Essentiality of conserved amino acid residues in β-lactamase

Chikunova, A.

Chapter 3
The N214-D233-D246 motif is essential for correct positioning of the active site residues in BlaC

Abstract

Evolutionary adaptability is restricted by essential amino acid residues, therefore their number in proteins is minimized. Highly conserved residues around active site of enzymes are believed to play an essential role in the precise positioning of the active site residues. Here, we examined a triad of the second-shell residues of the β-lactamase BlaC, Asn214-Asp233-Asp246. Introducing substitutions in these residues allowed to probe their involvement in activity and stability of the protein. It is shown that all variants affect the activity of the enzyme. NMR data suggests the presence of two conformations that are differently populated, depending on the mutation. We propose that the triad is responsible for the positioning of active site residue Thr216 and Arg220 and that substitutions in this motif increase dynamics and lead to displacements of these substrate-binding residues.

Introduction

Enzymes consist of a three-dimensional frame of secondary structure elements to arrange the positions of catalytic amino acid residues very precisely in the active site. Enzymes achieve this with a relatively small number of essential amino acids, residues that cannot be replaced without a detrimental effect on protein. The conservation of a residue among orthologous proteins is often used as a proxy for essentiality[34–36,108]. High conservation is generally observed for amino acids in the active site, because they are crucial for ligand binding and catalysis. Some residues in the layer around an active site (the second shell) are also conserved (see chapter 2).

The work described in this chapter focuses on a triad of highly conserved second-shell residues, Asn214, Asp233 and Asp246, which we believe play an important role in the function of β-lactamases. The active site of class A β-lactamases contains essential amino acids Ser70[168], Lys73 and Glu166[115,169] (Figure 3.1). Ser70 acts as nucleophile in the acylation reaction and Lys73 and Glu166 act as proton donor/acceptors during the reaction. Also the motifs S130-D131-(N/G)132[125] and K234-(T/S)235-G236[118,119] are conserved because of their role in regulation of the hydrolysis of β-lactam antibiotics (Figure 3.1a). Furthermore, several other residues are involved in making interactions with substrates.

Asn214 is a residue with a conservation of 93% among 494 class A β-lactamases (Chapter 2). It is found in a small loop between two α-helices (Figure 3.1b). In β-lactamase BlaC from *Mycobacterium tuberculosis*, this residue makes a hydrogen bond with another conserved residue, Asp233, as well as with Thr216, which is involved in substrate binding. Asn214 also interacts with an active site residue Lys234 via a water molecule. The homologous protein TEM-1, originating from *Escherichia coli*, has an Asp in position 214 instead. It was shown for TEM-1 that the interaction between Asp214 and Asp233 is important for a stability of the enzyme, while the water bridged by residues Asp214, Lys234 and Thr235 plays a role in activity[170]. In PER β-lactamases residue 214 is a threonine[171,172].

Residue Asp233 is 97% conserved and is located in a β-sheet[173] (Figure 3.1c). Next to the H-bond with Asn214, it forms an H-bond with the conserved residue Asp246. In TEM-1, Asp233 makes a salt bridge with Arg222, contributing to protein stability[170]. In some class A β-lactamases glutamate (GES β-lactamases[174–178]) and histidine (CepA from *Bacteroides fragilis*[179], DES-1 from *Desulfovibrio desulfuricans*[180] and PER β-lactamases[171,172]) are found as natural variants at position 233.

Residue Asp246 is 96% conserved. It is part of the same β-sheet as Asp233, located in the neighboring strand (Figure 3.1c). These two residues are known to have a carboxyl-

carboxylate interaction[181,182] forming a low-barrier H-bond that was hypothesized to play a role in protein stability[183]. Furthermore, Asp246 interacts with the amide nitrogen of Ile221.

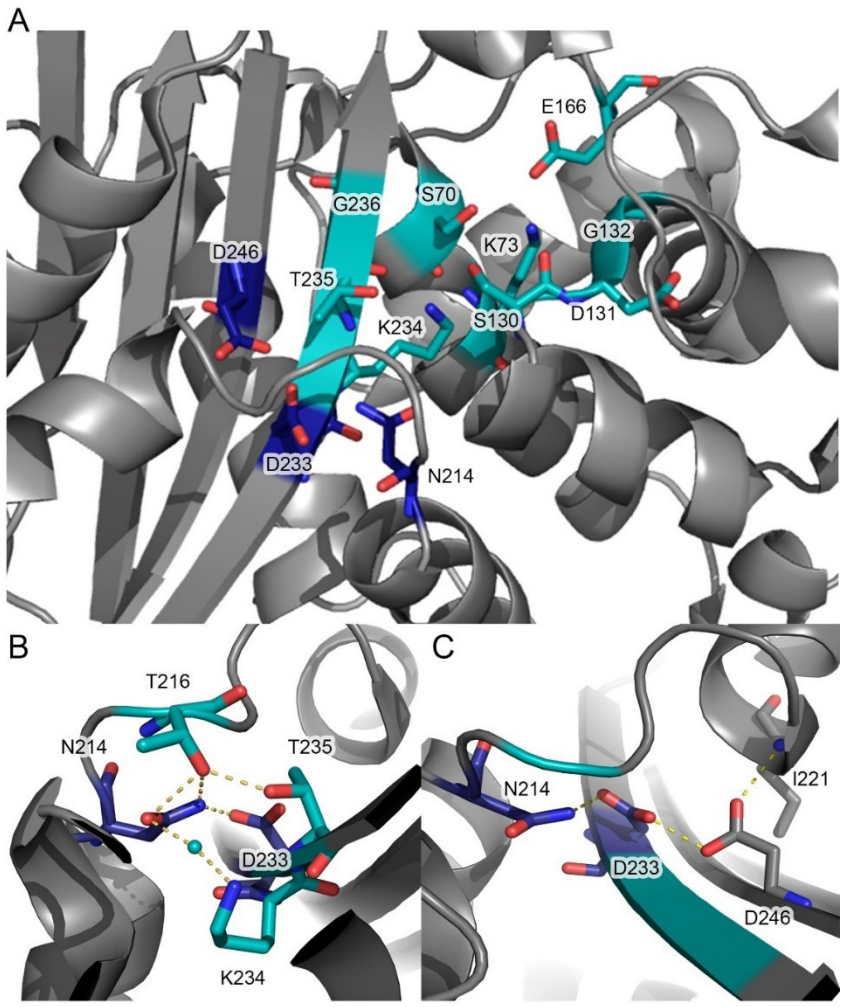

Figure 3.1. (A) The active site residues (in cyan) and the Asn214-Asp233-Asp246 triad (in dark blue) in BlaC (PDB entry 2GDN[101]); (B) Interactions made by the side chain of Asn214 shown in yellow dashed lines; (C) Interactions made by Asp233 and Asp246 shown in yellow dashed lines.

