Inhibition and dynamics of a β-lactamase

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Chapter 1

Introduction

Background

Tuberculosis

The disease that we know as tuberculosis has been killing humans since ancient times. Nevertheless, infections are believed to have been merely incidental until the increasing population density in relatively recent times allowed the disease to gain epidemic proportions. At its height in Europe in the 16th through 19th centuries, the so-called "Captain among the Men of Death" was responsible for up to a quarter of all deaths. Improvements in public healthcare and understanding of the disease led to a decline in the death toll during the 19th and early 20th century, but it was only by the discovery of streptomycin in 1946 and other highly effective antibiotics soon after that tuberculosis became a curable and controllable disease. In fact, the introduction of antibiotics was so effective that by the 1970's, it was widely believed that the disease had been defeated and would soon be eradicated completely.[1–3] Such hopes were not long-lived. In the 1980's, the appearance of drug resistance heralded a resurgence of tuberculosis that is becoming increasingly difficult to control. Today, the disease kills almost 2 million people per year, making it again the most lethal of all infectious diseases.[4–6]

Tuberculosis is caused by *Mycobacterium tuberculosis*, a bacterium that is notoriously hard to kill. Its thick, slimy cell wall slows diffusion of antibiotics into the cell. Coupled with its relatively slow generation time of about 1 day, this means that to stop an active infection, the concentration of medicine needs to remain high for a relatively long period of time. Moreover, the bacterium has the ability to encapsulate itself inside a host macrophage and subsequently stay inactive but alive for years. Such a macrophage will attract other host immune cells and form a little clump called a granuloma, which hinders the diffusion of antibiotics even more. To completely cure a tuberculosis infection, therefore, an extensive drug program must be followed, which continues for months after the symptoms have subsided. The completion rate of this treatment is poor. Especially in regions where education and finances are limited, not all patients are inclined to continue taking expensive medication after their symptoms are over. Bacteria that survive an incomplete antibiotic treatment are far more likely to be resistant against these antibiotics and so, when these are allowed to multiply again, the problem of drug resistance

increases dramatically. The World Health Organisation published an official definition of multidrug-resistant tuberculosis in the early 1990's. Tuberculosis was declared a global health emergency in 1993 and global initiatives were started to combat the disease. Nevertheless, by 2006 the problem had grown to the point that another classification had to be recognised: extensively drug-resistant tuberculosis.[7,8] The final step towards totally drug-resistant tuberculosis, although surprisingly difficult to define officially,[9] was reached soon after.[10,11]

The rise of drug resistance in *M. tuberculosis* poses a serious threat to public healthcare. New medicines to combat the disease are urgently needed. There are currently several new tuberculosis medicines in development,[12,13] but their numbers are limited and it may take many years before they reach the clinic. Another option that is worth looking into is the repurposing of antibiotics that are used to treat other diseases. By far the largest group of antibiotics that we know is that of the β-lactams. Their effectiveness in treating a wide range of bacterial infections as well as their safety for use in humans, have been proven beyond a doubt. Production processes have been scaled up to the extent that over half of the antibiotics that are being used are β-lactams. However, none of these compounds have been used historically to treat tuberculosis. This is because *M. tuberculosis* produces an enzyme that provides it with resistance to virtually all β-lactam antibiotics.[14–17] This enzyme is called BlaC.

BlaC

BlaC is the β-lactamase that is encoded by the *blaC* gene on the chromosome of *M. tuberculosis*. It is an extended spectrum β-lactamase (ESBL), which means that it can hydrolyse β-lactams, not only those of the penicillin group, but also those of the other two main groups: cephalosporins and carbapenems.[18] Like in all serine β-lactamases, its active site serine (Ser70) performs a nucleophilic attack on the carboxyl in the β-lactam ring, to form a covalently bound intermediate that is subsequently hydrolysed through a new nucleophilic attack by a water molecule, sitting in a conserved location in the active site. Some substrate structures and the general reaction mechanism are displayed in Figure 1.1 and Figure 1.2, respectively.

