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Inhibition and dynamics of a β-lactamase

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Chapter 5

General conclusions and perspectives

"*The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.*" – Alexander Fleming, 1945

When the man who discovered the first antibiotic received his Nobel prize, he predicted the problem of antibiotic resistance. While his discovery heralded a healthcare revolution that allowed us to live without fear of infections for many decades, we are currently discovering the severity of his warning. It is up to us to find to find solutions. The research line in the Ubbink group, as described in Chapter 1, is geared towards the potential design of drugs that are more able to avoid the emergence of resistance. We do not know if this is possible at all, so that is what we are trying to find out. A question like this may take many years of research to answer and could lead to the conclusion that, after all, it is not possible to design such drugs. Yet even if this were to be so, it is important to realise that in fundamental research, much will be learned in the process – about the nature of the model system, as well as about the nature of evolution itself. Moreover, it is my strong belief that if enough people embark on projects like this, a small percentage of them will succeed in finding novel opportunities. Then if these opportunities are useful enough, the small percentage will be all that is needed. I am convinced that if this research line ever leads to an understanding of how to design drugs such that they are less prone to the development of resistance, it will be worth everything that was invested into it and much more. In this chapter, I will try to describe if and how the work presented in this thesis brings us closer to this goal. Subsequently, I will give a short overview of how our research fits in the more general progression of the field.

Overarching research line

The work presented here describes the first steps that were taken in the research line in the Ubbink group. The first steps mainly concern understanding of the model system itself. As it will be necessary to learn much about the model system, a model system was chosen that is interesting by itself. The most common method through which infectious bacteria develop resistance to antibiotics is through the production of a β-lactamase. The β-lactamase of *Mycobacterium tuberculosis* (Mtb) was chosen as model system. This

bacterium causes the most lethal infectious disease worldwide. Moreover, inhibition of its β-lactamase, BlaC, has recently emerged as a potential treatment option. Furthermore, the high promiscuity of BlaC makes it a suitable model system to study substrate adaptation.

Inhibition of BlaC, like other β-lactamases, is most commonly achieved with the substrate-like β-lactamase inhibitor clavulanic acid. In Chapter 2, inhibition of BlaC by clavulanic acid is shown not to be irreversible. Rather, BlaC is able to slowly hydrolyse clavulanic acid. This reaction is promoted by the presence of phosphate, which binds in the carboxy binding pocket of the BlaC active site. These findings contribute to the understanding of the BlaC–clavulanic acid interaction that is required to determine the conditions of future experiments on the system under study. Clearly, this is vital for the research line described here. An example of how the knowledge from this chapter influenced future experiments is that it identifies conditions in which it is possible to perform the type of lengthy experiments that are presented in Chapter 3.

Proteins do not exist in a single static conformation, but rather in an ensemble of dynamic conformations. The range, frequency and relevance of such conformational exchanges may differ from protein to protein. In related β-lactamases, motions in the millisecond time scale have been identified that could be correlated to the function of the enzyme[31] as well as to its evolution[109]. In order to fully understand the function of BlaC, therefore, an understanding of its conformational dynamics may be required. In Chapter 3, NMR dynamics experiments were used to show that like related β-lactamases, BlaC is very rigid on pico- to nanosecond time scale but shows significant dynamics in the millisecond timescale. This conformational exchange occurs at a rate of *ca*. 860 s^{-1} and is located in the active site of the protein. For the first time in any β-lactamase, the effect of inhibition on the conformational dynamics were also probed. Inhibition of BlaC with clavulanic acid leads to a major increase in the active site low millisecond dynamics and potentially destabilises the hydrophobic core of the α-domain.

These results improve upon the existing knowledge of the interaction between BlaC and inhibitor, but yield no information on the ability of BlaC to develop resistance to inhibition. To probe this, a large library of semi-random BlaC mutants was generated and tested for improved resistance against inhibition.

One might wonder if it is wise to purposely develop novel drug resistance. I would argue not only that the biosafety in our laboratory is relatively controlled, but also that in the host which was used here, *Escherichia coli*, inhibitor-resistant β-lactamases are unfortunately not novel at all. The notion of introducing novel resistance therefore does not apply. Furthermore, should this research progress towards introducing mutated *blaC* genes into *M. tuberculosis*, then indeed the biosafety level at which such experiments

would be performed will be much higher, to ensure that we cannot induce the spread of novel resistance.

