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Site-Saturation Mutagenesis of Position V117 in OXA-1 β-Lactamase: Effect of Side Chain Polarity on Enzyme Carboxylation and Substrate Turnover†

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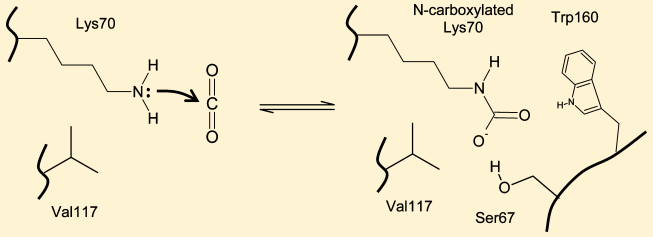
ABSTRACT: Class D β-lactamases pose an emerging threat to the efficacy of β-lactam therapy for bacterial infections. Class D enzymes differ mechanistically from other β-lactamases by the presence of an active-site N-carboxylated lysine that serves as a general base to activate the serine nucleophile for attack. We have used site-saturation mutagenesis at position V117 in the class D β-lactamase OXA-1 to investigate how alterations in the environment around N-carboxylated K70 affect the ability of that modified residue to carry out its normal function. Minimum inhibitory concentration analysis of the 20 position 117 variants demonstrates a clear pattern of charge and polarity effects on the level of ampicillin resistance imparted on *Escherichia coli* (*E. coli*). Substitutions that introduce a negative charge (D, E) at position 117 reduce resistance to near background levels, while the positively charged K and R residues maintain the highest resistance levels of all mutants. Treatment of the acidic variants with the fluorescent penicillin BOCILLIN FL followed by SDS−PAGE shows that they are active for acylation by substrate but deacylation-deficient. We used a novel fluorescence anisotropy assay to show that the specific charge and hydrogen-bonding potential of the residue at position 117 affect CO_2 binding to K70, which in turn correlates to deacylation activity. These conclusions are discussed in light of the mechanisms proposed for both class D β-lactamases and BlaR β-lactam sensor proteins and suggest a reason for the preponderance of asparagine at the V117-homologous position in the sensors.



β-Lactam antibiotics provide a tremendous array of therapeutic options for infections caused by both Gram-positive and Gram-negative species. Depending on the different structural features attached to the core β-lactam ring, these drugs can be classified as penicillins, cephalosporins, carbapenems, and monobactams.[1] Despite this structural diversity, all β-lactams act by inhibiting the transpeptidase enzymes that cross-link the peptidoglycan cell wall. The efficacy of these agents has long been threatened by resistance mechanisms, most notably the action of β-lactamases. These enzymes can be encoded chromosomally or carried on plasmids and may be constitutively expressed or inducible. In the latter case, highly sensitive signal transduction systems have been identified in *Staphylococcus aureus*[2] and *Bacillus licheniformis*[3] for sensing the presence of β-lactam antibiotics and turning on the transcription of β-lactamase genes.

Chemically, β-lactamase enzymes hydrolyze the β-lactam bond, rendering the drug incapable of transpeptidase inhibition.[4] These enzymes are subdivided by sequence homology into four classes (A−D). Classes A, C, and D share a similar serine-nucleophile covalent catalysis mechanism, while the structurally unrelated class B enzymes use a Zn^{2+} ion to activate a water molecule for attack.[5] Class D members are named OXA enzymes (i.e., OXA-1, OXA-24, etc.) because of an unusually high activity against the semisynthetic penicillin oxacillin. This oxacillinase activity is not robust in all class D enzymes however, and most notably it is lower in many (though not all) enzymes that have a strong activity against carbapenems.[6]

The active sites of class D β-lactamases contain two unusual features. They are highly hydrophobic compared to those of class A and class C enzymes, with nonpolarity conserved at positions 117 (V/I/L), 160 (W), and 161 (L/I)[a] among others. Second, a highly conserved lysine (K70) is modified by CO_2 to form a carbamate functional group (Figure 1). These two features are related to each other, as a hydrophobic environment is thought to promote the deprotonated state of the lysine side chain that is necessary for the attack of the lysine amine on the CO_2.[7] The carbamate group, normally quite labile, is stabilized by hydrogen bonds with the side chains of W160 and S67 (and in the case of OXA-1, S120). Functionally, it serves as a general base to activate the alcohol of S67 for attack on the substrate's lactam carbonyl.[7] This action leaves the drug transiently attached to the enzyme, but the carbamate subsequently activates a water for hydrolytic deacylation. In support of this, substitutions that lead to destabilization or elimination of the carbamate greatly slow the acylation and (even more effectively) the deacylation step.[8−11]

The conservation of nonpolar branched aliphatic residues at position 117[12] contrasts to the near ubiquity of asparagine at

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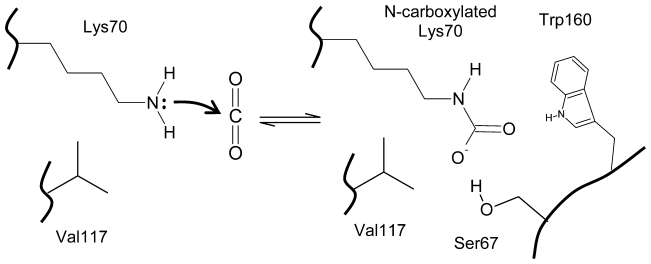

