Essentiality of conserved amino acid residues in β-lactamase

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Chapter 7
General conclusions

Overview and Perspectives

Changing conditions cause changing evolutionary selection pressures that lead to adaptations in organismal phenotypes. The underlying biochemical processes and thus the involved proteins need to be able to adapt to the changed environment. Proteins evolve by acquiring semi-random amino acid substitutions.

β-Lactam antibiotics are the largest and one of the best researched group of antibiotics. Due to introduction of new compounds in the clinic and cattle breeding, bacteria experience an environment with changing selection pressures. Throughout history, β-lactamases, the enzymes that are responsible for the breakdown of β-lactam antibiotics, were able to evolve and hydrolyze novel β-lactam antibiotics. These enzymes are excellent systems to study protein evolution because of their adaptability, and, furthermore, the changes in phenotype can be easily detected by antibiotic screening. For those reasons β-lactamases, especially TEM-1, often has become a focus in evolutionary studies (e.g. [17,127,136,237,252]). Saturation mutagenesis was performed on TEM-1, yielding the fitness effect of substitutions of every amino acid residue to all other 19 amino acids[15,253]. These extensive works showed that robustness and evolvability of TEM-1 are dependent on the strength of purifying selective pressure[15], and that mutational effects on protein thermodynamic stability shape the distribution of fitness effects of mutations[253]. Another study on ~ 12,500 single amino acid mutants of TEM pointed out the importance of epistatic interactions in the enzyme, as they showed that the frequency of mutations exhibiting epistasis increases along the evolutionary pathway[254]. The saturation mutagenesis approach differs from the approach presented in this thesis, and it might be interesting to carry out a similar study on β-lactamase BlaC in order to compare the evolutionary pathways taken by these two β-lactamases from the same class.

Evolutionary adaptability is restricted by essential amino acid residues because their substitution will likely render the protein less functional. Thus, evolution will strive to minimize the number of such essential amino acids. It is interesting to know what the exact reason for essentiality is. Being an essential residue is often linked to conservation of residue type in a sequence family[34–36]. We set a goal to describe the roles of highly conserved residues in enzymes in relation to their position in a structure relative to the active site. Whereas catalytic residues, or the first-shell residues, are usually extensively studied and their functions known, non-catalytic residues are addressed less often. In Chapter 2, the roles of the highly conserved, non-active site residues of the BlaC were determined. β-Lactamases evolve rapidly under the constantly changing selection pressures of different classes of these compounds, yet 11% of the residues outside active site stayed unmodified throughout history. The reason for such conservation was investigated with an extensive mutational study.

We observe striking differences between the effect of mutation on second-shell residues (close to the active site) and third-shell residues (more distant from the active site). Second-shell residues are shown to influence the entire active site and mutations in the second-shell residues lead to a changes sensed throughout the entire core of the protein, which can be picked up by NMR spectroscopy as chemical shift perturbations. The spread of such changes far from the mutation site indicates a network of correlated interactions throughout the region around the active site. Second-shell residues are not significant for folding and production of mutants often yields folded but inactive enzyme. Thus, these residues are relevant for stabilizing the active conformation from within the ensemble of conformations that the enzyme can visit in its folded state. Some second-shell conserved residues are also shown to contribute to the overall thermal stability of the enzyme. Substitutions in those lead to a shift of the balance of folding and unfolding toward the latter, by destabilizing the folded form, as soluble, unfolded protein is found coexisting with the folded form.

An unexpected effect is shown for some second-shell residues for which non-conservative substitutions are preferable to conservative substitutions (Chapter 2 and Chapter 3). This observation supports the concept of a complex and extensive web of interactions inside the functional enzyme, especially around active site. Substitutions that influence the interactions of the side chain provoke changes in the whole web of interacting residues and cause a dramatic effect for the activity or stability of the protein. Substitutions that eliminate the interactions of the side chain affect the enzyme less. While the conserved second-shell residues are shown to play a role in ability of a protein to perform its function, substitutions in those residues sometimes occur and persist (Chapter 3 and Chapter 6). Such substitutions might change the substrate profile of the enzyme[101] and some, due to the extreme importance of the residue, require a number of changes in the neighboring residues. Chapter 3 gives the example of Asn214 of said co-evolution, as in multiple β-lactamases the change in Asn214 is accompanied by a change in another conserved residue, Asp246, and a few first-shell residues. Although it is difficult to conclude what the exact order of mutation events was in the evolutionary process, the pattern in co-evolution is obvious. The role of the highly conserved triad discussed in chapter 3 needs more examination. Unfortunately, our attempts to crystallize BlaC mutants of this triad were not successful. Nevertheless, if the model of the mobile loop and helix that we describe is accurate, it is still possible that those changes would not be picked up in the crystal structure. Perhaps a better way to validate this model could be with paramagnetic NMR[255–257], a tool allowing for the determination of the three-dimensional structure of proteins and conformational heterogeneity.