The residues Asn214, Asp233 and Asp246 form a triad of residues interacting *via* a chain of hydrogen bonds in the protein core (Figure 3.1c). Given the high conservation of these interactions we wondered what the function of the triad is. As this motif is found just outside the active site, it is hypothesized that it is crucial for enzyme activity. This study aimed to investigate the structural and functional role of this conserved motif by mutagenesis and characterization of the variants *in vivo* and *in vitro*. The contribution of the triad to the precise positioning of important residues is discussed, and it is argued that in enzymes in which the triad is not conserved, such as TEM-1, adaptations are made to ensure the positioning of the catalytic residues in another way.

Results

In order to understand the roles of interactions between the conserved residues Asn214, Asp233 and Asp246, single point mutations were introduced to these residues. Substitutions were chosen to sample various scenarios, in which interactions are modified due to modified length or polarity of the side chain or abolished entirely.

Wild type BlaC and variants were first tested for their activity against ampicillin and carbenicillin in cells by spotting cell cultures as drops of different dilutions on agar plates containing various concentrations of antibiotics. BlaC mutant S70A, which produces intact enzyme that is catalytically dead, was used as a negative control. Cell cultures producing BlaC mutants showed different degrees of activity loss (Figure 3.2, Figure 3.3a, Figure S3.1). BlaC D233N, N214A and N214S show activity comparable to the wild type BlaC, whereas BlaC N214Q and D233A/E/H exhibited a 5-to-10-fold reduction in activity. None of the D246 mutants was able to hydrolyze these antibiotics, and neither were BlaC variants N214D, N214L and D233L.

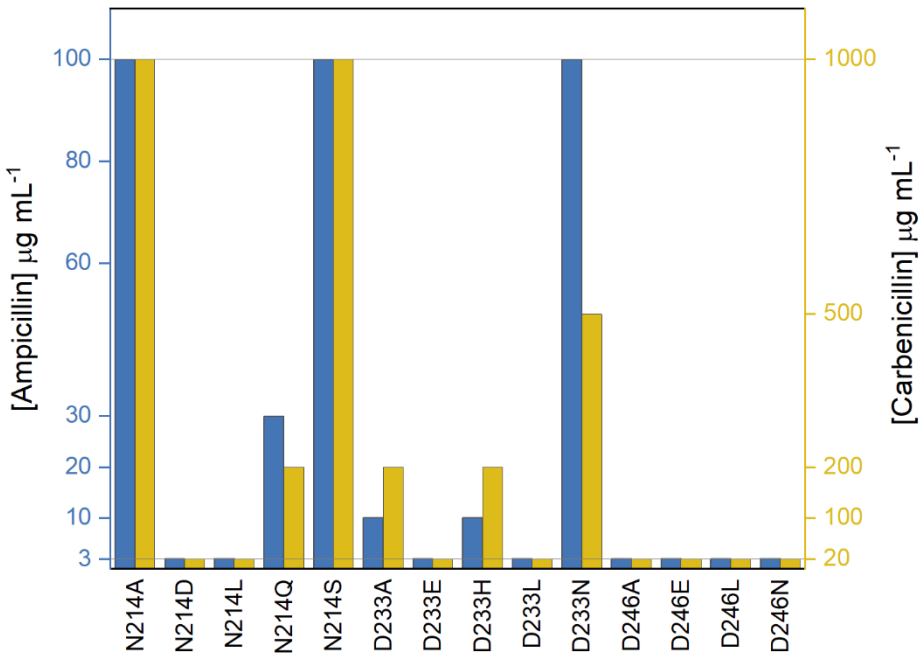

Figure 3.2. MICs of ampicillin and carbenicillin for BlaC variants. The horizontal lines represent MIC of wild type BlaC and BlaC S70A used as a negative control.

All BlaC variants were recombinantly produced in *E. coli* and purified, except for BlaC D233H that did not yield sufficient soluble protein to proceed with *in vitro* characterization. The degree of folding was determined by CD spectroscopy. Most mutants are folded and have a secondary structure composition comparable to the wild type protein. BlaC D233E, D233L and N214L are partially unfolded (Figure 3.3b) and these variants were not included in further *in vitro* studies. The thermal stability of BlaC variants was analyzed with a thermal shift assay using hydrophobic fluorescent dye SYPRO Orange[184] (Figure 3.3c, Table 3.1). All N214 mutants displayed a melting temperature similar to that of wild type BlaC. In contrast, BlaC D233A and all D246 mutants showed a decrease in melting temperature, up to 8 degrees.

Table 3.1. Kinetic parameters for the hydrolysis of nitrocefin and melting temperatures of wild type BlaC and BlaC variants. SD – standard deviation of triple measurements. NA – no activity detectable.

	Nitrocefin activity			Thermal stability	
	k_{cat} (SD) (s^{-1})	K_M (SD) (µM)	k_{cat}/K_M (SD[a]) ($x10^5\ M^{-1}s^{-1}$)	T_{melt} [b] (˚C)	ΔT_{melt} [c] (˚C)
Wild type	95 (3)	296 (18)	3.2 (0.2)	52	-
N214A	60 (8)	177 (49)	3.4 (1.0)	52	0
N214D	NA	NA		53	1
N214Q	19 (1)	47 (10)	4.0 (0.9)	50	-2
N214S	37 (4)	138 (38)	2.7 (0.8)	53	1
D233A	58 (2)	263 (17)	2.2 (0.2)	47	-5
D233N	24 (2)	102 (21)	2.3 (0.5)	52	0
D246A	8 (1)	134 (16)	0.6 (0.1)	47	-5
D246E	NA	NA		44	-8
D246L	NA	NA		46	-6
D246N	25 (4)	145 (30)	1.7 (0.5)	44	-8