Figure 1. N-Carboxylation of a conserved active site lysine in class D β-lactamases. The presence of several hydrophobic residues (most notably Val117) lowers the pK_a of the highly conserved K70 and thus activates it for N-carboxylation by CO_2. Once formed, the carbamate is stabilized by hydrogen bonds from Ser67 and Trp160.

the homologous position in class A (N132) and class C (N152) β-lactamases.[5,13] The asparagine side chain forms a hydrogen bond with the side chain amide carbonyl of penicillin and cephalosporin substrates.[14,15] It also hydrogen-bonds to carbapenem substrates but does so through the hydroxyethyl group that takes the place of the penicillin side chain amide.[16,17] In class D β-lactamases, the valine that is typically found at position 117 also approaches close to both the side chain amide of penicillins and the hydroxyethyl group of carbapenems, even though it is not able to form a hydrogen bond with either.[11,18−20]

The importance of having a hydrophobic residue at position 117 in class D β-lactamases has been illuminated by studies of a structurally related but nonenzymatic protein family. The β-lactam sensor proteins such as BlaR (from *Bacillus licheniformis*) and MecR (from *Staphylococcus aureus*) share a similar fold with OXA-1[21−23] and use an N-carboxylated lysine as a general base for serine-nucleophile attack on β-lactam substrates.[24,25] In line with their function as initiators of signal transduction pathways, sensor proteins differ from β-lactamases in being unable to efficiently deacylate the covalent intermediate. Interestingly, sensor proteins have a polar residue (N or T) in place of the hydrophobic residue typical of position 117 in the otherwise highly similar class D β-lactamases active sites. This notable difference led to the discovery that after acylation by β-lactam substrates, the BlaR sensor's N-carboxylated lysine modification is lost by spontaneous decarboxylation.[21,25,26] The loss of the general base that normally activates the deacylating water explains the stability of the acyl-enzyme intermediate. In support of this, Cha et al. used a chemical modification approach to replace the N-carboxylated lysine residue of BlaR with a standard carboxylate group and turned the sensor protein into an enzyme capable of full turnover of β-lactam substrates.[27] Conversely, two groups have demonstrated that substitution of polar residues for V117 in two different class D β-lactamases destabilizes the N-carboxylated lysine moiety, yielding various degrees of deacylation deficiency.[11,20]

In order to better understand the overall mechanism of class D β-lactamases and more specifically the role of V117 in that mechanism, we carried out site-saturation mutagenesis at this site. By revealing the chemical characteristics required at position 117 for proper binding and turnover of various substrates, we expected to illuminate how the wild-type valine regulates carbamate formation and function. Given that substitution for the sequentially homologous asparagine in class C β-lactamases has been shown to give major alterations in substrate specificity,[28] we also aimed to determine whether similar shifts would result in class D enzymes.

MATERIALS AND METHODS

Mutagenesis. Single amino acid mutations were generated using the PCR overlap extension method[29] using the bla_{OXA-1} gene subcloned into the pHSG398 vector.[9] All constructs were sequenced and then retransformed into DH10B *E. coli* cells for further analysis.

Antibiotic Susceptibility. Minimum inhibitory concentration (MIC) analysis was carried out as before, with minor modifications.[9] A Steers replicator was used to deliver a 10 μL spot of each of the *E. coli* strains containing the complete V117X set on Luria−Bertani agar plates at pH 7.2 in the absence or presence of 25 mM $NaHCO_3$. The plates contained varying concentrations of one of the antibiotics to be tested (ampicillin, 1−16384 μg/mL; cefotaxime, ceftazidime, doripenem, meropenem, 0.031−32 μg/mL). Each MIC value represents the median of a minimum of three replicate assays.

Detection of Acyl Intermediates. Acyl-enzyme intermediates were detected using the fluorescent penicillin BOCILLIN FL.[30] *E. coli* cultures (50 mL) containing plasmids expressing wild-type OXA-1, each of the 19 V117 variants, the K70D mutant, or a vector control were grown overnight at 37 °C. Cells were centrifuged, frozen, and lysed in 50 mM NaH_2PO_4, pH 7.0, 1 mM EDTA, using lysozyme and DNase I. After clarification by centrifugation, samples (21 μL) of lysate were brought to 100 μM BOCILLIN FL and incubated for 1 min. Reactions were quenched by addition of 10 μL of SDS−PAGE loading buffer. Samples were subsequently separated on a 10% SDS−PAGE gel and visualized using 365 nm light. After documentation of the fluorescence intensity, the steady-state expression level of OXA-1 protein in each lysate was measured by Western blot following a previous method.[9] The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane and then probed with 1 μg/mL anti-OXA-1.[9] The reactive protein bands were detected with horseradish-peroxidase-linked protein G and visualized by chemiluminescence.