Third-shell residues are shown to affect folding vs. aggregation rates or the thermodynamic balance between folded and unfolded (Chapter 2). Most mutations result in a heavily reduced amount of soluble protein. The folded fraction of such third-shell variants is remarkably

similar to wild type enzyme in stability, structure and activity (Chapter 4). Some mutations cause a fraction of soluble protein to remain unfolded, and the folded protein of these mutants often exhibits reduced thermostability. Effects of mutations in conserved residues on production and stability are well-known and they were also demonstrated for other proteins (e.g. [40,150,152]).

In contrast to the second-shell variants, substitutions in third-shell residues lead to localized structural effects, as observed both with NMR spectroscopy and crystallography. As the third-shell residues are involved in the formation of the correct three-dimensional fold, many of them are localized near the edges of the secondary structure elements and have interactions with nearby conserved residues, "stapling" the secondary structure elements together. The exact nature of the side chain of such residues is critical for the interactions they need to fulfill and, therefore, conservative substitutions in the third-shell residues are shown to be more beneficial than non-conservative ones.

Residues that were the focus of this study are highly conserved in class A β-lactamases. Harmful effects of substitutions in such essential residues were expected, still a few mutations did not cause a severe negative response in the enzyme. Two BlaC variants even displayed improved characteristics (Chapter 5). It is not obvious why these (nearly) neutral or beneficial substitutions are not found in natural variants of β-lactamases. It is possible that some residues are conserved for their essential role in most class A β-lactamases, but not necessarily all, as even closely related enzymes can take different evolutionary pathways. In that way, residues that are essential in most β-lactamases but not in BlaC can be considered an evolutionary rudiment in BlaC. Admittedly, based on our study conclusions can be made only about the effect of substitution on a limited number of enzyme properties. Furthermore, the changes in enzyme *in vitro* cannot be directly translated into changes that will take place in a living organism exposed to a pathogen carrying mutated enzyme. Zebrafish embryos are commonly used to study *Mycobacterium tuberculosis* infection (e.g. [258–261]), as they provide an immune response similar to that in humans. Thus, the idea to test these improved BlaC variants in a zebrafish embryo seems intriguing and most of all achievable. Such experiments are being started in the Ubbink group, in collaboration with the Meijer group at Leiden University. Moreover, it is not rare, that no change or improvement of one trait comes at a cost of another trait (e.g. [82]). In Chapter 2, we discuss a possibility of evolvability being one of such traits not tested within the scope of this research. For β-lactamases high evolvability is a crucial property, so high evolvability itself may have been optimized by evolution[15]. Some residues might be highly conserved to allow residues around it to be variable. As mentioned in Chapter 1, there is no consensus yet about the idea of evolution of evolvability. To date, it is stated that stability promotes evolvability[24,25,33], however, it is not known yet if the presence of specific residues can be one of the features benefiting or hindering evolvability via the

mechanism of influencing the variability of surrounding residues. That is a highly speculative idea that nevertheless is certainly interesting to test. One way to do that can be to perform laboratory evolution with wild type BlaC and an improved BlaC variant as a starting point to examine if a new function can be easier evolved in a wild type BlaC.

The work presented here offers the reasons for amino acid conservation, and it is our believe that these principles are general and applicable to essential amino acid residues in other enzymes. According to our data, the possible effects of mutations in conserved amino acid residue can be linked to their position in the structure (Figure 7.1a). Conserved residues of the first shell form a catalytic center of a protein, and therefore, substitutions are likely to result in an inactive protein or protein with changed activity. Conserved residues of the second shell establish a functional core of the protein, where they form a web of interactions around the active site, fine-tuning the structure of the active site. Changes in these residues result mostly in impaired stability, or stable enzyme with altered active site. The third-shell residues ensure the overall fold of an enzyme. Substitutions in these residues often have a dramatic negative effect on production of folded protein. Another possible effect that can be attributed to the mutations in the distant residues is an allosteric effect. The distance between allosteric ligand-binding sites and active sites was shown to range from 20 Å in hemoglobin to 60 Å in glycogen phosphorylase[38,262]. These distances point to the third-shell residues being the ones involved in possible allostery. Activity modulation by a allosteric site was attributed to Trp229 in TEM-1 β-lactamase[131,132]. Although the scope of this research did not concern protein dynamics, the importance of enzyme dynamics on its activity was demonstrated by various studies[252,263,264]. A study by Elings *et al*. demonstrated how single substitutions in BlaC (in the first and the second-shell residues) can affect enzyme dynamics[83]. And a few conserved residues from the third-shell were hypothesized to be involved in dynamics in BlaC in Chapter 2 of this work, however that requires further testing.

