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Mutation of the N^{ζ}-Carboxylated Lysine of OXA-1 β-Lactamase Results in Deacylation Impaired Enzyme and Build-Up of Acyl-Intermediate

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Abstract

β-lactamases hydrolyze penicillin, cephalosporin, and carbapenem β-lactam antibiotics. The class D β-lactamase OXA-1 has a catalytic serine (position 67) thought to be deprotonated and thereby activated by an unusual N^{ζ}-carboxylated lysine (position 70). We have made several mutations of OXA-1 at both K70 and S67 to help elucidate the role of these two critical residues in the catalytic mechanism. We have used the fluorescent substrate BOCILLIN FL to demonstrate that the K70 mutants can acylate but are severely impaired for deacylation of various substrates. Interestingly, deacylation rates vary depending on the identity of the substituting residue, from $t_{1/2}$ = 40 min for K70A to undetectable deacylation for K70D. We have used tryptophan fluorescence spectroscopy to confirm that these results are applicable to natural (i.e. non-fluorescent) substrates.

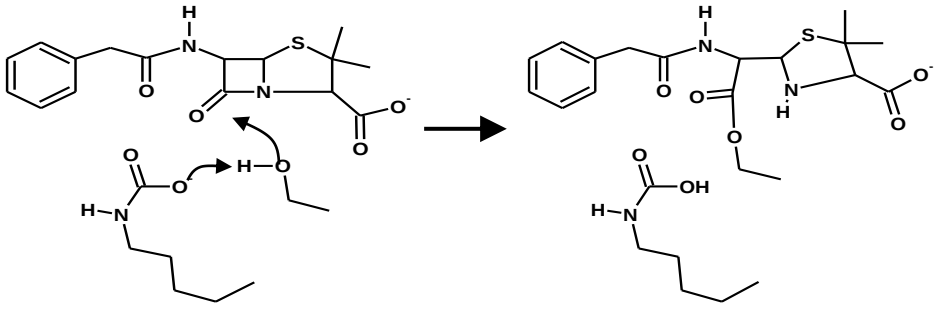
Introduction

β-lactams make up one of the widest ranging classes of clinically utilized antibiotics today, encompassing penicillins, cephalosporins, and carbapenems, among others. The drugs selectively target and inhibit the crucial bacterial cell wall enzymes, penicillin binding proteins (PBP), preventing them from cross-linking the D-alanyl-D-alanine peptide of the cell wall, subsequently resulting in cell death. β-lactamases, believed to share a common ancestor with PBP's (Kelly, et al. 1986), are the primary bacterial enzymes responsible for resistance to the β-lactam class of antibiotics. These enzymes are separated into four distinct classes (A-D), differing by mechanism, active site architecture, and substrate specificity. Class B β-lactamases are metallo-enzymes, typically requiring a zinc molecule in the active site (Fisher, et al., 2005). Classes A, C, and D rely on a nucleophilic serine to break open the β-lactam ring before rapid hydrolysis and regeneration of free enzyme.

Extensive research has been conducted on the two step mechanism of the serine based classes. Like in PBPs, the serine forms an acyl-intermediate via nucleophilic attack of the β-lactam ring carbonyl. Unlike PBP's, however, β-lactamases are able to undergo rapid hydrolysis and deacylation, allowing for efficient destruction of antibiotics (Fisher, et. al., 2005), thereby conferring resistance to the bacterial cell. Many studies have focused on the importance of a general base residue and its role in the acylation and deacylation mechanism of the enzyme. Class A enzymes utilize a Ser-X-X-Glu motif where the glutamate acts as a general base and deprotonates the nucleophilic serine three residues away and activates it for catalytic attack of the carbonyl (Fisher, et. al., 2005). The glutamate also has been shown to be crucial for proper alignment of the water molecule for hydrolysis of the acyl bond and loss of the general base results in severely impaired deacylation and a buildup of acyl-intermediates (Chen and Herzberg, 2001; Shimamura, et al. 2002). A similar result has been found in Class C enzymes regarding a tyrosine proximal to the nucleophilic serine. Though it is still not well understood how the serine becomes deprotonated, the tyrosine is known to play a key role and studies have shown mutation of this residue impairs the enzyme's ability to turnover substrate and also can result in build-up of acyl intermediates (Patera, et. al., 2000).