[a] Propagated standard deviation; [b] Error 0.5 ˚C; [c] Error 0.7 ˚C

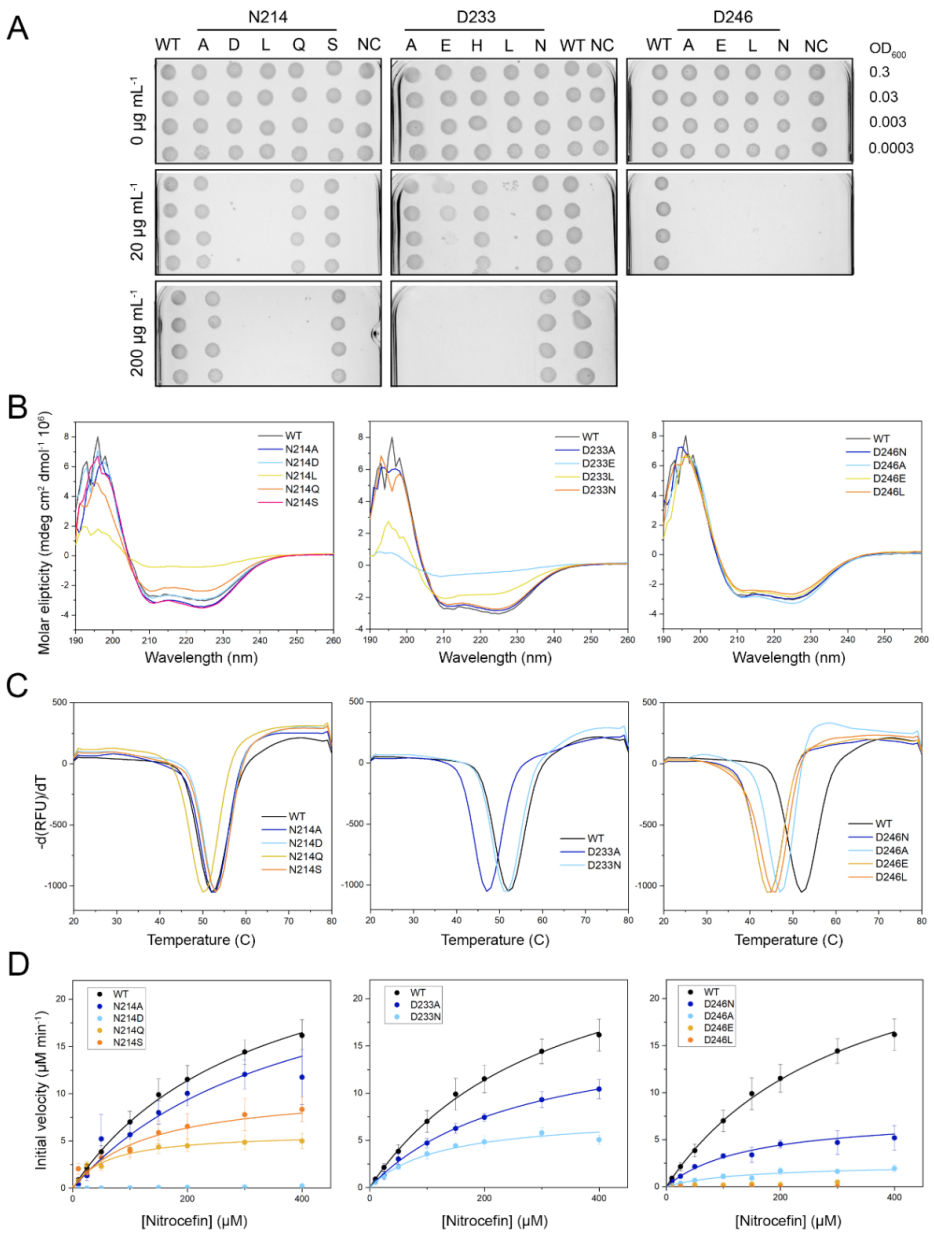

Figure 3.3. (A) Plates showing growth at 37 ˚C of *E. coli* cells expressing wild type (WT) or mutant *blaC* genes (negative control, NC is the mutant S70A) with no antibiotics, 20 or 200 µg mL^{-1} of carbenicillin (MIC for wild type BlaC is 1000 µg mL^{-1}); (B) Circular dichroism profiles of wild type BlaC and mutants of Asn214, Asp233 and Asp246. D233L, D233E and N214L are partially unfolded; (C) Normalized negative derivatives of thermal shift assay (SYPRO orange binding). The minimum in the curve represents melting temperature; (D) Michaelis-Menten kinetic curves of reaction with nitrocefin. The lines represent the fits to Michaelis-Menten equation. Error bars represent the standard deviations of triple measurements.

The catalytic efficiency of wild type BlaC and its variants was determined using the chromogenic substrate nitrocefin (Figure 3.3d, Table 3.1). The kinetic parameters demonstrate that all mutants suffer from activity loss to some extent. BlaC N214A and D233A exhibit a turn-over rate constant, k_{cat}, that is 35% lower than that of the wild type enzyme, while BlaC N214Q, N214S, D246N and D233N display a reduction of 60-83%. No activity could be measured for two Asp246 mutants and BlaC N214D. The specificity constant k_{cat}/K_M was reduced up to about 2-fold for the mutants showing activity. Only for BlaC D246A, the reduction was 6-fold.

Interestingly, the mutations modifying the length of the side chain cause a larger effect on enzyme than the mutations that completely remove the functional group of the side chain. For Asn214, the mutations to Ala and Ser were less damaging than the mutation to Gln, as both BlaC N214A and BlaC N214S were shown to be more thermostable and more active *in vivo* than BlaC N214Q. The same effect can be noted for the Asp246, as BlaC D246A is more active and stable than BlaC D246E.

To probe the effects of the mutations on the structure of the protein, TROSY-HSQC spectra were recorded with ^{15}N labeled mutants. All mutants show changes in chemical environment of the backbone amides not only surrounding the mutation site but throughout the whole protein with prominent changes around catalytically important residues (Figure S3.2). For some amide groups in Asn214 and Asp233 mutants two resonances were present, indicating that there is a second state present for these mutants (Figure 3.4). An interesting observation was made for Asp246 mutants. The CSPs of some amide hydrogen and nitrogen resonances increased approximately with the same ratio for the different mutants, placing peaks on a vector, in the order WT-D246N-D246A-D246E-D246L (Figure 3.5a). The activity loss of Asp246 mutants followed a similar trend but with different magnitude (Figure 3.5b). These findings suggest the existence of a second state, which is most likely catalytically inactive, and which is populated by the mutants to a different degree. The linear trend was visible for many residues throughout the structure (Figure 3.5c). For many other amides, mostly localized around the active site and right above the mutation site, peaks could not be assigned