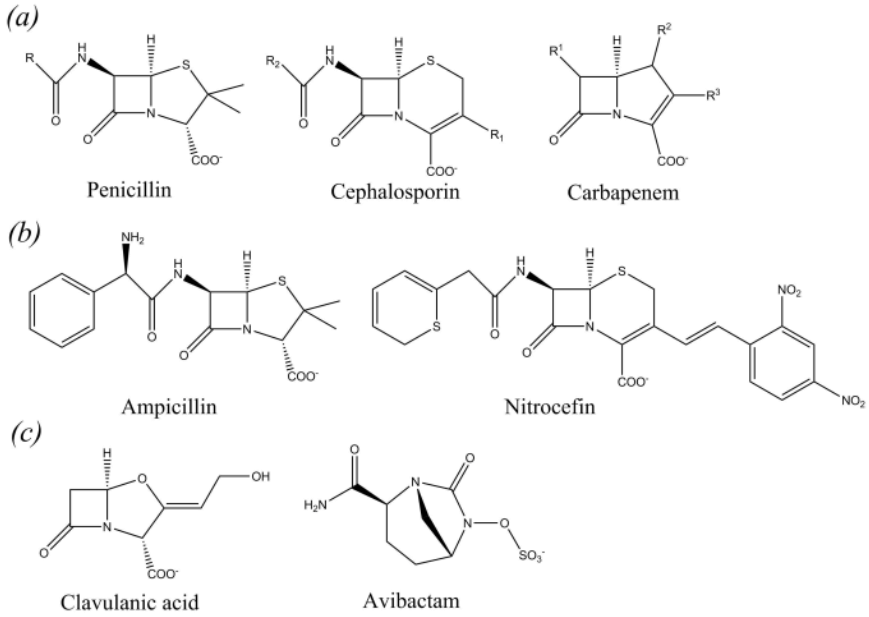

Figure 1.1: *(a)* Core structures of the three main groups of β-lactams. The four-membered ring, containing a nitrogen and a carbonyl, is the β-lactam ring. Note that the increasing number of rest groups in the core structures indicates an increasing level of variability within the groups. *(b)* Structures of two substrates that were used in this work. *(c)* Structures of two inhibitors that are used and/or discussed in this work.

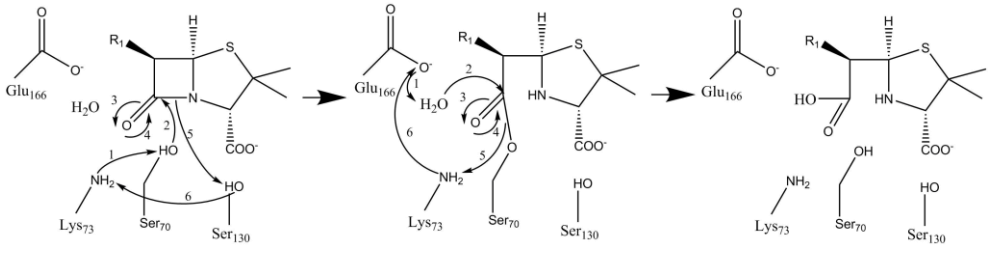

Figure 1.2: Proposed mechanism of penicillin hydrolysis by class A β-lactamases. Activated Ser70 performs a nucleophilic attack on the β-lactam carbonyl group. The β-lactam nitrogen is protonated, resulting in formation of the acyl-enzyme. Subsequent nucleophilic attack of the activated conserved active site water molecule leads to deacylation, yielding the active enzyme and the inactive β-lactam. The key steps of this enzymatic cycle are well characterised, but debate still exists over which residue activates Ser70 prior to acylation and which residues are involved in the proton shuffling (e.g. [19,20]). Shown is the proposed mechanism for BlaC.[21,22] The number labels are included to help the reader appreciate the general order of events, not as absolute and discrete steps.