β-Lactamase Expression and Purification. Several V117 variant genes were amplified by PCR (excluding the export sequence), ligated into the NdeI and BamHI sites of pET24a, and transformed into BL21(DE3) *E. coli* cells. Expression and purification of the mutant proteins were carried out as described previously.[9] Purified fractions (>95% pure) were combined and concentrated using a Centricon ultrafiltration device (10 000 MWCO). Protein concentration was determined from A_{280} values using an extinction coefficient of 42 065 M^{-1} cm^{-1} [31] and stored at −80 °C after snap freezing in liquid nitrogen.

Kinetic Assays. Kinetic analysis was conducted at room temperature in a Beckman DU-800 spectrophotometer. The assays were carried out in 50 mM NaH_2PO_4, pH 7.4, supplemented with enough $NaHCO_3$ to ensure full carboxylation (25 mM for wild-type, V117K, and V117T; 100 mM for V117N). Initial velocities for ampicillin hydrolysis were determined using the $\Delta\varepsilon$ (molar absorption coefficient) of −900 M^{-1} cm^{-1} (λ = 235 nm). The average of three measurements was plotted as a function of substrate concentration. K_M and k_{cat} values were determined by nonlinear regression of the data to the Michealis−Menten−Henri equation.

Determination of CO_2 Affinity to OXA-1 and Its Variants. Wild-type and V117 mutants were dialyzed against 50 mM sodium acetate, pH 4.5, under vacuum (4 °C, >10 h) to

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decarboxylate K70. Samples of each protein were incubated for 10 min in degassed 50 mM NaH_2PO_4, pH 7.4, or the same buffer containing varying concentrations of $NaHCO_3$ up to 100 mM. Enzyme activity was measured after adding ampicillin to these samples up to 500 μM. CO_2 concentrations were calculated using the Henderson−Hasselbach equation and a pK_a adjusted for ionic strength.[9] Initial velocities (v_0) were measured in triplicate, fit to the following single binding site model, and then normalized to v_{max}.

$$v_0 = \frac{v_{max}[CO_2]}{K_d + [CO_2]} \quad (1)$$

Anisotropy Measurements. Anisotropy was measured with a Photon Technology International QuantaMaster 7 fluorimeter in the T-format. BOCILLIN FL (100 nM) was added to 50 mM NaH_2PO_4, pH 7.4 (either degassed or supplemented with varying amounts of bicarbonate). Anisotropy was monitored over time, and then aliquots of enzyme were added to a final concentration of 400 nM (K70A) or 200 nM (V117D). After an initial rise of anisotropy that lasted ~2 min, wild-type OXA-1 was added (200 nM) to rapidly hydrolyze any unbound BOCILLIN FL. The decay in anisotropy that followed was fit to a single exponential equation to generate a deacylation time constant.

RESULTS

In order to assess the function of the nonpolar residue V117 and its impact on substrate binding and turnover in OXA-1, we carried out site-saturation mutagenesis at that position. All 19 substitutions were prepared by PCR in a bla_{oxa-1} gene cassette in the plasmid pHSG-398.[9] The collection of variants, hereafter referred to as the "V117X set", were individually transformed into the *E. coli* strain DH10B. Minimum inhibitory concentration values were determined for a number of different penicillin, cephalosporin, and carbapenem antibiotics in the absence and presence of 25 mM sodium bicarbonate.

As expected for a narrow-spectrum penicillinase, low MIC values were observed for cefotaxime (≤1 μg/mL), ceftazidime (≤0.25 μg/mL), doripenem (≤0.06 μg/mL), and meropenem (≤0.06 μg/mL) for wild-type OXA-1 and all V117X mutant strains whether or not bicarbonate was present. When challenged with ampicillin, however, the V117X set displayed a wide range of MIC values, suggesting the 117 position has a large effect on substrate binding and/or turnover (Table 1). In the absence of bicarbonate, variants possessing negatively charged side chains (aspartate and glutamate) showed the lowest level of resistance with MIC values near background. Conversely, substitution with the positively charged residues arginine and lysine (but not histidine) led to the highest MIC levels observed (2048 μg/mL) aside from wild-type (8192 μg/mL). High MIC values were also noted for the other two branched chain aliphatic side chains leucine (1024 μg/mL) and isoleucine (512 μg/mL), as well as the valine isostere threonine (2048 μg/mL). Bulky aromatics and small polar uncharged residues (except T) generally displayed relatively low MIC values, though none as weak as aspartate and glutamate. As observed before for other OXA-1 active site mutations, the addition of 25 mM bicarbonate to the MIC plates raised the MIC level of almost all V117X variants between 2- and 8-fold.[9]

The positive effect of bicarbonate on MIC levels and the proximity of the side chain of V117 to the N-carboxylated K70 are consistent with previous observations that position 117 substitutions can alter the affinity of CO_2 for K70. Previously, we and others have observed that class D mutants for which CO_2 binds weakly are typically acylation competent but deacylation deficient.[8,10,20] We therefore sought to determine which of the V117X substitutions were deacylation-deficient by using the fluorescent penicillin BOCILLIN FL in an SDS−PAGE assay previously developed to observe covalent acyl intermediates.[10] Lysates from overnight cultures of each V117X variant strain, along with wild-type and vector-only controls, were treated with BOCILLIN FL. After incubation for 1 min, the samples were separated by 10% SDS−PAGE and visualized on a UV transilluminator. The results, shown in Figure 2, show a striking pattern of the variants that do accumulate acyl intermediates. Most notably, the two residues that showed the lowest levels of resistance to ampicillin (aspartate and glutamate) were the most strongly labeled by BOCILLIN FL. The asparagine variant was also significantly labeled, while histidine, cysteine, glycine, methionine, and serine were weakly modified. Those variants that maintained strong resistance such as lysine, arginine, leucine, isoleucine, and threonine all showed no evidence of acyl-enzyme accumulation.