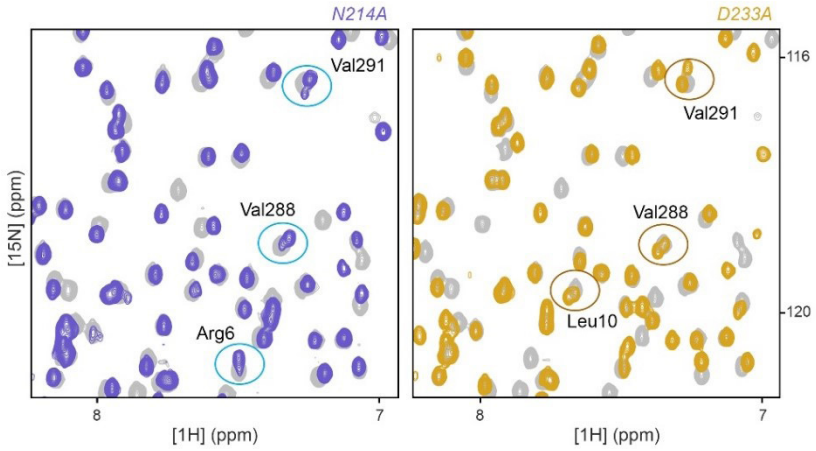

Figure 3.4. A few examples of double peaks found in NMR spectra of BlaC N214A/S (left) and BlaC D233A/N (right). Spectra of wild type BlaC are in grey.

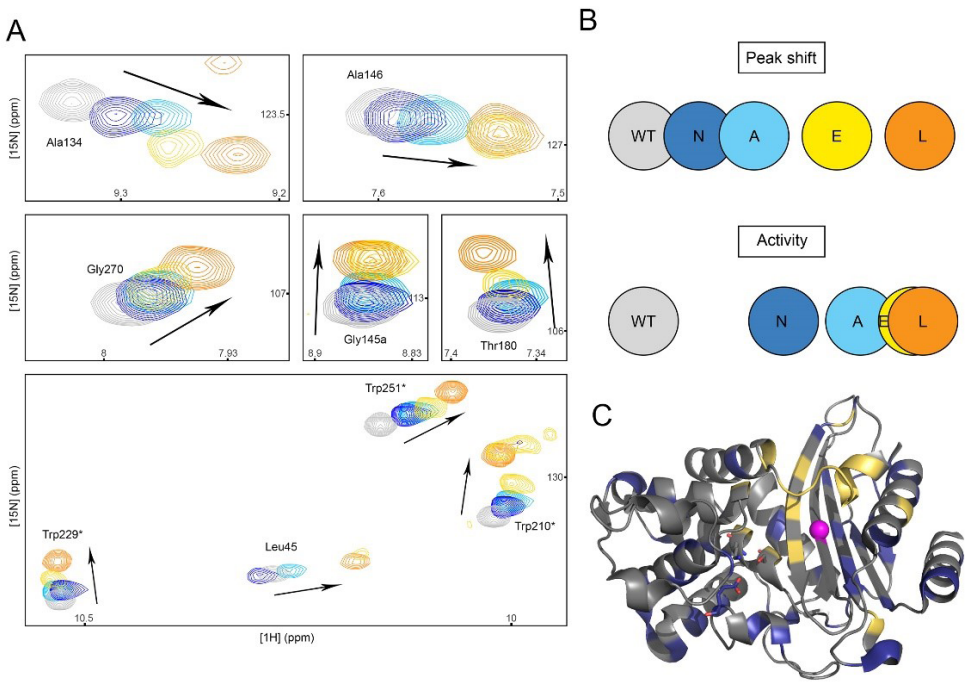

Figure 3.5. (A) Examples of the dependence of the peak shift from mutation in Asp246; (B) Schematic representation of the effect of the mutations in Asp246 on NMR spectrum and activity (k_{cat}/K_M); (C) Residues for which peaks are following the trend from panel A are shown in blue, residues for which peaks could not be assigned for all variants are shown in yellow. Cα of Asp246 is shown in magenta sphere. Active site residues are shown in sticks.

Discussion

The triad Asn214-Asp233-Asp246 is conserved in most class A β-lactamases. Our data indicate that this motif plays crucial role in the catalytic activity of the protein. Many mutations lead to activity *in vivo* and *in vitro*. All Asp214 mutants displayed little or no change in melting stability and secondary structure, while Asp246 and Asp233 mutants were more affected. That is not surprising, giving that Asn214 is located on the surface of the protein and Asp233 and Asp246 are buried. However, that means that the H-bond between residues 214 and 233 that was shown to be essential for protein stability in TEM[170], is not essential in BlaC, as N214A is not able to make that interaction and N214S is unlikely to, due to the expected increased distance between the Ser214 and Asp233 side chains. Most likely, residue Asp233 contributes to enzyme stability by maintaining the interactions with the loop on top of it. The thermal stability data indicated that the length of the residue at position 233 is of great importance, because only the mutation to Asn did not affect the melting temperature. At the same time mutation to Glu, yielding a side chain that is only one carbon atom longer, has a dramatic effect on stability. Mutation to Ala decreased melting temperature, also indicating a less stable tertiary structure. Thus, even though conservation of interactions is preferable, the complete removal of the side chain is less detrimental than its elongation. The same trend was observed for the other residues. For Asp246 all mutants showed stability and activity loss, however mutation to Glu resulted in the least stable protein, as judged by NMR spectroscopy and thermal shift assay. Out of all soluble Asn214 mutants BlaC N214Q displayed the lowest melting temperature, however, catalytically it performed better than BlaC N214D. This observation indicates that the exact nature of the side chain is vital for residue 214, but length and charge are important for different reasons.

We believe that the Asn214-Asp233-Asp246 motif is responsible for two features of BlaC. First, Asp246 in BlaC and many other β-lactamases makes a hydrogen bond with the backbone amide of Ile221. Ile221 is located in a small helix that is placed directly above the β-sheet domain. Another variation of this motif that is found in TEM and SHV β-lactamases is Asp214-Asp233-Ile246. As can be seen in Figure 3.6a the α-helix in structures with Ile246 is placed at least 1.9 Å further away than this helix in structures with Asp246, due to lost 246-221 connection. The same helix shift can be observed in a β-lactamase from *Vibrio*, which also carries Ile246 naturally. This small helix in BlaC contains a residue important for substrate/inhibitor binding – Arg220[185]. In TEM and SHV Arg244 plays this role. The guanidine group of Arg244 in TEM or SHV occupies the same space as Arg220 in BlaC (Figure 3.6b). It was shown for TEM that mutations in Arg244 result in a lower affinity for inhibitors and thus a higher resistance. Also penicillin hydrolysis is compromized[186]. Exactly the same effect was shown for Arg220 in PER-2[187]. It has been proposed for both Arg244 and Arg220 that they make interactions with the substrate[80,188,189]. That suggests that for TEM,

the precise positioning of the small α-helix is not as crucial, because residue 244, not 220 is involved in substrate binding, thus conservation of an aspartic acid in position 246 is not required for TEM and SHV β-lactamases.