The BlaC protein is exported through the *M. tuberculosis* cell membrane by the Twin-arginine translocation (Tat) pathway. The protein, with an N-terminal tat-signal peptide, is folded inside the cell and exported afterwards. The signal peptide contains a putative lipoprotein lipid attachment site. This likely tethers the protein to the cell wall after

export, as was derived from the relatively high cell-association of full-length BlaC when expressed in *Mycobacterium smegmatis* and compared to its native β-lactamase, BlaS.[23,24]

β-lactamases have been classified according to their substrate specificities[25–27] and according to their amino acid sequence similarities.[17] In the former, the Bush-Jacoby-Medeiros classification, BlaC is classified in class 2 which contains all serine penicillinases, carbapenemases and broad-spectrum β-lactamases. It has not been classified in a subgroup, however, as the broad substrate spectrum, including β-lactams from all three classes, is unique.[18] BlaC is a member of class A in the latter, the Ambler classification system. This class contains the majority of all β-lactamases that have been identified to date, but it is nevertheless slightly more specific than Bush-Jacoby class 2, as that class contains all Ambler classes A and D β-lactamases. Here, we will therefore use the Ambler classification system to indicate related β-lactamases. An additional advantage is that the Ambler system is arguably less ambiguous and more relevant in evolutionary and structural comparisons.

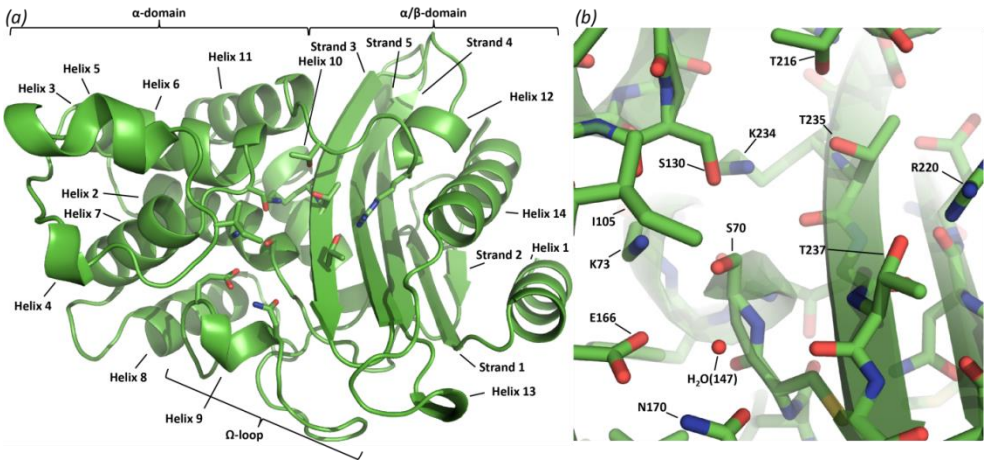

Figure 1.3: Crystal structure of BlaC (subunit A of PDB entry code 5NJ2).[28] *(a)* Cartoon representation with indication of α-helices, β-strands and the Ω-loop. Several active site residues are shown in stick representation. *(b)* Detail of the active site, showing both stick representation and transparent cartoon representation for clarity. Several active site residues and the conserved active site water molecule are indicated.

The structure of BlaC, including the major structural elements and active site residues, is displayed in Figure 1.3. The enzyme is structurally very similar to other class A β-lactamases. Nevertheless, a few structural features can be identified that are specific for BlaC. In the general class A β-lactamase models, the carboxylate group of the substrates interacts through hydrogen bonds with a pocket formed by the conserved Lys234, Thr235, Gly236 (KTG) motif and Arg244. BlaC harbours an alanine at position 244, and the spatial position of the arginine group near the active site is occupied instead by Arg220. This