To ensure that the observed effects were not simply due to differences in expression levels of the various mutants, we carried out Western blot analysis on the same samples used in the BOCILLIN FL assay. While expression levels vary slightly for individual mutants, the differences are not extensive and do not correlate to the fluorescence pattern observed (Figure 2).

It appeared that polar substitutions that are neutral or negatively charged lead to deacylation-deficient enzymes that contribute little or nothing to ampicillin resistance in *E. coli*. Conversely, long, positive side chains or nonpolar branched residues allow OXA-1 to complete the catalytic cycle, resulting in significant ampicillin resistance. Given the proximity of position 117 to K70, a carboxylate side chain at 117 would

Table 1. Minimum Inhibitory Concentration Values for Ampicillin

	MIC values (μg/mL)		
V117X	bicarbonate −	bicarbonate +	ratio +/−
A	256	1024	4
C	128	*a*	
D	4	16	4
E	8	16	2
F	64	128	2
G	32	128	4
H	16	128	8
I	512	2048	4
K	2048	4096	2
L	1024	4096	4
M	64	512	8
N	64	128	2
P	512	1024	2
Q	32	128	4
R	2048	4096	2
S	128	128	1
T	2048	8192	4
V (WT)	8192	16384	2
W	32	128	4
Y	64	256	4
DH10B	4	16	4

[a]The addition of bicarbonate to the MIC plates led to high variability in minimum inhibitory concentration values for V117C.

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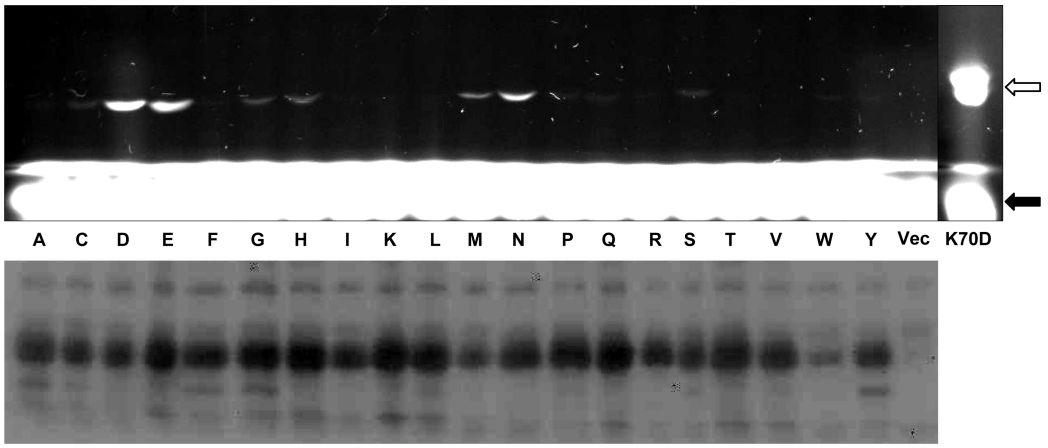

Figure 2. Detection of BOCILLIN FL acyl-enzyme intermediates in all OXA-1 V117 variants. *E. coli* lysates containing each of the 20 OXA-1 V117X variants (indicated by single letter code) were incubated with BOCILLIN FL for 1 min and then separated by 10% SDS−PAGE. BOCILLIN FL-enzyme acyl intermediates (open arrow) and free BOCILLIN FL (solid arrow) were visualized by transillumination at 365 nm (top panel). Controls were DH10B *E. coli* cells containing either the pBCSK vector alone (negative) or the deacylation-deficient OXA-1 K70D mutant (positive). The separated proteins were then transferred to a PVDF membrane and probed with anti-OXA-1 antibody to estimate the relative amounts of expressed variants.

discourage N-carboxylation of K70 by repelling the resulting negative carbamate and stabilizing the unmodified and presumably protonated amine.[21] A neutral side chain capable of hydrogen bonding (e.g., asparagine) would have a similar though possibly weaker effect. Without the active site carbamate, OXA-1 is known to be deacylation-deficient.[10] A lysine or arginine at position 117, on the other hand, could possibly stabilize the carbamate, with consequent effects for maintaining higher resistance levels.

