes. In comparison, a k_{inact}/K_i value of 16 M^{-1} s^{-1} was calculated for *Citrobacter freundii* class C GN346 cephalosporinase from the data reported by Yamaguchi et al. (33). In contrast, higher k_{inact}/K_i values of 220 M^{-1} s^{-1} and 2,300 M^{-1} s^{-1} were calculated for *Proteus morgana* cephalosporinase and *Proteus vulgaris* cephalosporinase, respectively, based on the data reported by Sawai and Tsukamoto (34). Measurements of kinetic constants for the inhibition of class B and D β-lactamases by sulbactam do not appear to have been published previously.

Sulbactam turnover numbers for inhibition of β-lactamases. The sulbactam turnover number of TEM-1 measured here agreed closely with the value of 10,000 reported by Imtiaz et al. (18), Merouehet al. (19), and Therrien et al. (35). Delaire et al. (20) and Labia et al. (30), however, reported much lower values of 415 and 525, respectively. Our value of 2,300 for the sulbactam turnover number with KPC-2, after a 1- to 2-h preincubation, was similar to the values of 1,000 and 1,500, following a 15-min incubation, reported by Papp-Wallace et al. (15, 36).

Some other reported β-lactamase turnover numbers with sulbactam include 2,280 (30) and 5,200 (33) for the class A enzyme TEM-2, 1,225 for the class A NMC-A carbapenemase from *E. cloacae* (32), 13,000 for SHV-1 (22), 13,000 for the class A SGM-1 β-lactamase (37), 40,000 for the class A OHIO-1 β-lactamase (38), 131 for the class A PSE-4 (CARB-1) β-lactamase (35), and 80 for the *Citrobacter freundii* class C GN346 cephalosporinase (33). Except for the finding for PSE-1, these published values are consistent with our observations of high turnover numbers for class A enzymes and much lower turnover numbers for class C enzymes. No sulbactam turnover number measurements for class D β-lactamases appear to have been published previously.

Sulbactam is considered to be useful primarily as a class A β-lactamase inhibitor (3). Indeed, potent inhibition of the class A enzymes CTX-M-15, SHV-5, and TEM-1 was observed (Table 2), although KPC-2 was much more weakly inhibited. However, relatively high catalytic efficiencies for hydrolysis of sulbactam by SHV-5 and TEM-1 (Table 1), as well as a very high turnover number for TEM-1, were also seen. From these measurements, the utility of sulbactam against class A β-lactamase-expressing clinical strains ought to be limited to a subset of class A enzymes. Since sulbactam is used clinically in combination with ampicillin, cefoperazone, and ceftriaxone, the sensitivity of these β-lactams to hydrolysis by the β-lactamases in clinical strains must also be considered.

Based on the observations reported here, it might be expected that sulbactam would be effective as an inhibitor of class C enzymes, if *A. baumannii* ADC-7, *P. aeruginosa* AmpC, and *E. cloacae* P99 are representative. The catalytic efficiencies of these enzymes for sulbactam hydrolysis were relatively low, which should result in relatively slow degradation of sulbactam, and the relatively low turnover numbers should contribute to effective inhibition. However, the inhibitory efficiencies (k_{inact}/K_i) were also relatively low, leading to low rates of inactivation of the enzymes by sulbactam. A sufficiently high level of class C β-lactamase expression, low outer membrane permeability to the β-lactam partner, and/or a sufficient degree of active efflux may override the capacity of sulbactam to inhibit β-lactamase activity. Moreover, clinical strains often express multiple β-lactamase enzymes, which could overcome the effectiveness of sulbactam by combining an enzyme that degrades it effectively with an enzyme that degrades its β-lactam partner effectively.

The class B metallo-β-lactamases studied here showed high catalytic efficiencies for sulbactam hydrolysis and weak or no inhibition by sulbactam. Thus, sulbactam lacks utility against metallo-β-lactamases, and the expression of class B enzymes can be expected to reduce or to eliminate the utility of sulbactam as either a β-lactamase inhibitor or an antibacterial drug.