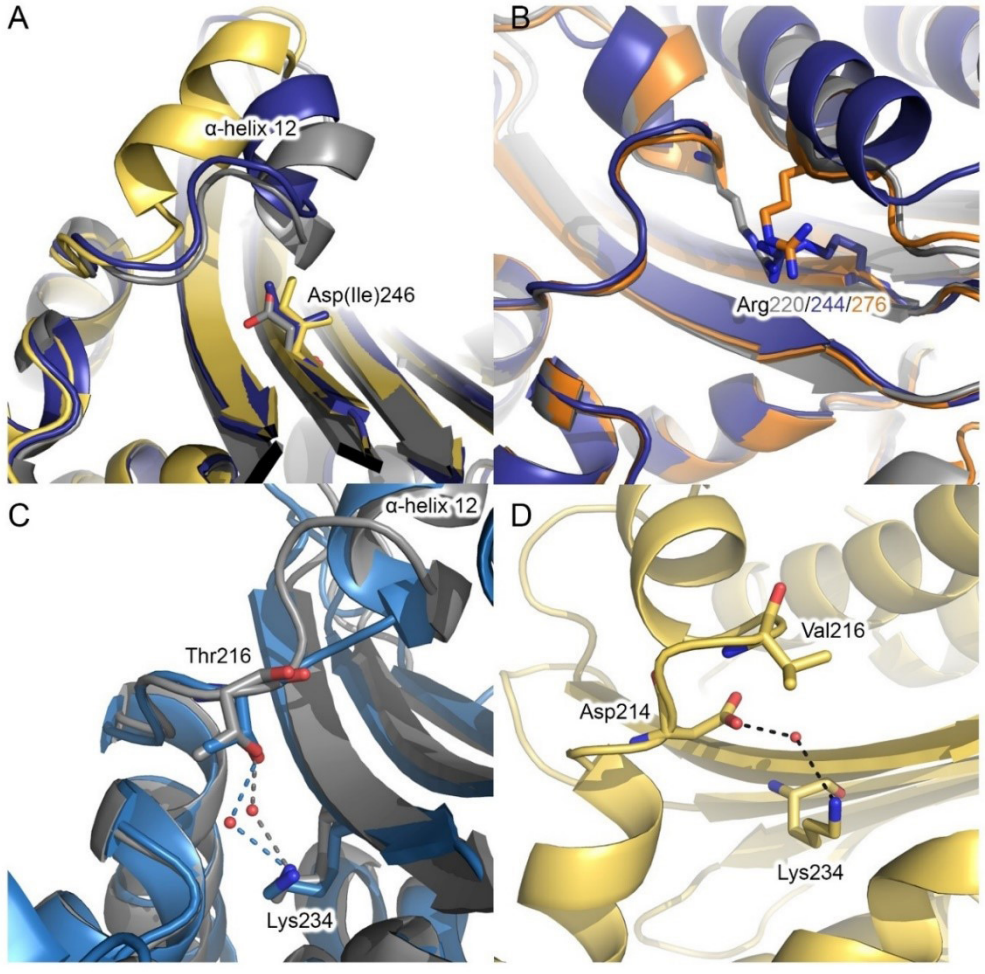

Figure 3.6. (A) Structural alignment of BlaC in grey (2GDN[101]), SHV in yellow (2ZD8[190]) and TEM in blue (1BTL[191]). Residues at position 246 are shown in sticks, label represents residues in BlaC with residues in TEM and SHV in brackets; (B) Guanidino group of Arg220 in BlaC, Arg244 in TEM and Arg276 in Toho-1 (in orange, 1IYS[192]) occupy the same space; (C) Position of Thr216 in BlaC and β-lactamase from *Mycobacterium fortuitum* (in light-blue, 2CC1[193]). Despite different length of the linker to α-helix 12, Thr216 occupies the same place, allowing it to interact with the conserved water (shown as spheres); (D) Structure of SHV. Asp214 and Val216 are present instead of Asn214 and Thr216 but the water position is still conserved.

The second feature that we believe is ensured by Asn214-Asp233-Asp246 motif is "locking" Thr216 residue in place. Residue Thr216 in BlaC and many other β-lactamases is involved in a chain of H-bonds involving Thr216-Asn214-water-Lys234. Lys234 is part of the active site KTG motif (Figure 3.1b). Most likely, the precise position of Thr216 is influenced by both the H-bonds between Asn214 and Asp233 and between Asp246 and Ile221, discussed above. The position of Thr216 is very conserved (94%) among many class A β-lactamases. Even in case of β-lactamase from *Mycobacterium fortuitum*, which has a shorter linker between α-helices 11 and 12 α-helix 12 is mounted on top of the β-sheet in the same fashion as in BlaC and Thr216 is locked in place, allowing it to interact with Lys234 via a water molecule (Figure 3.6c). A few β-lactamases, however, do not carry threonine at position 216. Interestingly, these enzymes have Asp rather than Asn at position 214 (Figure 3.6d). It seems that water bridging in such structures can be achieved solely by Asp214. However, as shown in this study, in BlaC mutation N214D results in completely inactive protein. We believe that this effect appears because of repulsion between Asp214 and Asp233, shifting Thr216 away from its natural site. The repulsion effect was demonstrated before for TEM, in MD simulations performed for a fully deprotonated Asp214-Asp233 pair[170].

Thus, in summary, the motif Asn214-Asp233-Asp246 ensures the correct positioning of Thr216 and Arg220, which are important for catalysis. The combination of this motif together with Thr216 is a conserved feature. Another variant of this motif is Asp214-Asp233-Ile246, which occurs less frequently and is coupled with no threonine at position 216 and no arginine at position 220.

Marciano and colleagues performed an extensive analysis of β-lactamase sequences in order to determine the evolution of the positive charge near an active site. They discussed the presence of arginine either at position 220, or 244, or 276 and named Arg220 as an ancestral position[189]. To link the position of arginine to the nature of residue at position 246, we built a phylogenetic tree using 310 class A β-lactamase sequences (Figure 3.7). The evaluation of the phylogenetic tree revealed that arginine at position 220 was initially coupled with the aspartate at the position 246. A switch to arginine 244 or arginine 276 appeared at some point independently in non-related branches. However, these sequences still carried Asp246 (Asn in some rare cases) as well as Thr216. The only exception was a relatively small group of sequences including TEM, SHV and β-lactamase from *V. parahaemolyticus*, which carry Arg244 and lost both Asp246 and Thr216.