In order to determine how V117 substitutions affected the kinetic properties of OXA-1 and whether or not they affected the stability of N-carboxylated K70, several key mutants were transferred into the expression vector pET24a. OXA-1 mutants V117D, V117N, V117K, and V117T were subsequently overexpressed in *E. coli* strain BL21(DE3) and purified by cation-exchange chromatography. These four variants, along with wild-type OXA-1, were stripped of their carbamate modifications by vacuum dialysis in 50 mM sodium acetate, pH 4.5. This treatment reduced wild-type OXA-1, V117K, and V117T ampicillin hydrolysis activity by ≥70%, while the V117N and V117D variants were devoid of ampicillinase activity. Titration of bicarbonate at various levels up to 100 mM was used to restore CO_2 concentrations and resulted in a hyperbolic increase in ampicillinase activity for all mutants except V117D (Figure 3). K_d values determined from these plots indicate that CO_2 affinity was essentially identical for wild-type and V117K (0.019 and 0.020 mM, respectively), slightly reduced for V117T (0.044 mM), and highly reduced for V117N (4.0 mM). Ampicillin K_M and k_{cat} values were determined for each variant in the presence of saturating amounts of bicarbonate. Interestingly, K_M values remained nearly unchanged for wild-type (30 ± 3 μM), V117K (20 ± 1 μM), V117T (17 ± 4 μM), and V117N (12 ± 1 μM) (Table 2), suggesting that mutation of that position does not affect binding of ampicillin. Values of k_{cat}, however, were greatly reduced even in the presence of bicarbonate, from 564 ± 14 s^{-1} for wild-type to as low as 4.5 ± 0.1 s^{-1} for V117N.

We have previously shown that the loss of N-carboxylation on K70 eliminates catalytic turnover in OXA-1 and that this effect was due to a disproportionate reduction of the

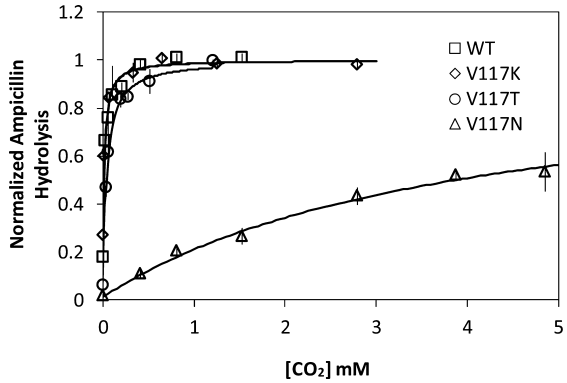

Figure 3. Ampicillinase activity of several OXA-1 V117 variants as a function of CO_2 concentration. Wild-type OXA-1 and four V117 variants were dialyzed at pH 4.5 under vacuum to strip CO_2 from N-carboxylated K70. Samples were incubated in degassed 50 mM NaH_2PO_4 buffer supplemented with 0−100 mM $NaHCO_3$ and then assayed for β-lactamase activity against ampicillin. All velocities were normalized to a v_{max} of 1.0. OXA-1 V117D has no detectable activity with or without bicarbonate supplementation (data not shown).

Table 2

OXA-1 variant	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\cdot s^{-1}$)
WT	30 ± 3	546 ± 14	18 ± 2
V117K	20 ± 1	92 ± 1	4.5 ± 0.2
V117T	17 ± 4	201 ± 12	12 ± 3
V117N	12 ± 1	4.5 ± 0.1	0.37 ± 0.05
V117D	nd[a]	<0.002	nd[a]

[a]nd: not determined.

deacylation rate.[10] We therefore sought to measure deacylation rates for position 117 mutants. Previously, we developed two independent assays to measure deacylation rates. In the first, SDS−PAGE was used to detect covalent acyl-enzyme intermediates between the enzyme and the fluorescent penicillin BOCILLIN FL. While quantitation of the fluorescence signal over time allowed determination of exponential deacylation rates, this assay suffered from low sensitivity and a lack of time-resolution. Deacylation could also be measured by

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following the quench of OXA-1 tryptophan fluorescence by the dye ligand Cibacron Blue 3GA as the hydrolyzed β-lactam product left the active site.[10] This assay greatly increased the time resolution for measuring deacylation rates but was dependent on the unusually high environmental sensitivity of the class D β-lactamase's tryptophan fluorescence to Cibacron Blue. In order to overcome some of these drawbacks, we developed a novel deacylation assay based on the fluorescence anisotropy of BOCILLIN FL. Anisotropy serves as a proxy of the rotational mobility of a fluorescent compound and is therefore expected to vary markedly as the small BOCILLIN FL molecule binds to and later releases from the large β-lactamase proteins.

To test this system, we first used OXA-1 K70A, a variant that is known to form highly stable acyl-enzyme intermediates.[10] Samples of K70A and BOCILLIN FL were mixed and incubated for 5 min to allow binding and acylation to occur. Deacylation was initiated by the addition of wild-type OXA-1, which rapidly breaks down any free BOCILLIN FL. As shown in Figure 4 (top panel), these complexes had high anisotropy values that decayed over time. This decay fit well to a single exponential curve with a time constant of 0.0042 ± 0.0001 s^{-1} (white overlay, with residuals shown on the x-axis). That this decay rate is equivalent to the deacylation rate is supported by several lines of evidence. First, the time constant matched closely to that determined previously for this mutant and BOCILLIN FL in the SDS–PAGE assay (0.0056 ± 0.0004 s^{-1}), an assay that presumably measures the deacylation event itself rather than product release.[10] Second, the rate of deacylation was strongly accelerated by the addition of propionate, which we previously showed to be able to act as a small molecule complement of the N-carboxylated lysine moiety that is missing in this mutant (Figure 4, lower panel). Lastly, the addition of wild-type OXA-1 prior to the addition K70A to BOCILLIN FL almost completely eliminated the higher anisotropy values observed (Figure 4, lower panel).