Similar to the Class A β-lactamases, Class D enzymes possess a lysine in Ser-X-X-Lys motif where the lysine has been shown to be the general base involved in catalysis. Of greater significance, the lysine has been shown to be N^{ζ}-carboxylated in most Class D enzymes (Golemi, et al. 2001; Sun, et al. 2003). This N^{ζ}-carboxylated lysine carries out deprotonation of the serine, and likely aids in the positioning of a water molecule in hydrolysis of the acyl-intermediate

Scheme 1



(Scheme 1) (Fisher, et.al., 2005; Leonard, et.al., 2007), though its importance on the deacylation mechanism has not been sufficiently investigated.

To explore the precise requirements for side-chain size, orientation, and chemical reactivity of the key active site residues and also to serve as a comparison to other classes, we have synthesized three mutants of K70 (alanine, aspartate, and glutamate) and one mutant of S67 (glycine). These mutants have provided valuable insight into the mechanism of the enzyme and also to the crucial importance of the N^{ζ}-carboxylated lysine to the deacylation of acyl β-lactams.

Materials and Methods

Mutagenesis. Amino acid substitutions at position 67 (serine) and 70 (lysine) were carried out using the Polymerase Chain Reaction (PCR) Overlap Extension (Higuchi, et.al., 1988). Two rounds of PCR were conducted using mutant oligonucleotides and Phusion High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) to produce the desired point mutation OXA-1 gene. PCR products were subcloned into the BamHI and NdeI sites of pET24a(+) (Novagen, Madison, WI) and mutant plasmids were transformed into NEB 5-α competent *E.coli* cells (New England Biolabs). Mutant constructs were transformed into BL21 DE3 (K70E and S67G) or BL21 DE3 pLysS (K70D and K70A).

Expression and Purification. *E.coli* cells containing the WT or mutant bla_{OXA-1} genes were grown in Luria-Bertani media containing 25 μg/mL to an optical density (D_{600}) of ~ 0.80. Protein expression was induced with 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 hours. Cells were harvested by centrifugation (7500 rpm for 20 min 25^{o}C; Sorvall SLA-3000 rotor) and frozen at -20^{o}C to improve cell lysis. Frozen cells from 1-2 liters of culture were thawed in ~ 20 ml 50 mM NaH_2PO_4/1 mM EDTA, pH 7.0 supplemented with 100 μL HALT

Protease Inhibitor Cocktail (Thermo Fischer Scientific, Rockford, IL). In the case of K70E and S67G, a final concentration of 1.0 mg/mL lysozyme (Sigma, St. Louis, MO) was also added to achieve lysis. Chromosomal DNA was eliminated using a final concentration of 2.5 µg/mL DNAse I and 2 mM $MgCl_2$. The resulting lysate was clarified by centrifugation (15000 rpm for 30 min 4^oC, Sorvall SS-34 rotor) and dialyzed against 4 liters of 5 mM NaH_2PO_4, pH 5.8. The dialysis retentate was purified according to a previous method (Leonard, et.al., 2007). The lysate was applied to a CM-32 CM-cellulose (Whatman, Kent, UK) column (1.5 cm x 15 cm) equilibrated with 5 mM NaH_2PO_4, pH 5.8. A linear gradient of 5 mM NaH_2PO_4, pH 5.8 to 50 mM NaH_2PO_4, pH 7.0 was used to elute the protein. Pure fractions (>95% purity by SDS-PAGE) of eluted OXA-1 were combined and concentrated to <5 mg/mL (Sun, et.al. 2003) using Amicon Ultra 10 kDA MWCO (Millipore, Billerica, MA) centrifugal filtration devices. Aliquots of protein were snap frozen with liquid nitrogen and stored at -80^oC until use.

Cibacron Blue 3GA Titrations. Tryptophan fluorescence quench of OXA-1 by Cibacron Blue 3GA (CB3GA) experiments were carried out in a Photon Technology International QuantaMaster^{TM} UV-Vis fluorimeter (PTI). Proteins (0.4 µM) were titrated with increasing aliquots of CB3GA from 0-8 µM. Tryptophan quench was fit to an inverted hyperbola:

$$F_I = F_{max} - F_{change} \cdot [CB3GA] / (K_d + [CB3GA])$$

where F_{max} is the initial tryptophan fluorescence, F_{change} is the fluorescence quench, and K_d is the dissociation constant.