Using the semi-random mutagenesis method, several mutants with improved resistance to clavulanic acid were identified. Of course, the identification of resistant mutants is only the first step towards understanding the mechanism through which resistance is achieved. The next step was to identify which (combinations of) mutations, amongst the multiple mutations in the resistant mutants, were responsible for the phenotype. In the mutants that were identified here, like in that which was engineered by Soroka *et al.*,[74] a single amino acid mutation was enough to reach a clavulanic acid-degrading phenotype. These two mutations, K234R and G132N, have been extensively characterised kinetically by others[73–75] while we were optimising our cloning pipeline for library generation. These biochemical characterisations yielded the information that at least the G132N mutation causes increased hydrolysis of the clavulanic acid BlaC acylenzyme adduct, but they provided no chemical models as to how this works for either mutation. In an attempt to gain more insight into this question, the tools described in Chapter 3 were used to study the dynamic behaviour of these two single amino acid mutants. These experiments showed that the two mutants, with similar functional phenotypes, displayed very different dynamic behaviour. While the relaxation dispersion measurements showed a striking increase of active site dynamics in the G132N mutant, they showed a severe decrease in the K234R mutant. This led to the conclusion that the active site dynamics are not required for enzymatic function of BlaC.

Unfortunately, while the results in Chapter 4 allowed speculation on possible models, no evidence for one or another chemical mechanism of clavulanate hydrolysis was obtained. Further clues could potentially be found through comparison of the two conformational states that were observed for BlaC G132N. For example, if only one of the states binds clavulanic acid, a rate-limiting step of 70 s^{-1} might be identified using time-resolved, pre-steady state kinetics between BlaC G132N and clavulanic acid. An assigned ^{1}H-^{15}N TROSY-HSQC spectrum of BlaC G132N bound to clavulanic acid could help to identify which of the states binds clavulanic acid, though this may be experimentally difficult to achieve due to the ability of this mutant to hydrolyse the inhibitor faster than the wt enzyme. Naturally, another approach would be to solve the crystal structures of these mutants. The insights derived from such structures could aid tremendously in the development of a chemical model for inhibitor hydrolysis, especially if, like the dynamics data of BlaC K234R seem to suggest, conformational dynamics are not essential for the reaction. In practice, however, past results have shown that it is challenging to explain the effects of many mutations in terms of structural changes, as the differences between crystal structures of variants with significantly different activities are often extremely subtle.[174] In such cases, it would be interesting to combine the cryogenic, static structures obtained from crystallography with NMR dynamics data at ambient temperature and molecular dynamics simulations. Such

combinations have proven valuable in the past (e.g. [102,104,106]) and this may also be applicable here. Currently, steps in all these directions are being undertaken within the Ubbink group.

Although the mechanisms of clavulanic acid hydrolysis by the two mutants described here have yet to be elucidated, it would nevertheless be interesting to investigate the effects of combinatorial mutations. The identification of combinatorial mutations has been an important argument for the employed experimental design, and indeed, such combinations have been shown to be capable of improving the clavulanic acid hydrolysing phenotype considerably.[73] The library generation pipeline described in Chapter 4 can also be exploited further to probe the existence of pathways towards inhibitor resistance that have not been explored yet.

For validity of any identified pathways in the model system itself, finally, the results will have to be tested in *M. tuberculosis*. This bacterium has a very different morphology and growth rate than *E. coli*, and past results have shown that results obtained through *in vitro* experiments do not always translate to this host in a straightforward manner.[29] The translation from resistance mechanisms to the potential design of drugs that can avoid these mechanisms will be even more challenging. A parallel research project within the Ubbink group focuses on residues that are conserved in BlaC and other class A β-lactamases. Through the combined understanding of which evolutionary routes are available and which parts of the enzyme are not available for mutation without loss of function, it may be possible to target inhibitors specifically to the latter parts.

Context of the field

Due to technological advances in areas such as saturation mutagenesis and deep sequencing, the understanding of the mutual effects of protein sequences and populations of organisms has grown rapidly over the past years.[175] Protein fitness landscapes have yielded insights into protein structure, stability and function which were recently reviewed by Gupta and Varadarajan[118], while those into epistasis were reviewed by Storz[114] and those into evolution by Canale *et al*.[129] as well as by Bastolla *et al.*[176] Here, I will shortly mention the recent insights that are most relevant for the current BlaC research line.