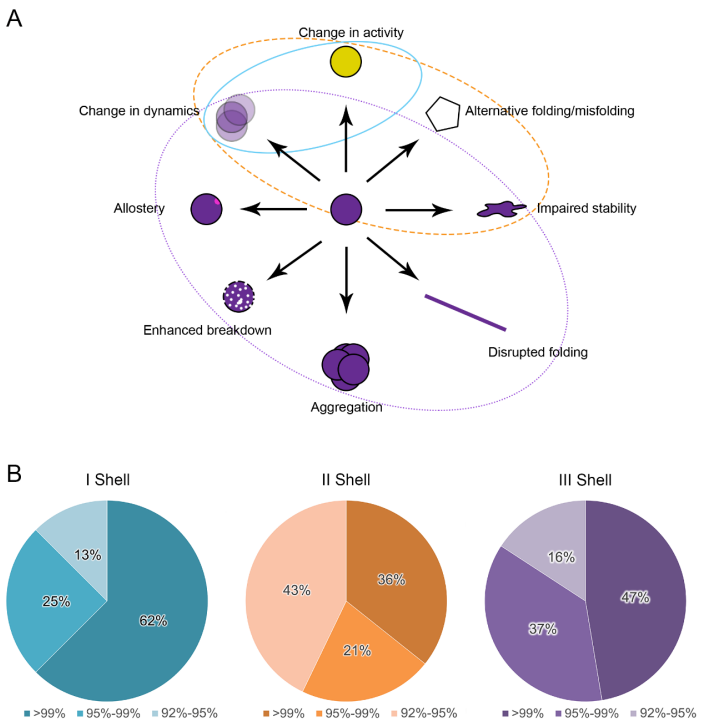

Figure 7.1. (A) Schematic representation of possible effects of mutation in a conserved residue. Circles show possible effects attributed to a mutation in the first (cyan), second (orange) and third (purple) shell; (B) Percentage of residues with conservation >99%, 95-99% and 92-95% in first (cyan), second (orange) and third (purple) shells.

Conserved residues of the second shell contribute to a high evolvability of the protein, as they protect the integrity of the active site, while random mutations can accumulate in and around the active site, possibly leading to a new trait. The conserved second-shell residues can in some instances mutate as well, leading to rearrangements in the binding site. One of the examples of such successful substitution is Gly132 in BlaC, which is believed to contribute to the broad antibiotic spectrum of this enzyme[101], while most β-lactamases of class A carry Asn132. Conserved residues of the third-shell are frequently found at the edges of the secondary structure elements. In that way they contribute to a high evolutionary robustness of the protein, as they ensure the correct position of the secondary structure elements in three-dimensional space, allowing for substitutions to occur inside the secondary structure elements. These principles are also reflected in the conservation patterns of the residues of the different shells (Figure 7.1b). The conserved first-shell residues are mostly invariable with less conserved residues being the substrate-binding residues. While they are still highly conserved a certain degree of variability is likely responsible for adaptation of some

β-lactamases to specific substrates. The conservation of second-shell conserved residues is less extreme and as stated above that can leave some freedom for an active site evolution. The conserved third-shell residues are in general more conserved than the second-shell residues, as they are responsible for the three-dimensional frame formation, and it normally does not require any further adaptation.

Concluding remarks

The enzyme used in this study, BlaC from *Mycobacterium tuberculosis*, represents a good model system, but certainly it does not advocate for all β-lactamases. Although we consider our results as the general trend relevant for other β-lactamases and even other enzymes, this notion is based on the observed overall patterns and comparison with patterns described in literature. The results concerning the exact roles of amino acids in β-lactamases might hold some degree of ambiguity. Phenomena such as epistasis make it virtually impossible to use one enzyme to assign all specific functions of residues in related enzymes. Furthermore, experiments performed *in vivo* in this study were carried out in a model set-up in *Escherichia coli*, which clearly differs from *Mycobacterium tuberculosis*, as well as it differs from an "actual" *in vivo* set-up in a model organism. Nevertheless, the comparison of BlaC variants to unmodified BlaC made it possible to draw conclusions about the changes brought upon mutations.

The studies presented in this work serve a basis for our understanding of protein evolution. Surely such a topic cannot be fully clarified with one model system and limited set of tested properties. This thesis is merely a modest contribution to modern science, still, I believe, all small accomplishments will one day act as the building blocks for our comprehensive knowledge of such fundamental field as evolution.