Fluorescent SDS-PAGE Assay for Acyl-Enzyme Intermediates. Acyl-enzyme intermediates were detected using the fluorogenic substrate BOCILLIN FL (Invitrogen, Carlsbad, CA). Aliquots of 2.2 µg of each protein were incubated with 50 µM BOCILLIN FL in 50 mM NaH_2PO_4, pH 7.0 for 5 min, followed by a 250-fold excess of ampicillin (13mM). A second set of the same

proteins were treated the same way, except the order of addition for ampicillin and BOCILLIN FL were reversed. Following the incubations, SDS sample buffer was added to each tube and all samples were separated by 10% SDS-PAGE. Gels were illuminated at 365 nm and imaged using UVP Bioimaging Systems Epichemi 3 Darkroom with Biochemi Camera Kit (Ultraviolet Products, Upland, CA). Gels were subsequently stained and imaged with Coomassie Brilliant Blue G.

Active Sites Titration. The acylation stoichiometry of K70D was assessed by treating the substrate ampicillin as an irreversibly binding inhibitor following previous methods (Birck, et.al. 2004; Bauvois, et.al, 2005). Aliquots of K70D protein at 13 µM were incubated with varying levels of ampicillin ranging from 1.3-130 µM for 2 hours to assure complete acylation. BOCILLIN FL was added to samples at 160 µM and incubated for 1 hour, at which point the reactions were quenched with SDS sample buffer. Samples were separated by 10% SDS-PAGE and the resulting gel was illuminated at 365 nm and imaged. Fluorescent bands were quantified with VisionWorks LS (Ultraviolet Products) and the [substrate] to [enzyme] ([S]/[E]) ratio was plotted versus time.

Michaelis-Menten Kinetics. Kinetic analysis was carried out in 50 mM NaH_2PO_4, pH 7.0, at room temperature in a Beckman DU-800 spectrophotometer. Measurements were carried out for nitrocefin and ampicillin using the $\Delta\varepsilon$ [molar absorption coefficient ($M^{-1} \cdot sec^{-1}$)] values of 17400 (λ = 472 nm) and -900 (λ = 235 nm), respectively. The average of three measurements of initial velocity was plotted as a function of substrate concentration, and the K_m and k_{cat} were determined by non-linear regression to the Michaelis-Menten equation.

Deacylation Rates Determination. Deacylation rate constants (k_3) were determined by tryptophan fluorescence [excite = 280 nm (0.4 nm slit width), emission = 330 nm (26 nm slit

width)]. Mutant proteins (0.4 μM) were incubated with excess substrate in a 1 cm pathlength quartz cell and allowed to acylate fully (2-10 min). A trace amount of WT enzyme was added (~20 nM) to hydrolyze excess substrate and initiate deacylation. The reporter dye CB3GA was added prior to WT addition at 3.7 μM. Samples were incubated at 25^{o}C and fluorescence intensity was measured at varying time intervals. Fluorescence change was fit to a first-order exponential rate equation:

$$F_I = F_{base} + F_{max} \cdot e^{-kx}$$

where k is the deacylation rate constant (k_3), F_{max} is the amount of acyl-enzyme intermediate at time 0, and F_{base} is the baseline fluorescence intensity.

Results and Discussion

When utilizing site-directed mutagenesis of key active site residues, it is critical for the enzymes to be properly folded and, unless they're unable to do so due to the mutation itself, be able to interact with its environment in a comparable fashion to the native protein. To demonstrate mutant integrity, the reversible inhibitor CB3GA was analyzed with the mutants and compared to WT. CB3GA has long been known to tightly bind to the active site of OXA enzymes (Monaghan, et. al., 1982). This characteristic has been extended to tryptophan fluorescence. The dye substantially quenches trypthophan fluorescence when bound to OXA-1 with a relatively low K_d value for WT of 0.86 μM (Figure 1a). The serine and lysine mutants demonstrated similar dissociation constants to WT (Figure 1b-c), demonstrating the active site of the mutants likely retain their proper folding and hydrophobicity allowing us to (quantitatively?) study the catalytic mechanism disruptions caused by each mutation.

Of interest for the S67G mutant, the tryptophan fluorescence quench can be extended to natural substrates. The serine mutant is unable to acylate substrates, therefore β-lactams can be treated as reversible binders and dissocation constants can be measured in a similar fashion. Anaylsis of the penicillin class substrate ampicillin yielded a K_d of 235 μM. The K70 mutants, however, show nearly no measurable quench when saturated with substrate, even over long periods of time. Though it's possible these mutants do not bind β-lactams tightly (we show herein that this is not the case), it is also highly probable that loss of the N^{ζ}-carboxylated lysine causes a change in fluorescence of W160, likely a key residue in substrate and mechanism interactions.