One use of the measurements described is to ascertain which β-lactamases pose the greatest threat to the use of sulbactam as an antibiotic (as opposed to a β-lactamase inhibitor) for the treatment of *A. baumannii* infections. Sulbactam has antibacterial activity against *A. baumannii* due to its inhibition of PBP3 (10, 39), but this activity is compromised in current clinical strains due to the expression of multiple β-lactamases, including class D enzymes (40). This study provides some of the first measurements of the kinetic constants for sulbactam hydrolysis by class D β-lactamases and for inhibition of those enzymes by sulbactam. The relatively high catalytic efficiencies of class D enzymes such as OXA-23 and OXA-24 (which are often found in *A. baumannii* clinical strains) with sulbactam as a substrate, the relatively low efficiencies of inactivation, and the very high turnover numbers combine to make sulbactam a good substrate for hydrolysis by these enzymes and a poor inhibitor. Thus, the expression of these enzymes by bacteria at sufficient levels should effectively degrade sulbactam, lowering its antibacterial potency. An effective class D β-lactamase inhibitor, especially one that is a poor substrate for these enzymes, is needed to counter this problem. ETX2514, a broad-spectrum diazabicyclooctenone β-lactamase inhibitor (11), likely satisfies this requirement, since ETX2514 inhibits many class D β-lactamases in addition to class A and C enzymes and is not significantly degraded by them. Indeed, the addition of 4 mg/liter ETX2514 significantly restored the activity of sulbactam against a global collection of 1,131 clinical isolates of *A. baumannii* collected in 2014, reducing its MIC_{90} from $>$32 mg/liter to 4 mg/liter (41). The combination of sulbactam and ETX2514 is currently in clinical development for the treatment of *A. baumannii* infections.

MATERIALS AND METHODS

Materials and experimental conditions. β-Lactamases were purified as described in reference 11 and references therein. ADC-7 (42) was a gift from the laboratory of Robert A. Bonomo of Case Western Reserve University. The free acid of sulbactam was from U.S. Pharmacopeia (Rockville, MD). The sodium salt of sulbactam was from Dr. Friedrich Eberth Arzneimittel GmbH (Ursensollen, Germany). The latter was used when high concentrations of sulbactam were required, in order to avoid acidification of the buffer with the acid form of sulbactam. All serine β-lactamase experiments were performed at ambient temperature in a buffer composed of 0.1 M sodium phosphate (pH 7.0), 10 mM sodium bicarbonate, and 0.005% Triton X-100. All metallo-β-lactamase experiments were performed at ambient temperature in buffer composed of 50 mM HEPES-NaOH (pH 7.0), 1 μM $ZnSO_4$, and 0.005% Triton X-100.

Sulbactam hydrolysis by β-lactamases. Hydrolysis of sulbactam was monitored as an increase in absorbance at 235 nm (17). Assays were performed in a volume of 100 μl in clear 96-well acrylic plates (Corning Life Sciences, Tewksbury, MA). Absorbance was measured at 3- to 6-s intervals for 10 min, with a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). Progress curves from triplicate wells were averaged. Slopes were measured from the linear initial phase of each progress curve. Two-fold serial

dilutions of sulbactam were used, varying the range of concentrations as needed. The enzyme concentration was also varied as needed (see Fig. S1 in the supplemental material). Enzyme concentrations were relatively high, to permit substantial product formation prior to the onset of inhibition by sulbactam. K_m and V_{max} values for each enzyme were obtained by fitting a Michaelis plot of initial rate versus sulbactam concentration to the Michaelis-Menten equation. V_{max} was converted to k_{cat} by using the enzyme concentration and a measured extinction coefficient difference between hydrolyzed and intact sulbactam, for the path length of the 100-μl assay format, of 416 M^{-1}. For TEM-1, the K_m was estimated by extrapolation and was consistent with published measurements (17, 18, 20). A Michaelis plot could not be obtained for SHV-5 due the low sulbactam K_m. V_{max} was calculated for SHV-5 based on the rate of hydrolysis of 50 μM sulbactam by 500 nM SHV-5.