Based on results presented in this research together with structural studies, we propose a model in which mutations in residue 246 lead to the loss of the connection between the β-sheet and α-helix 12. This lost connection introduces dynamics in which the helix is mobile, and activity is reduced, due to changed Thr216 and Arg220 positions. However, the protein

still populates the native state for a fraction of the time that differs for different mutants. This conformational freedom leads to chemical exchange in the NMR resonances of backbone amides, resulting in the linear pattern of the CSPs. To characterize the exchange, CPMG experiments were performed with BlaC D246A, however, no significant differences were observed with the wild type protein. Most likely, the exchange is occurring with a rate that is too fast to detect (k_{ex}> 1000 s^{-1}). The linear shift of the peaks upon mutation was observed before by multiple research groups. Jensen and colleagues reported linear correlation between the chemical shifts of the wild type and two variants of SH3 domain of JIP1[194]. They proved that the variants are in fast to intermediate exchange between two conformations governed by an aromatic ring flipping inside the protein core. Schütz, Rennella, and Kay showed a peak position of one of the p97 residues titrating as a function of mutation in a near linear fashion indicating a rapid exchange between two conformers[195]. A similar trend was observed in TEM β-lactamase Zimmerman et al., where resonances were found to display a linear shift pattern from the wild type protein and the least stable variants to the most stable variants[196]. The authors propose that chemical shifts close to the wild type represent nuclei in a more loosely packed interface and those closer to the stabilized variants represent nuclei in a more tightly packed interface. Although the packing of the structure and strength of interaction do not directly explain the shift of the amide resonances in a linear manner, it is possible that the effect observed by Zimmerman and colleagues also indicates the interconversion between different states. In this case, however, the wild type enzyme is the one that undergoes these changes.

It was shown for TEM[170] that the pair Asp214-Asp233 share a proton, but most of the time, Asp214 is protonated in this pair. Analogously, in BlaC, the pair Asp233-Asp246 needs to share a proton to avoid repulsion. Considering our results, we propose that predominantly Asp246 is protonated.

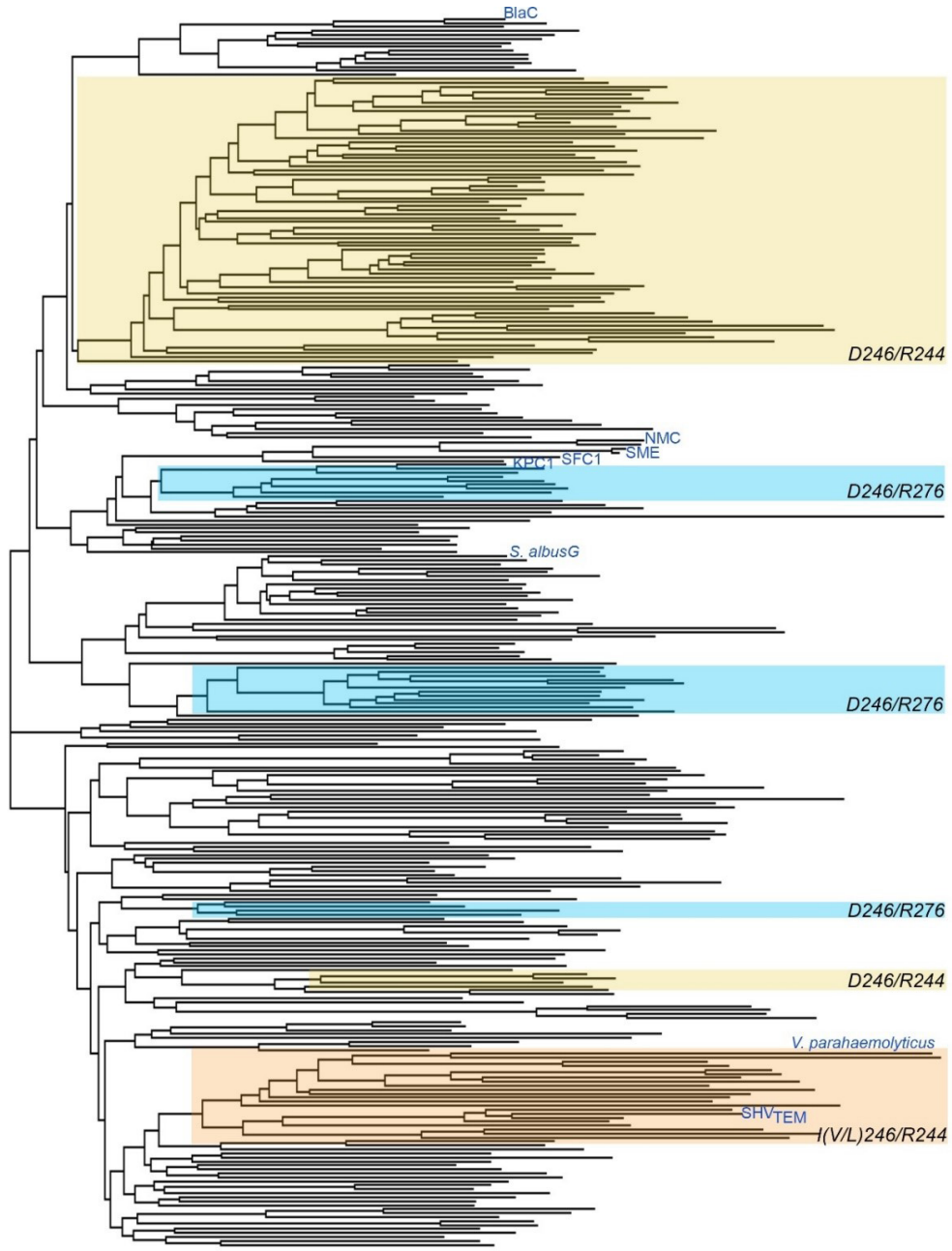

Figure 3.7. Phylogenetic tree of class A β-lactamases. A total of 310 sequences were used to compose a tree. Some well-known β-lactamases and structures discussed in the text are labeled. Blue highlights represent enzymes that carry Asp246 together with Arg276; yellow represents sequences with Asp246 and Arg244; orange color represents sequences with Ile(Val/Leu)246 and Arg244. All the rest carry Asp(Asn)246 and Arg220. Only sequences in orange do not carry Thr216.