As suggested, the mutations of S67 and K70 result in enzymes that can be considered 'dead,' they are unable to measurably hydrolyze substrate by conventional means. Michaelis-Menton kinetics could only be determined using the highly sensitive chromogenic substrate nitrocefin (Table 1). Turn-over efficiency (k_{cat}) for nitrocefin was reduced over 2500-fold for S67G compared to WT, while turn-over for natural substrates (ampicillin) was undetectable. Likewise, the K70 mutants saw a reduction of over 5000-fold when compared to WT, and ampicillin turn-over was also undetectable.

Often, mutagenesis of the catalytic serine of enzymes results in residual turn-over as a result of trace WT contamination a small number of natural back mutations in the genetic sequence (Peracchi, 2001). The low turn-over for S67G and the K70 mutants with nitrocefin, in this case however, can be attributed directly to the mutants and not to any WT contamination since their K_m values are very different than that for WT. In the case of S67G, it's likely that a small molecule, such as a water, is able to act as nucleophile and break the sensitive lactam ring of the properly situated substrate. Also as expected, the K70 mutants are greatly affected by loss

of the general base. Previous studies have shown that the Class D β-lactamase OXA-10 is completely inactivated when the general base lysine is mutated to an alanine, illustrating the importance of the carboxylate on the enzyme's mechanism and function (Golemi, et al. 2001). In the closely related membrane sensor protein BlarR1 which contains a N^{ζ}-carboxylated lysine acting as a general base, the protein has been shown to be unable to turn-over β-lactam substrates after decarboxylation of the lysine (Cha and Mobashery, 2007). Though decarboxylation in BlaR1 leads to its function, it's clear the carboxyl group is a critical part in the mechanism of many Class D and related sensor proteins, so much so that loss of the CO_2 results in near complete arrest of the proteins' ability to hydrolyze substrate.

Though the enzymes are for all purposes 'dead,' the K70 mutants are actually able to acylate substrates. In effect, they are stuck half way through the two step acylation and deacylation mechanism. When the K70 mutants are incubated with BOCILLIN FL for 5 min, they fluoresce under ultraviolet light after separation on SDS-PAGE. This effect cannot be reversed by subsequent addition of ampicillin and preincubation of ampicillin prior to BOCILLIN FL results in complete loss of signal (Figure 2), demonstrating this effect is specific to the active site. Furthermore, WT and S67G show no fluorescence as expected since WT can hydrolyze BOCILLIN FL efficiently and S67G is unable to acylate and form a covalent complex which remains in SDS-PAGE. It is unclear how the K70 mutants acylate substrate but the inability to deacylate suggests the N^{ζ}-carboxylated lysine plays a crucial role in the deacylation mechanism of OXA-1 enzymes, keeping up with earlier work on the general bases in β-lactamases (Chen and Herzberg, 2001; Shimamura, et al. 2002; Patera, et. al., 2000).

Despite being severely impaired, however, the deacylation rates vary substantially by indentity of the residue. As measured by SDS-PAGE, the deacylation rates of BOCILLIN FL

were assessed over a period of several hours (Figure 3). Substitution of aspartate (K70D) for the lysine results in near complete deacylation deficiency, with no significantly measureable rate, whereas the alanine mutant (K70A) is able to deacylate BOCILLIN FL with a half-life ($t_{1/2}$) near 40 min. K70E is drastically impaired like K70D, possibly due to their similar residue structure, though it does have some residual turn-over with a half-life over 3 hours.

Measurement of natural penicillin class substrates such as ampicillin could not be done by SDS-PAGE, so analysis was conducted by tryptophan fluorescence. Ampicillin binding in the K70 mutants yield little change in fluorescence when bound or acylated as stated earlier, but the tightly binding reversible inhibitor CB3GA does. In this novel assay, K70A is allowed to fully acylate and a trace of WT is added to hydrolyze excess substrate. An amount of CB3GA was added at greater than 4x the dye's K_d to ensure every open (deacylated) active site will be immediated filled by a CB3GA molecule and its fluorescence thereby quenched. Thereby, tryptophan quench by CB3GA could used to measure the deacylation rate of ampicillin. The rates for the mutants were close to those of BOCILLIN FL in SDS-PAGE, with the half-life for K70A at 100 min (Figure 4). It is of interest to note that rates were found to be dependent on temperature. The ampicillin deacylation experiments were kept at 25^{o}C, the SDS-PAGE experiments were done at room temperature (~22^{o}C), so further studies are being conducted with temp variation.