In addition to the traditional, initial rate-based analysis of the reaction kinetics, progress curve analysis was employed, when feasible, using Global Kinetic Explorer software (KinTek) to perform numerical integration. The set of progress curves at a range of sulbactam concentrations for 10-min reactions with each enzyme were fit globally to either a simple model (CTX-M-15 [Fig. S2] and class B enzymes), depicted by E + S ↔ ES (with rate constants for forward and reverse reactions of k_{+1} and k_{-1}, respectively) and ES ↔ E + P (with rate constants for forward and reverse reactions of k_{+2} and k_{-2}, respectively), with $K_m = (k_{-1} + k_{+2})/k_{+1}$ and $k_{+2} = k_{cat}$, or a more complex model incorporating one or more of the following, in order of increasing complexity: formation of an inhibited state or covalent inhibition of the enzyme by sulbactam (class D enzymes [see Fig. S3 for OXA-23]) plus resolution of the covalent complex by hydrolysis (KPC-2 [Fig. S4]) plus substrate inhibition due to sulbactam binding to the inhibited state or covalent complex (AmpC and P99 [Fig. S5] and ADC-7), depicted by E + S ↔ ES, ES ↔ E + P, ES ↔ EI (formation of covalent complex or inhibited state), EI ↔ E + P (k_{off} for covalent complex), and EI + S ↔ EIS (substrate inhibition). There was close agreement between the two methods regarding the values of K_m and k_{cat} (Table 1), and the progress curve method allowed estimation of kinetic constants for CTX-M-15 (Fig. S2), which could not be measured by the initial rate method because of the very high K_m. In the above description, E, S, P, and I represent the enzyme, the substrate (sulbactam), the product of sulbactam hydrolysis, and inhibitor, respectively.

Inhibition of β-lactamases by sulbactam. The second-order rate constants for time-dependent inhibition (k_{inact}/K_i) of serine β-lactamase-catalyzed nitrocefin hydrolysis, or in some cases the equilibrium inhibition constant (K_i) instead, for sulbactam were measured as described in reference 11, with 100 μM nitrocefin as the substrate. For ADC-7, the nitrocefin K_m was 400 μM under the same conditions as used for the other enzymes, and the enzyme concentration used was 16 pM. The maximal sulbactam concentration tested was 5 mM.

For the metallo-β-lactamases IMP-1, NDM-1, and VIM-1, the nitrocefin K_ms were 6.0, 3.2, and 12.4 μM, respectively (data not shown). The substrate was 100 μM nitrocefin. The enzyme concentrations used were 300 pM IMP-1, 1.8 nM NDM-1, and 200 pM VIM-1. The maximal sulbactam concentrations tested were 5 mM for IMP-1 and NDM-1 and 66.7 mM for VIM-1. The K_i for inhibition of VIM-1 by sulbactam was calculated by using the formula for a competitive inhibitor, $K_i = IC_{50}/(1 + [S]/K_m)$, where IC_{50} is the 50% inhibitory concentration and [S] is the nitrocefin concentration.

Sulbactam turnover numbers for inhibition of serine β-lactamases. Each enzyme was incubated at either 3 μM (CTX-M-15, SHV-5, *P. aeruginosa* AmpC, *E. cloacae* P99, and *A. baumannii* ADC-7) or 0.3 μM (KPC-2, TEM-1, and class D enzymes) with a set of 2-fold serial dilutions of sulbactam, with the highest sulbactam concentration being either 1 or 10 mM, respectively. After 1 to 2 h or 5 to 6 h at ambient temperature, the enzyme-sulbactam mixtures were diluted 1:333,000 from 3 μM or 1:33,000 from 0.3 μM into reaction mixtures with 100 μM nitrocefin. The triplicate 45-μl reaction mixtures in clear polystyrene 384-well plates were monitored at 490 nm for 10 min with a SpectraMax Plus plate reader. Control wells in which enzyme was replaced with buffer were included for background subtraction. Data for the triplicate wells were averaged, the background was subtracted, the initial rate of absorbance increase was measured, and the percent inhibition was calculated for each sulbactam/enzyme ratio. The extrapolated point of intersection with the horizontal axis of the percent inhibition versus sulbactam/enzyme ratio curve was taken to be the turnover number.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01612-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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