Materials and Methods

Production of BlaC variants

Site-directed mutagenesis was performed using whole plasmid site-directed mutagenesis. The primer sequences can be found in Table S2.3. Wild type and mutant proteins were produced in *E. coli* BL21 (DE3) pLysS strain transformed with pET28a plasmids containing the *blaC* gene with an N-terminal His-tag and TEV cleavage site. Cells were cultured in LB medium at 250 rpm at 37 ˚C until the optical density at 600 nm reached 0.6, at which point protein production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by incubation of the cultures at 18 ˚C for 16 hours. For the production of isotope labeled proteins for NMR experiments, M9 medium was used with ^{15}N ammonium chloride as the sole nitrogen source for ^{15}N-labeled samples. Components of the M9 medium can be found in Table S2.4. Cells were harvested by centrifugation and lysed with French Press in 20 mM Tris-HCl buffer, pH 7.5 with 500 mM NaCl. The first purification was conducted with 5 mL HisTrap Nickel column (GE Healthcare) in 20 mM Tris-HCl buffer, pH 7.5 with 500 mM NaCl. Protein was eluted with the same buffer with 125 mM imidazole. Proteins carried an N-terminal His-tag that was removed by overnight incubation with 0.2 mg mL^{-1} His-tagged Tobacco Etch Virus (TEV) protease at 4 ˚C in 25 mM Tris-HCl buffer, pH 8.0 with 100 mM NaCl, 1 mM EDTA and 5 mM DTT. After cleavage the enzyme was purified further by another run over a HisTrap Nickel column.

In vivo activity studies

For *in vivo* experiments in *E. coli* cells proteins encoded on pUK21 were produced with an *E. coli* twin arginine transporter (Tat)-signal at the N-terminus, to ensure translocation to the periplasm, mimicking the location of the protein in *M. tuberculosis*[147]. The survival of the *E. coli* cells carrying pUK-based plasmids with wild type or mutant *blaC* genes was tested on LB-agar plates with various concentrations of ampicillin or carbenicillin. All plates contained 50 μg mL^{-1} kanamycin and 1 mM IPTG. Cells were applied on the plates as 10 μL drops with OD_{600} values of 0.3, 0.03, 0.003 or 0.0003 and incubated for 16 h at 37 ˚C before photography.

Circular dichroism

CD profiles were recorded using Jasco J-815 spectropolarimeter with a Peltier temperature controller (Jasco, MD). Measurements were performed at 25 ˚C with 10 μM protein in 100 mM sodium phosphate buffer, pH 6.4. Spectra were acquired in 1 mm quartz cuvette at a scan rate of 50 nm/min.

Thermal stability

Thermal stability of the proteins was analyzed by thermal shift assay (TSA) with SYPRO Orange dye (Invitrogen). The measurements were performed in triplicate in two independent experiments using the CFX 96 Touch Real-Time PCR Detection System from Bio Rad with 2x dye and 10 μM protein in 100 mM sodium phosphate buffer, pH 6.4 with the temperature range 20-80 ˚C. Melting temperatures were determined as an average of 6 measurements.

Kinetics

Determination of the Michaelis-Menten kinetic constants was done by measuring the absorption change at 486 nm for nitrocefin ($\Delta\varepsilon$ = 11300 M^{-1}cm^{-1} [145]) in a PerkinElmer Lambda 800 UV-Vis spectrometer at 25 ˚C in 100 mM sodium phosphate buffer, pH 6.4. All measurements were performed in triplicate. The reactions were carried out at various concentrations of substrates and 5 nM of BlaC, and initial rates of the hydrolysis were plotted against concentration of substrate and fitted separately for each experimental set to the Michaelis-Menten equation 3.1 using OriginPro 9.1, where v_0 is the initial reaction rate, $[S]_0$ the initial substrate concentration, V_{max} the maximum reaction rate and K_M the Michaelis-Menten constant. v_0 and $[S]_0$ are the dependent and independent variables, respectively, and K_M and V_{max} are the fitted parameters. $V_{max} = k_{cat}[E]$, k_{cat} is the rate-limiting conversion rate, and $[E]$ is the concentration of BlaC.

$$v_0 = \frac{V_{\max}[S]_0}{[S]_0 + K_{\mathrm{m}}} \tag{3.1}$$

NMR spectroscopy

TROSY-HSQC spectra of all BlaC variants were recorded on a Bruker AVIII HD 850 MHz spectrometer at 25 ˚C in 100 mM phosphate buffer (pH 6.4) with 6% D_2O. Data were processed in Topspin 3.2 (Bruker). Spectra were analyzed with CCPNmr Analysis V3[167] software. Peaks of the mutant spectra were assigned by comparison to peaks in the wild type BlaC spectrum and average chemical shift perturbations (CSP), $\Delta\delta$, of the ^{1}H ($\Delta\omega_1$) and ^{15}N ($\Delta\omega_2$) resonances of backbone amides were calculated using equation 3.2. Peaks that could not be assigned with certainty were assigned based on the smallest possible CSP (Figure S3.2).

$$\Delta\delta = \sqrt{\frac{1}{2}\left(\Delta\omega_1^2 + \left(\frac{\Delta\omega_2}{5}\right)^2\right)} \tag{3.2}$$