An intense area of antibiotic research now has focused on β-lactam sensors which have a similar active site structure and mechanism to Class D proteins (Fisher, et.al., 2005). These membrane proteins (such as BlaR1) are responsible for resistance in some 'superbugs,' including methicillin-resistant *Staphylococcus aeurorus* (MRSA). BlaR1 uses an N^{ζ}-carboxylated lysine as a general base like in OXA enzymes. After acylation, however, the lysine decarboxylates and

results in acyl-intermediate which begins a signal transduction pathway which leads to the bacteria's resistance (Cha and Mobashery, 2007). In effect, the K70 mutants of OXA-1 show a similar function. Loss of the carboxyl group at that exact position results in severely reduced ability to hydrolyze acyl-intermediate. Indeed, even substitution of other carboxylates such as aspartate or glutamate still prevents deacylation, suggesting the N^{ζ}-carboxylated lysine in both OXA-1 and BlaR1 is uniquely positioned to carry out deacylation of the substrate.

The mutagenesis studies on OXA-1 are also the first to show the crucial importance of K70 on the deacylation mechanism of the enzyme in Class D. It has been long known that the general base of Class A enzymes is important for deacylation, but these are the first results suggesting a similar function in the emerging class of enzymes. We would like to extend this result to most of Class D, and indeed early findings in the carbapenemase OXA-24 indicate a similar result (data not shown), yet we do not expect this to be a universal amongst the class since a study on OXA-10 has found the lysine to alanine mutant is completely unable to acylate at all (Golemi, et al. 2001), further demonstrating the intense diversity of β-lactamases even within the same class. This research adds to the depth of knowledge on these increasingly prevalent and challenging modes of antibiotic defense. Further studies on OXA class proteins and this unique lysine carboxylation will hopefully produce a clearer picture of mechanism and function of each active site residue and will lead to intelligent antibiotic or inhibitor drug design to circumvent these dangers.

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Table 1: Michaelis-Menton Kinetics

	Nitrocefin			Ampicillin		
Protein	$k_{cat,}$ sec^{-1}	$K_{m,}$ µM	$k_{cat}/K_{m,}$ μM^{-1} sec^{-1}	$k_{cat,}$ sec^{-1}	$K_{m,}$ µM	$k_{cat}/K_{m,}$ μM^{-1} sec^{-1}
WT	110	1.9	61	520	21	25
S67G	7.4	41	0.18	NM	NM	NM
K70A	0.023	0.72	0.032	NM	NM	NM
K70E	0.02	1.2	0.016	NM	NM	NM
K70D	NM	NM	NM	NM	NM	NM