The saturation mutagenesis analyses of TEM-1[120,121] have provided information on the fitness effect of every single mutation in that protein. As explained in Chapter 1, this presents an alternative approach to the analysis of mutational landscapes that is complementary to, rather than competitive with the approach presented here. One of the central conclusions of such studies is that the epistasis, or context-dependence, of single point mutations in β-lactamases is high.[122] It could therefore be interesting to perform a similar analysis in BlaC.

Computational approaches are also revolutionising the field of protein science. For example, Jacquier *et al.* showed that it is possible, to some extent, to predict epistatic effects of mutations in TEM-1.[177] Others have computationally predicted inhibition efficiencies[178] and other protein parameters.[179,180] Importantly, conformational dynamics can also be probed computationally. This has been done through molecular dynamics simulations for at least a decade already, leading to useful insights such as those into TEM-1 dynamics by Fisette *et al*.[102] More recently, the time scale constraint of traditional molecular dynamics simulations is being challenged by novel ensemble techniques such as Markov State Modelling.[181] This technique has been applied to β-lactamases to explore how activity can be explained by hidden conformations[174] and to predict stabilizing mutations.[182] It would be interesting to pursue such avenues as complementary approaches in the research line presented here. This would be especially helpful in the model selection step, as NMR dynamics data can be difficult to interpret structurally.

Over the last few years, it has become increasingly clear that conformational dynamics can play an important role in evolutionary trajectories, with evolutionary changes occurring through conformational selection.[83,109] In this light, however modest the results presented in this thesis are, it could be said that the extension of our knowledge of BlaC towards its dynamic behaviour puts the research on this protein and its evolution in a different light. Specifically, our observation that a single point mutation can have dramatic effects on the millisecond dynamics of the protein raises concerns about the conclusions regarding the conservation of these dynamics. Kinetics and molecular dynamics experiments on resurrected Precambrian β-lactamases have shown that these ancestral proteins combine a broader substrate profile with increased fast conformational dynamics relative to the modern enzymes.[108,109,183] However, Risso *et al.* performed NMR dynamics experiments on an ancestral and a modern β-lactamase and observed the fast dynamics to be similar, while only the millisecond dynamics were more pronounced in the ancestral form.[110] They concluded that the slow dynamics relate to promiscuity. Our results suggest that perhaps the conclusion should instead be that fast conformational dynamics are more conserved in proteins than slow conformational dynamics are.

General perspectives on β-lactamases have recently been reviewed by Bush,[184] while specific insights into their inhibition were reviewed by Bush and Bradford,[40] Tehrani and Martin[185] and van den Akker and Bonomo.[39] These developments include the identification of an allosteric inhibition site in TEM-1.[186,187] It is currently not known if such a site also exists in BlaC, but based on the crystal structures, the targeted π-stacking PWP triad in TEM-1 does not appear to have a conserved orientation in BlaC. The most important development in the field of β-lactamase inhibition has been the clinical approval of several new β-lactamase inhibitors such as avibactam, vaborbactam and relebactam. While the former has a relatively low affinity for BlaC,[75] it has nevertheless been shown to display potent sterilising activity against multi-drug-resistant *M.*

tuberculosis when combined with ceftazidime.[188] Furthermore, all these inhibitors were shown to enhance the potency of several β-lactams against at least *Mycobacterium abscessus*.[189–191] It is therefore interesting to expand the scope of the research line to these new inhibitors, which is currently being done within the Ubbink group.

Still, most studies on the treatment of tuberculosis with β-lactam / β-lactamase inhibitor cocktails use clavulanic acid. Several clinical results give hope for the effective use of clavulanic acid with meropenem or tebipenem,[47,48,50,52,53,57–59] strong antibiotics that are such slow substrates for BlaC that they are practically inhibitors themselves.[22,43] The gain of resistance to these combinations through mutation of BlaC may be more difficult to achieve than to the combination of clavulanic acid and ampicillin, which was tested here. It would be interesting to utilise the mutagenesis and screening approach presented here to test this hypothesis. These and other new treatment options for tuberculosis have recently been reviewed by Tiberi *et al.,* who came to the conclusion that the arrival of new drugs and new treatment strategies brings hope to this plagued field.[13]

In conclusion, the work presented in this thesis provides improvements in the basic understanding of the model protein BlaC and its inhibition by clavulanic acid, as well as several tools that can be used to study these phenomena. Alas, these are rather modest steps in the light of goals such as the understanding of protein evolution and the development of drugs with improved evolution resistance. Nevertheless, these modest steps may one day contribute to something greater. After all, no great discoveries can be made without the tools to study the phenomena they describe.