Supplementary materials

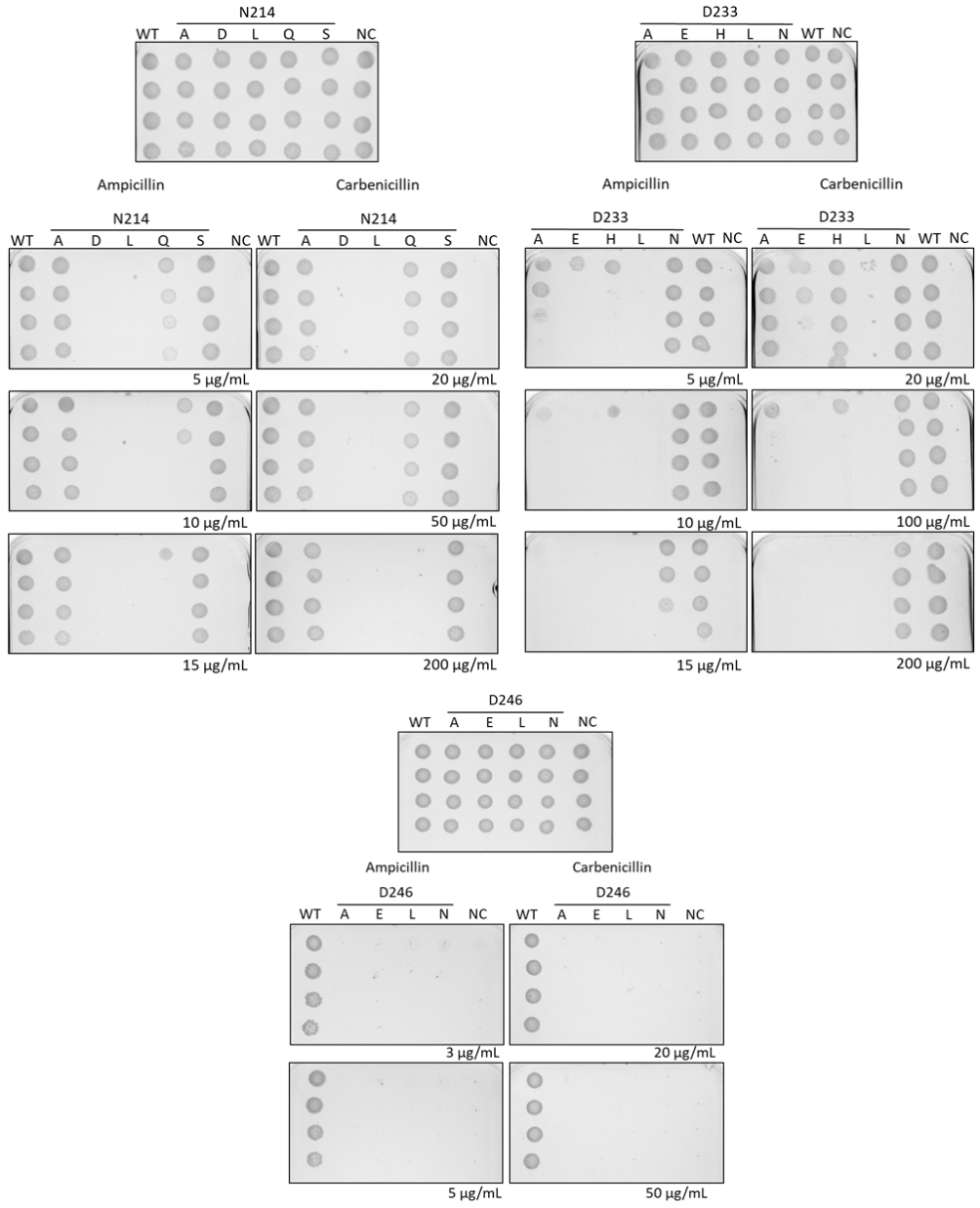

Figure S3.1. Plates showing growth at 37 °C of *E. coli* cells expressing wild type (WT) or mutant blaC (negative control, NC is the S70A mutant) with no antibiotics or various concentration of ampicillin and carbenicillin (MICs for wild type BlaC are 100 µg/mL and 1000 µg/mL respectively).

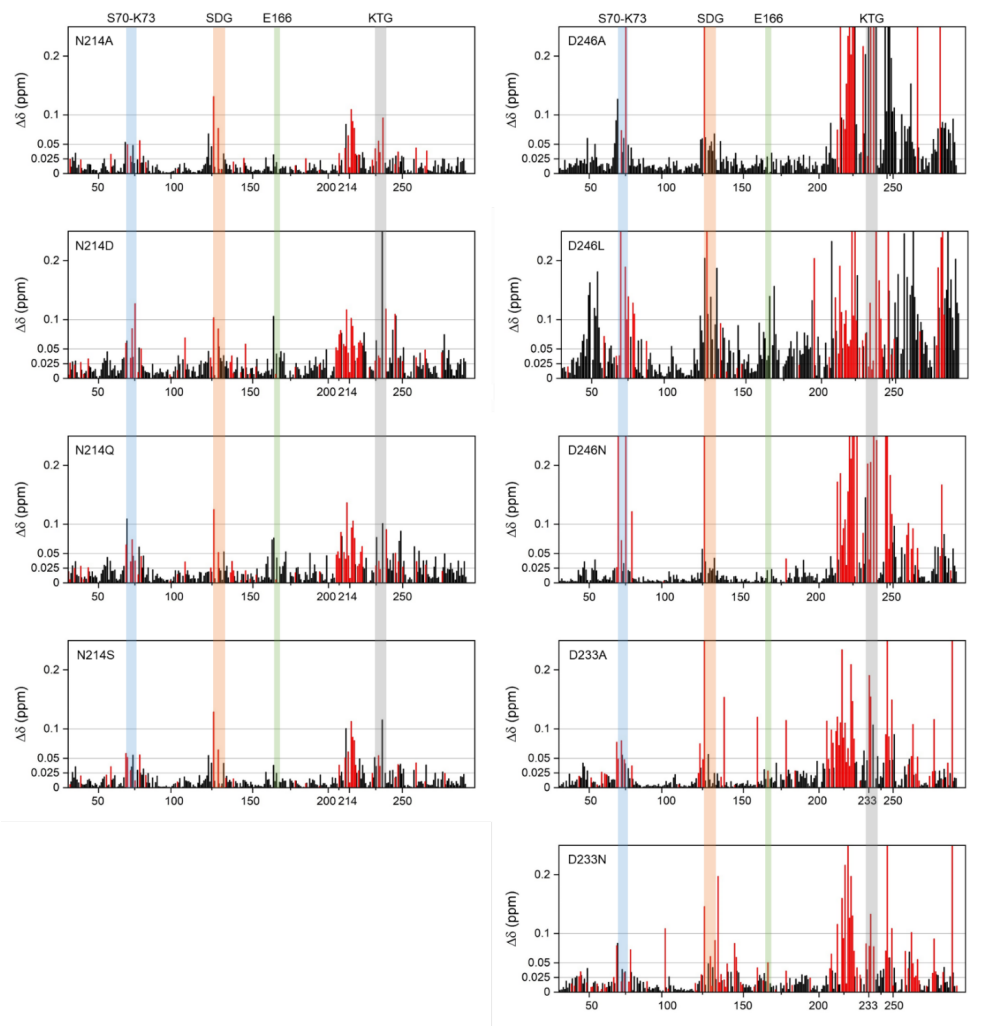

Figure S3.2. Average CSP for the backbone amides of the BlaC mutants. Residue numbers refer to Ambler numbering. Black bars represent CSP for residues assigned with certainty. Red bars show CSP calculated as the minimal possible CSP. Important catalytic residues and residues next to them are shown with colored highlights.