NM = not measurable

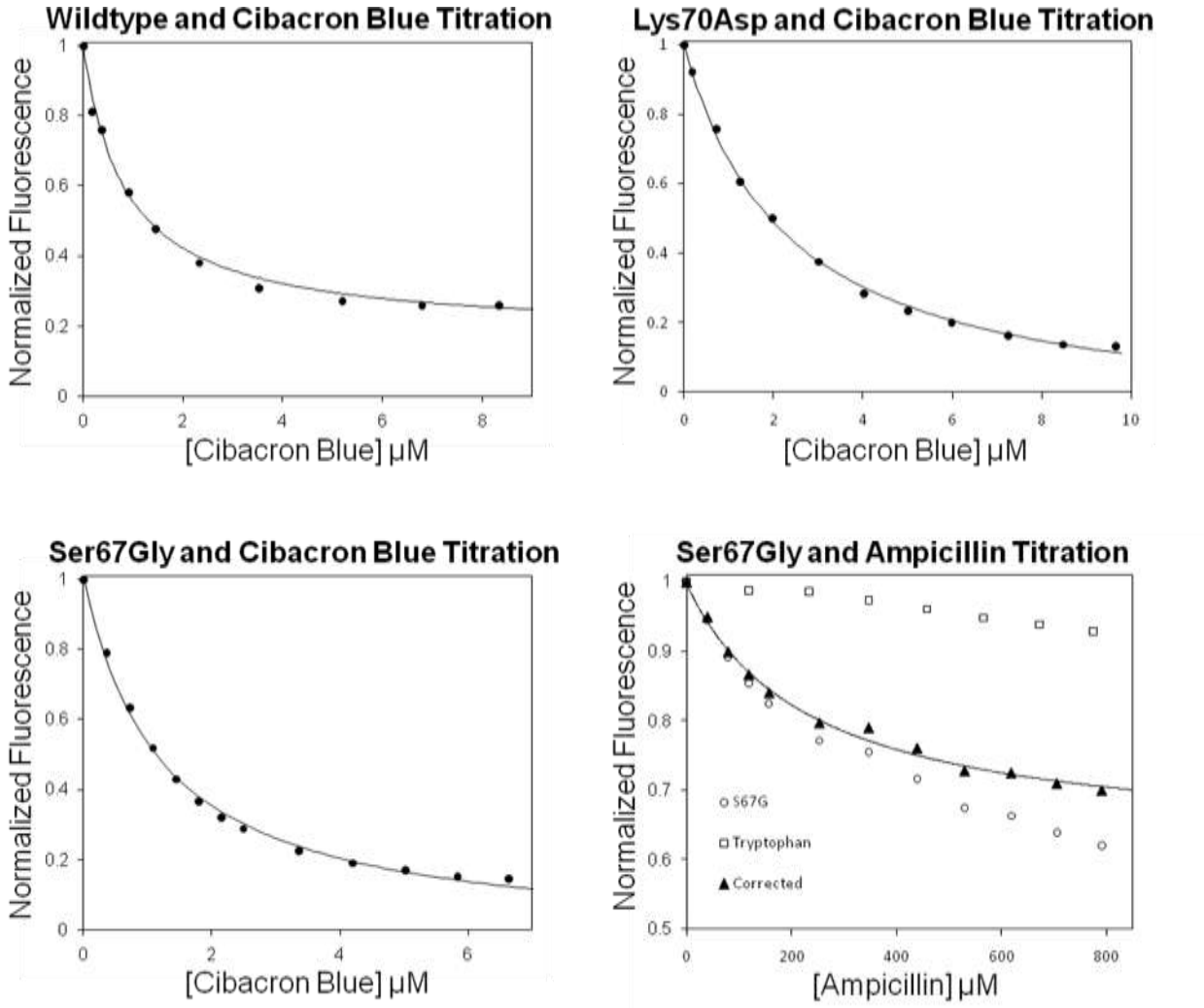

Figure 1. Active Site Titrations with Cibacron Blue and Ampicillin. Cibacron Blue was titrated onto wildtype and mutants (first 3 frames) and tryptophan quench was fit to an inverted hyperbola. Cibacron Blue K_d's were as follows: WT = 0.86 µM, S67G= 2.1 µM, K70A = 0.88 µM, K70E = 2.6 µM, K70D = 2.2 µM. Ampicillin was titrated with S67G and tryptophan (fourth frame). Corrected data was fit to an inverted hyperbola with a K_d of 235 µM.