The addition of OXA-1 V117D to BOCILLIN FL resulted in a similarly strong increase in the anisotropy of the β-lactam (Figure 5). If this assay was carried out in buffer that had been degassed by vacuum to remove residual CO_2, deacylation was almost nonexistent (Figure 5, top panel). If the assay was instead carried out in the presence of 100 mM sodium bicarbonate, the deacylation rate increased to 0.017 ± 0.001 s^{-1}. Interestingly, the rate of the rise in anisotropy at the beginning of the assay (presumably some combination of BOCILLIN FL binding and acylation) is also increased by the presence of bicarbonate. Bicarbonate supplementation also leads to a significant increase in the rate of acylation of the MecR sensor protein from *S. aureus*.[32] In order to further probe the role of polar residues in the active site of the BlaR sensors (which have either an asparagine or a threonine at this position), the anisotropy assay was repeated using OXA-1 V117N and V117T. As expected, BOCILLIN FL deacylation rates on V117N varied with CO_2 concentration (Figure 6), with a time constant ranging from 0.00038 ± 0.0001 s^{-1} for degassed buffer to 0.035 ± 0.001 s^{-1} for 50 mM $NaHCO_3$ (2.8 mM CO_2). No significant rise in anisotropy was observed with V117T, suggesting that the deacylation rate on this enzyme is too fast to isolate the acyl intermediate on the time-scale of the anisotropy assay (data not shown).

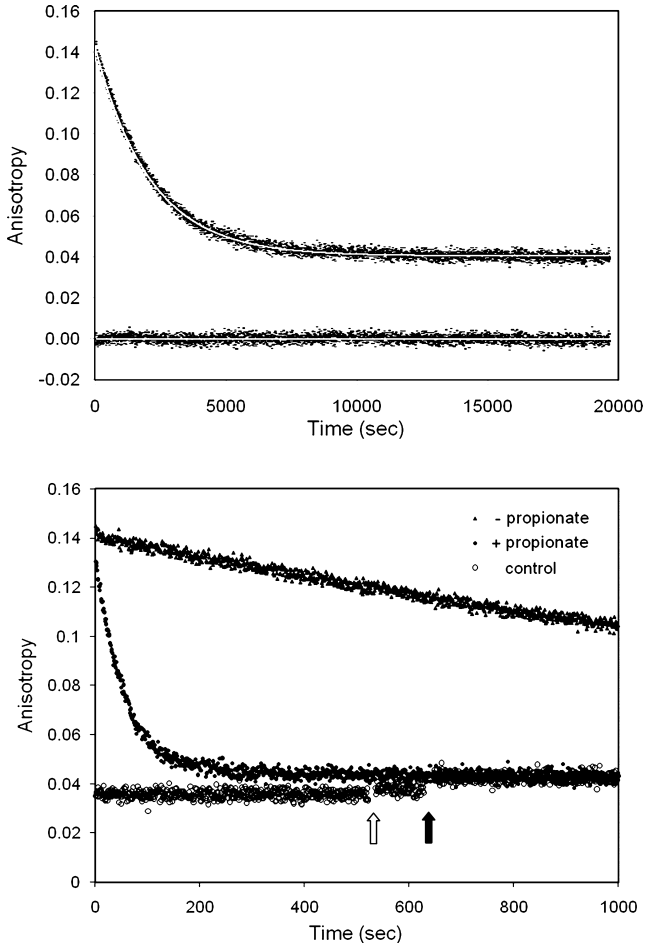

Figure 4. Interaction of BOCILLIN FL and OXA-1 K70A monitored by changes in anisotropy. The fluorescent penicillin BOCILLIN FL was combined with the slowly deacylating OXA-1 mutant K70A. After the addition of wild-type OXA-1 to hydrolyze any excess unbound BOCILLIN FL (at time = 0), anisotropy was monitored over time (top panel). The data points were fit to a single exponential curve (white overlay), with residuals shown on the x-axis. The early portion of the same data is shown in the lower panel (solid triangles) with two additional controls. OXA-1 K70A/BOCILLIN FL anisotropy was monitored after the addition of sodium propionate (time = 0), which serves as a mimic of the missing carbamate moiety and greatly increases the deacylation rate (solid circles). Anisotropy of BOCILLIN FL alone was monitored (open circles) with subsequent additions of wild-type OXA-1 (open arrow) and OXA-1 K70A (solid arrow).

