Essentiality of conserved amino acid residues in β-lactamase

Chikunova, A.

Summary

The subject of enzyme evolution has been a popular topic for decades because it conveys information about fundamental evolutionary principles and is useful for a variety of research areas, such as protein engineering and antibiotic resistance. Evolution acts via mutations in amino acid sequences, but substitution of essential amino acids can lead to a nonfunctional protein. Thus, the number of essential residues is limited by evolutionary pressure. From this notion the question arises which critical roles the remaining essential residues play. In this work an enzyme from *Mycobacterium tuberculosis,* β-lactamase BlaC, is used as a model protein to formulate the principles of amino-acid conservation. β-Lactamases are ancient proteins, which are extremely evolvable and therefore are widely used in evolutionary research. An introduction to protein evolution and β-lactamases is provided in chapter 1 of this thesis.

The roles of all non-catalytic essential residues in class A β-lactamases are discussed in chapter 2 of this thesis. It is demonstrated that residues close to the active site (second shell) and farther away (third shell) have different reasons for being essential. Substitutions introduced to the residues of the second shell are followed by the changes in stability and especially activity, showing their influence on the integrity of the active site. The active site exhibits high correlation and mutations in the second-shell residues lead to a changes sensed throughout the entire core of the protein. On the other hand, substitutions in the residues of the third shell result in a dramatic effect on the protein production, indicating the importance of these residues for the integrity of the overall protein structure. The protein frame exhibits low correlation. The structural changes are very localized and conservative mutations are preferable for the preservation of protein function.

These general patterns are supported with more focused studies of specific functions for several second- and third-shell residues. In chapter 3, a characterization of a triad of the second-shell residues of BlaC, Asn214-Asp233-Asp246 is presented. It is proposed that this triad is involved in the positioning of active site residues Thr216 and Arg220. Substitutions in Asn214, Asp233 or Asp246 affect the activity of the enzyme and lead to population of a second conformation of the enzyme, according to NMR data. We hypothesize that the substitutions enhance the mobility of a small α-helix that causes displacement of active site residues Thr216 and Arg220. Comparison of the sequences of class A β-lactamases shows that in enzymes in which the triad is not conserved adaptations are present to ensure the positioning of the active site residues in another way, a demonstration of co-evolution of residues.

The extensive characterization of the effects of the substitutions in the third-shell residues is presented in chapter 4. A severe decrease in the production of soluble, folded protein upon mutations in Glu37 and Trp229 is observed. However, the folded fraction is demonstrated to

be kinetically and structurally similar to wild type BlaC. The high conservation of these two residues is attributed to their involvement in the folding process.

Despite extreme conservation, some residues are shown to tolerate mutations. For two residues Asp179 and Asn245 some mutations are even found to improve phenotype. This is unexpected, because highly conserved residues are considered optimized by evolution. Chapter 5 provides a deeper characterization of mutants of both residues. Several substitutions of Asn245 result in elevated resistance to the β-lactamase inhibitor avibactam, which can be the consequence of an altered binding site. For BlaC D179N higher survivability can be attributed to a more stable enzyme. Thus, it appears that Asp is not the optimal residue at position 179 and may be considered an evolutionary rudiment in BlaC, because Asp179 seems to be optimal in other members of the enzyme family.

The subject of this study, BlaC, is of high medical relevance, as this enzyme from *Mycobacterium tuberculosis* is the main reason β-lactam antibiotics are not widely used to treat tuberculosis. The problem of antibiotic resistance was addressed by introduction of β-lactamase inhibitors, however, the reports of inhibitor-resistance variants found in laboratory set-up indicate the need to find a new solution. Insights into evolutionary pathways that cannot be taken by this enzyme due to essentiality of some residues, might lead to a new design of antibiotics or inhibitors to which resistance is not readily possible by mutations. Thus, understanding of inhibitor resistance mechanisms in BlaC is important. In chapter 6 of this thesis structural changes in inhibitor-resistant BlaC variants identified in the Ubbink group are discussed. Two BlaC variants displayed modifications in the binding site that are responsible for changed specificity of these mutants.

Chapter 7 offers an overview of this work and raises a few questions that remain unaddressed. This study aimed to contribute to our understanding of protein evolution and patterns of conservation of the non-essential residues. These insights are generally applicable to enzymes. In the case of β-lactamases, which are responsible for resistance against β-lactam antibiotics, understanding essentiality of residues can help prevent enzyme evolution against β-lactamase inhibitors, enhancing combination therapies.