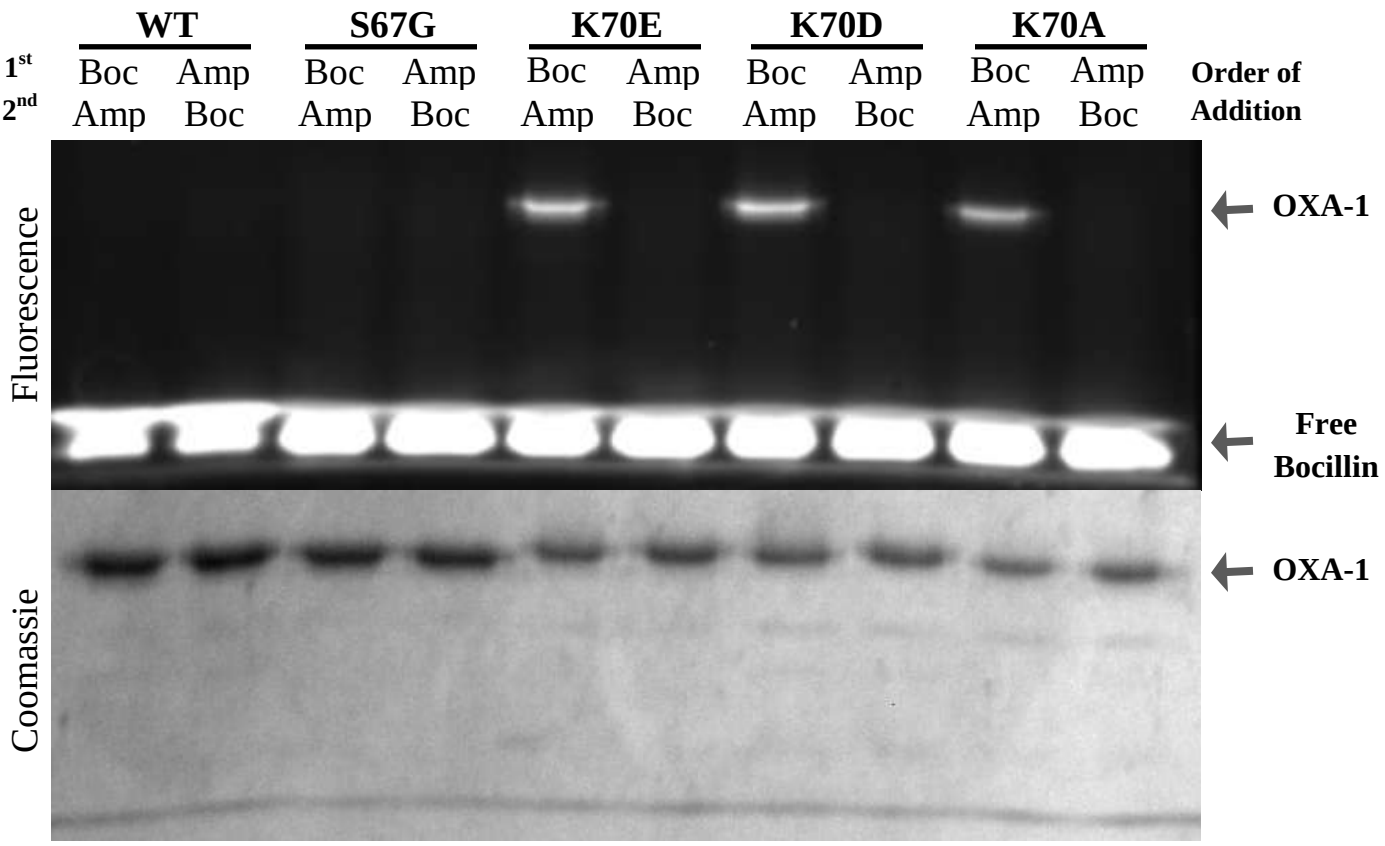

Figure 2. SDS-PAGE of OXA-1 Enzymes after Incubation with BOCILLIN FL. The OXA-1 enzymes wildtype, S67G, K70E, K70D, and K70A were incubated with BOCILLIN FL for 5 minutes and run on 10% SDS-PAGE. Excess ampicillin was added either before the 5 minute bocillin incubation (A) or after the BOCILLIN FL addition (B).

