Substrate adaptability of β-lactamase

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English Summary

Enzymes are essential biological molecules that catalyze and regulate biochemical reactions within living organisms. The study of enzyme evolution has been a prominent and enduring topic in molecular and evolutionary biology, providing valuable insights into fundamental principles of adaptation at the molecular level. β-Lactamases are enzymes that break down the β-lactam ring present in antibiotics, including penicillin, cephalosporins, and carbapenems, rendering them ineffective and allowing bacteria to survive the antibiotic treatment. These antibiotics target bacterial cell wall synthesis by inhibiting the activity of enzymes called transpeptidases, which are responsible for generating cross-links in the peptidoglycan layer in the bacterial cell wall. BlaC is a class A β-lactamases from *Mycobacterium tuberculosis* and is applied as the model for protein evolution in this work. ***Chapter 1*** provides an introduction to protein evolution and descriptions of BlaC and β-lactam antibiotics.

To explore the evolutionary adaptability of enzymes and the impact of temperature on protein evolution pathways, lab evolution was performed with BlaC as a model protein, described in ***Chapter 2***. Several amino acid substitutions in BlaC were identified that confer improved resistance to ceftazidime, which is the poor substrate for BlaC. The mutant P167S shows increased resistance to the antibiotic ceftazidime, accompanied by a reduction of the activity of hydrolysis of nitrocefin and ampicillin, indicating an activity trade-off between ceftazidime and other compounds. Structural analysis offered insight into the mechanism of enzyme adaptation. The introduction of the mutation of Ser167 causes the *cis* peptide bond between 166-167 to change into a *trans* bond, resulting in changes in the structure of the Ω-loop. The BlaC P167S shows open and closed conformations for the resting state of the enzyme due to changes in the Ω-loop. The open state gives ceftazidime better access to the active site. Because of the repositioned Ω-loop, the catalytic residue Glu166 is flipped to the outside of the enzyme and is located far away from the active site. Our results thus suggest a difference in the catalytic mechanism, underlying the trade-off in activities.

In ***Chapter 3*** the role of Glu166 was further investigated. Glu166 is assumed to have an important role in proton transfers during the catalytic reaction of serine β-lactamases. The removal of the functional group in the variant E166A had no negative effect on ceftazidime hydrolysis, confirming that the Glu is not needed for catalysis. Interestingly, the catalytic conversion of ceftazidime for WT was improved at a high pH. In the crystal structure of BlaC E166A in the resting state, a minor change in the backbone atom positions of Asn170 is observed compared to WT, due to the absence of the hydrogen bond between Glu166 and Asn170, and this effect extends far into the Ω-loop. NMR spectroscopy revealed that BlaC WT and E166A exist in two conformations in the resting states at high pH. In the case of WT, one of two states resembles the conformation seen in the BlaC P167S open state. The dynamics of the Ω-loop observed for BlaC P167S are triggered in WT BlaC by the deprotonation of the Asp172/Asp179 pair of residues, which share a low-barrier hydrogen bond. The results demonstrate that the Ω-loop is susceptible to destabilization even by changes in pH.

The mutation P167S enhanced the activity of ceftazidime hydrolysis and can be seen as a first step to an adapted substrate specificity of BlaC. However, the activity is still relatively low. ***Chapter 4*** explores the evolutionary adaptability of BlaC to enhance the activity of this antibiotic further, using directed evolution as a tool for simulating natural evolution to predict possible future mutations. Also, the role of temperature as selection pressure was analyzed. Enzymes are in a delicate balance between activity and stability, and the acquisition of new functions is often accompanied by trade-offs. Thus, it was expected that temperature, which directly relates to stability, would act as a force during evolution experiments. Using the mutant P167S/D240G as a template, variants were generated at both 23 °C and 37 °C. Through three generations of evolution, mutants originating from low-temperature conditions exhibited superior resistance at lower temperatures compared to those evolved under higher temperatures. This suggests that variants evolved at lower temperatures are better optimized for this specific condition. Considerable further increase in ceftazidime activity was obtained for the third generation mutants and across all mutants, enhanced ceftazidime activity was accompanied by a decline in activity on nitrocefin,

indicating a trade-off between activities on these substrates. The crystal structures of the evolved mutants displayed flexibility of the Ω-loop, which gives ceftazidime better access to the active site, as observed for BlaC P167S.

Chapter 5 summarizes the work presented in this thesis and offers an outlook for future research. The research gives insight into how readily enzymes can changes substrate specificity but also highlight the trade-offs involved. Ultimately, we hope that this knowledge could provide reference to the development of antibiotics. Additionally, the techniques used in this work may contribute to exploring more protein structures and mechanisms.