DISCUSSION

The high degree of nonpolar residue conservation observed at position 117 in class D β-lactamases, in contrast to the polar residues found in class A or C, suggests that this position plays an important role in the unique mechanism known to operate in class D enzymes. The results presented in this paper strongly support the hypothesis that the presence of a nonpolar residue at position 117 facilitates the formation of the carbamate general base formed when K70 reacts with CO_2. The fact that isoleucine and leucine substitutions at this position yield highly functional enzymes fits this assertion well. The strong dependence of activity on the charge at position 117, with negative substitutions having a deleterious effect and positive substitutions yielding strong activity, gives further insight into the nature of this interaction. The full negative charge of glutamate or aspartate is incompatible with the presence of the

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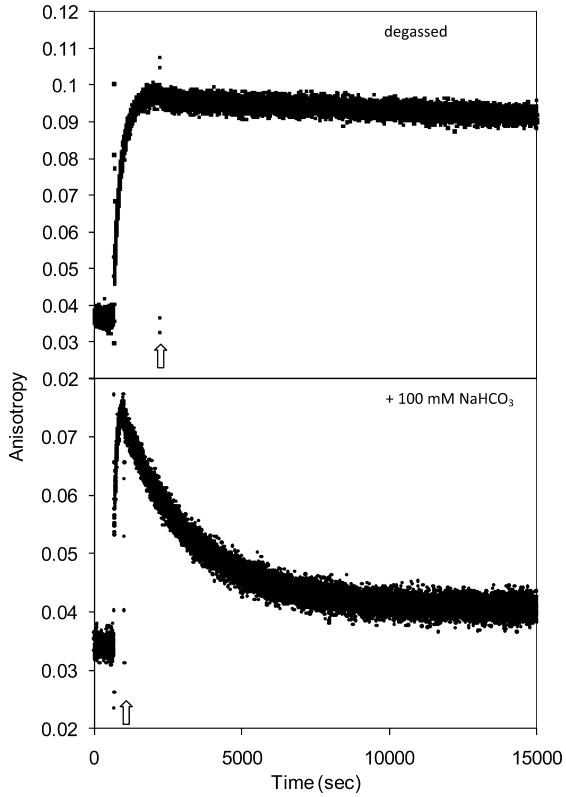

Figure 5. Effect of bicarbonate on the deacylation rate of OXA-1 V117D. The addition of OXA-1 V117D to BOCILLIN FL in degassed NaH_2PO_4 buffer (pH 7.4) led to a rapid rise in anisotropy (top panel). The addition of wild-type OXA-1 (to hydrolyze excess BOCILLIN FL and initiate deacylation) is shown by the position of the arrow. The experiment was repeated in the same buffer supplemented with 100 mM $NaHCO_3$ (lower panel).

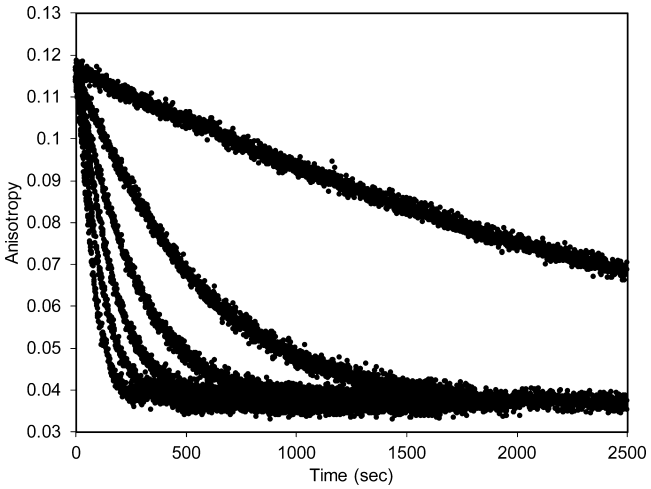

Figure 6. Effect of bicarbonate on the deacylation rate of OXA-1 V117N. Deacylation rates of OXA-1 V117N/BOCILLIN FL complexes in various concentrations of $NaHCO_3$ (0−50 mM, right to left) were determined by monitoring anisotropy as in Figure 5.

carbamate anion, as observed crystallographically with OXA-24.[11] Our bicarbonate titrations demonstrate that the full positive charge of lysine or arginine maintains the very high affinity of CO_2 for K70, as would be expected for a stabilizing ionic force. However, the k_{cat} of the V117K mutant is reduced significantly compared to wild-type, even under saturating bicarbonate conditions. It is likely that the full positive charge of the side chain in this case decreases the basicity of the carbamate and thereby reduces its activity as a general base. A similar effect may be responsible for the decreased activity of V117T in OXA-10, a variant in which the threonine side chain was shown to be hydrogen-bonded to the carbamate.[20]

The identification of several V117 substitutions that result in deacylation deficiency is broadly useful. The study of β-lactamase/substrate complexes is often dependent on the use of variants that arrest the catalytic mechanism at some midpoint. Indeed, knowledge gained from this study has already led to the structure determination of a class D enzyme (OXA-24 V130D) with the carbapenem doripenem bound as an acyl-enzyme intermediate.[11] Such variants that result in loss of carbamate stability may prove to be a more subtle method to achieve deacylation deficiency than mutation of the N-carboxylated lysine itself.