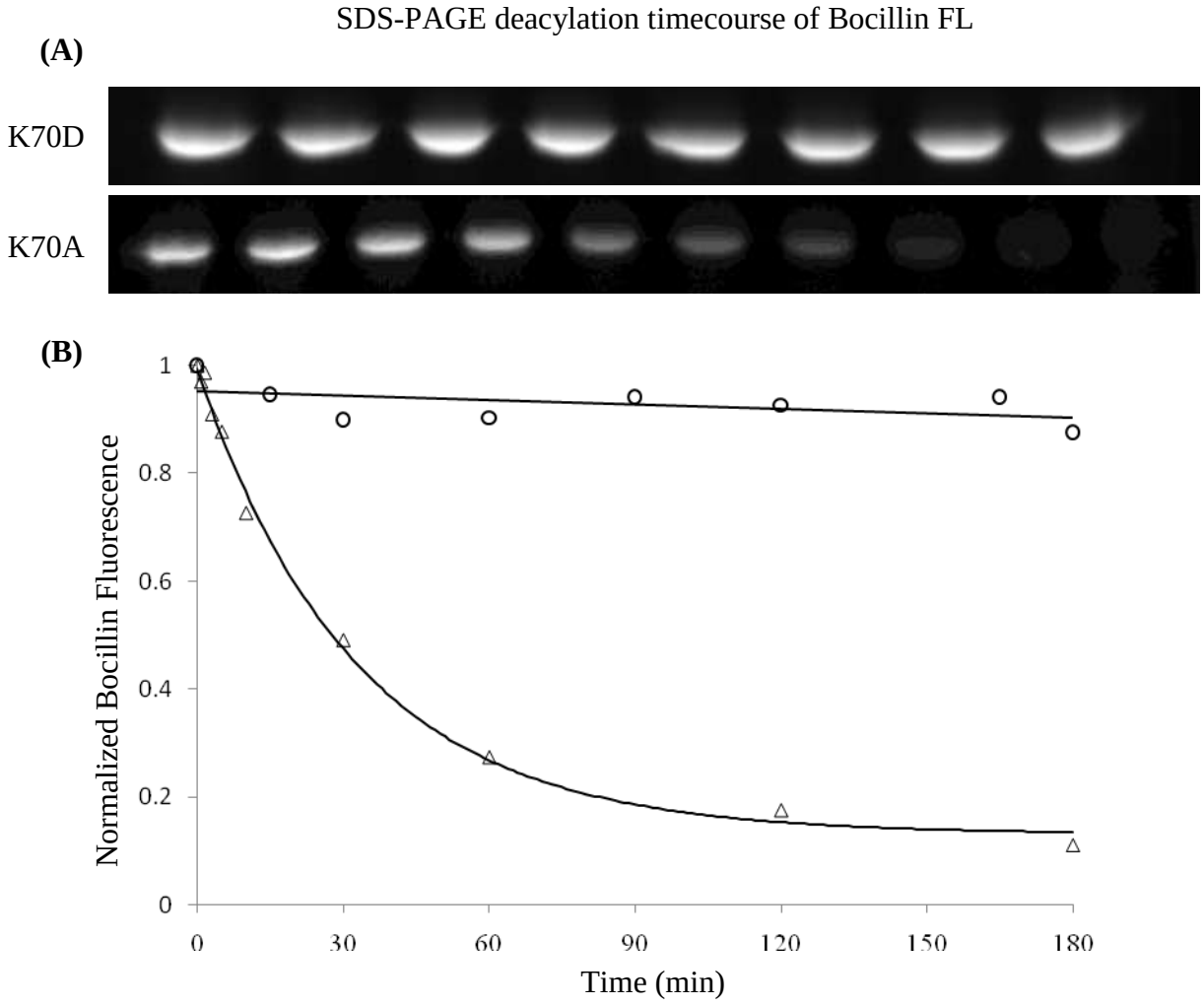

Figure 3. K70D vs. K70A Deacylation. Samples of K70D or K70A were incubated with 18-25 µM BOCILLIN FL for 5 minutes. Deacylation was initiated with 0.3 µg of WT to hydrolyze excess BOCILLIN FL. Aliquots were taken over time and quenched with SDS Buffer. BOCILLIN FL fluorescence was measure using 10% SDS-PAGE. Lys70Ala was fit to $y = a \cdot e^{-k \cdot x} + c$ where $k = k_3 = 0.017\ s^{-1}$ yielding an average half life ($t_{1/2}$) of 40 min.

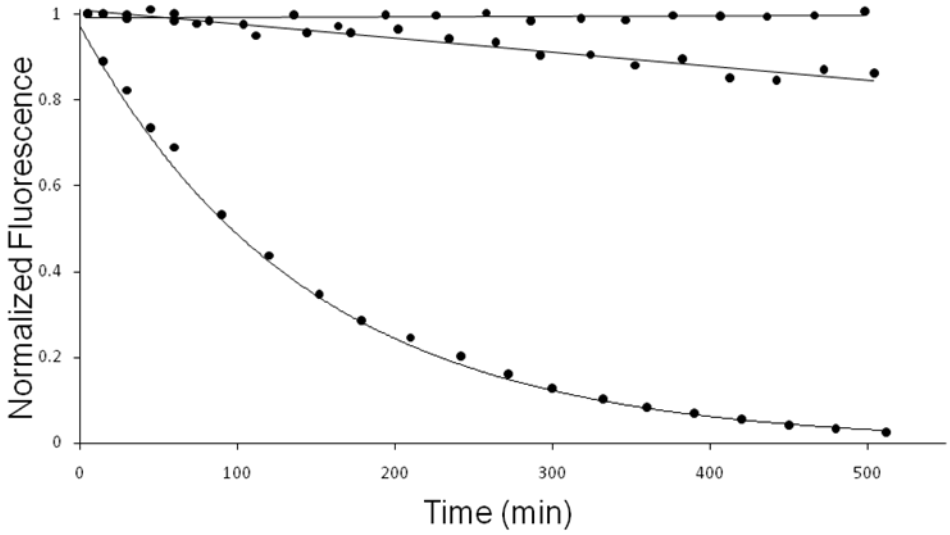

Figure 4. Lys70 Mutants Ampicillin Deacylation Rates by Tryptophan Fluorescence at 25°C. Mutant proteins (0.42 µM) were incubated with excess ampicillin (80 µM) for 5-10 min until full acylation. Cibacron Blue was added (3.7 µM) to quench fluorescence in any open active sites. A trace amount of wildtype (20 nM) was used to hydrolyze any excess ampicillin and initiate deacylation. Deacylation rate constants (k_3) were determined for Lys70Ala using the equation $y = a \cdot e^{-kx} + c$ where $k = k_3 = 0.0069\ s^{-1}$ yielding a half life ($t_{1/2}$) of 100 min.