When the position homologous to residue V117 is mutated in class A or class C β-lactamases, large changes in substrate specificity are observed.[28] It is therefore notable that we did not see any gain of function phenotypes with respect to third generation cephalosporins or carbapenems. It is possible that the reversible nature of the lysine N-carboxylation modification, and the importance of residue 117 in its formation, precludes the types of substitutions that are necessary for changes in substrate binding affinities or turnover rates. A similar situation exists with regard to the residue immediately preceding the catalytic serine, which when mutated yields advanced generation cephalosporin resistance in a class A lactamase (M69).[33] Mutation of the same residue in OXA-1 (D66) results in destabilization of the N-carboxylated K70 and only minor gains in hydrolysis of cefepime and cefotaxime.[9] These observations suggest that the carbamate moiety may serve as an "Achilles's heel" for class D β-lactamases, critical for activity but labile enough that the kinds of substitution necessary for substrate plasticity cripple the enzyme's basic turnover mechanism. In light of this argument, it is interesting to note that point mutations leading to activity against extended spectrum cephalosporins are rare in seven of the nine class D subfamilies (OXA-10 and OXA-2 subclasses being the exceptions).[12]

The results observed here for polar substitutions of V117 are particularly helpful in understanding how the presence of asparagine or threonine at the homologous position in the BlaR and MecR β-lactam sensor proteins contributes to the deacylation deficiency that is necessary for their function. It has been proposed that the hydrogen-bonding potential of these polar side chains in some way induces the postacylation decarboxylation of the sensors, thus preventing activation of the deacylating water.[21,22] The deacylation deficiency observed for OXA-1 V117N is consistent with this hypothesis, as is the greatly reduced affinity for CO_2 observed for this variant. It is possible that the presence of the amide side chain of asparagine favors the carbamate nitrogen acting as general base, which leads to a barrierless decarboxylation event.[21,27] Alternatively, a conformational change that occurs upon acylation could further decrease CO_2 affinity, as seen when moxolactam occupies the active site of OXA-10.[20] In either case, the asparagine likely hydrogen-bonds to the decarboxylated amine of K70 as observed for BlaR1.[25]

A recent report by Kumarasiri et al. describes the results of a similar but converse mutagenesis experiment to what we have reported here. In this study, they introduced an N439V

mutation into the BlaR1 sensor protein of *Staphylococcus aureus* and demonstrated that this variant is capable of full turnover of cephalosporin substrates.[34] The presence of the hydrophobic valine side chain precludes the hydrogen bond that normally forms between the asparagine and the N-carboxylated lysine of BlaR1 and thereby increases the ability of the latter to activate a water for deacylation. It is interesting to note that neither of these two reciprocal substitutions is enough to fully switch the functional profile of the two proteins: BlaR1 N439V mutant gains β-lactamase activity toward cephalosporin substrates (but not penicillins), and the OXA-1 V117N variant, unlike a sensor protein, maintains significant penicillinase activity in the presence of modest levels of bicarbonate. Clearly other amino acid differences between the two protein families will need to be identified to explain their different rates of deacylation. Wilke et al. suggest that the leucine at position 389 in BlaR1 may further destabilize the carbamate moiety and note the presence of polar residues in the spatially equivalent areas of class D β-lactamases (e.g., N73 in OXA-10, S120 in OXA-1).[22]

It is interesting to note that various polar substitutions at position 117 in OXA-1 yield vastly different biochemical properties, with a rank order of T > N > D for both CO_2 affinity and deacylation rate. If deacylation deficiency is central to the function of β-lactam sensor proteins, why is aspartate never observed at the homologous position in that family? One possibility is that the full negative charge of that residue's side chain slows both the deacylation and acylation reaction, as we observed for the V117D mutant of OXA-1. The loss of sensitivity caused by such a slower acylation rate may offset the longer signal lifetime achieved with the aspartate. A more important question is the following: How does the BlaR of *Bacillus licheniformis* achieve deacylation deficiency with a threonine at this position? V117T mutations in two different class D enzymes (OXA-10 and OXA-1), while yielding slight decreases in CO_2 affinity, maintain strong turnover rates under physiological conditions. The most likely explanation is that the *B. licheniformis* protein may have additional active site features that augment the slight carbamate-destabilizing effect of threonine. Cha et al. note that different sensor proteins can display vastly different acylation rates, and thus subtle differences in active site residue choice may also simply reflect how different sensor proteins are "tuned" to advantage under particular physiological conditions.[32] Ultimately, the more striking deacylation deficiency displayed by V117N over V117T is probably the reason that most sensor proteins have asparagine at this position.

Lastly, we note that our anisotropy assay represents a great advance for measuring deacylation rates for β-lactamase enzymes. The assay has much higher time resolution and is less cumbersome than the SDS−PAGE based assays previously used for the detection of BOCILLIN FL acyl intermediates.[10] The assay will also be highly suitable for determining the typically slow deacylation rates of penicillin-binding proteins but could also be adapted to stopped-flow measurements for the faster rates of wild-type β-lactamases.

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ABBREVIATIONS

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; EDTA, ethylenediametetraacetic acid

ADDITIONAL NOTE

[a]All residue references are OXA-1 numbering unless otherwise noted.

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