arginine likely performs a very similar function to Arg244 in other class A β-lactamases. However, BlaC additionally contains a threonine at position 237, which forms an extra hydrogen bonding partner in between the arginine side chain and the substrate. Despite these structural changes in the carboxylate binding pocket compared to other class A β-lactamases, substrates have been shown to bind in the same orientation. It is therefore unclear how these substitutions affect the substrate profile and reaction kinetics.[29] On the other side of the active site, in the usually conserved substrate-binding motif serine-aspartate-asparagine (SDN) at Ambler positions 130 – 132, Asp132 is replaced by glycine. This substitution widens the active site considerably, while decreasing the potential for stabilising enzyme-substrate interactions. This likely contributes to the broad substrate profile and relatively low activity of BlaC, compared to other class A β-lactamases.[16] Another structural feature of the BlaC active site that stands out compared to its relatives is the isoleucine at position 105. This residue hangs like a lid over the active site, restricting the access. For this reason, it has been named 'gatekeeper' residue.[30] Interestingly, most class A β-lactamases harbour an aromatic residue at this position. Site-saturation mutagenesis of this residue in TEM-1 revealed that at least in that enzyme, the aromatic character of the residue plays an essential role in substrate recognition.[31] The isoleucine in BlaC is smaller, which contributes to the active site entrance being ~3 Å wider than in other class A β-lactamases.[16] This may also contribute to the broad substrate profile of BlaC. A structural peculiarity of BlaC outside the active site is that it contains a small, glycine-rich insertion in the loop between helices 7 and 8. Despite these aberrant features, it is important to emphasize that BlaC is structurally in fact very similar to other class A β-lactamases. For example, the root mean square deviation of all C_α positions in a structural alignment of BlaC (PBD 5NJ2, subunit A)[28] with TEM-1 (PDB 1BTL)[32] or SHV-1 (PDB 1SHV)[33] is only 0.9 or 0.85 Å, respectively.

The catalytic efficiency of BlaC with most penicillins (e.g. [16,18,34]) is slightly lower than that of some related β-lactamases (e.g. [35–37]). This may be related to the long generation time of *M. tuberculosis*, which could decrease the importance of sheer conversion rates. The BlaC substrate spectrum, however, is very broad. BlaC is relatively efficient at hydrolysing cephalosporins and even has considerable activity with several carbapenem antibiotics.[16,18,34] Extensive research has gone into identifying compounds that can inhibit β-lactamases.[38–40] Classical β-lactamase inhibitors clavulanic acid, tazobactam and sulbactam, as well as modern variants such as avibactam and some boronic acid-based inhibitors, have all been shown to be able to inhibit BlaC.[18,41,42] Furthermore, some carbapenems such as meropenem, tebipenem and 6-methylidene penem 2 have been found to function as substrates that take so long to degrade that they also effectively inhibit BlaC.[22,34,43,44] Due to the availability of other convenient antibiotics, combinations of these compounds have not been used historically to treat tuberculosis. Considering the rise of resistance to these other drugs, however, β-lactam / β-lactamase inhibitor

combinations have been presented in recent decades as potentially effective treatment (e.g. [29,45,54,46–53]). The most potent and popular clinically approved β-lactamase inhibitor so far is clavulanic acid.[38] In BlaC, clavulanic acid inhibition was initially reported to be irreversible[18] and later found to be slowly reversible.[41] The most used combination is that of clavulanic acid with amoxicillin. For tuberculosis, this combination was shown to be effective at least for early bactericidal activity.[55–57] An even more promising combination is that of clavulanic acid with meropenem, which was suggested to be especially suitable for treatment of difficult-to-treat cases.[47,48,50,52,53,58,59] Other potentially effective β-lactam / β-lactamase inhibitor strategies include the use of clavulanic acid and tebipenem[57] or faropenem.[60] As the use of β-lactamase inhibitors in the treatment of tuberculosis becomes more prevalent, it is high time to consider the possibility that *M. tuberculosis* may in turn develop resistance to these medicines.

Possibility of clavulanate resistance

Drug resistance at the bacterial level can arise through many pathways, amongst which are mutations in the drug target, upregulation of efflux pumps and other diffusion-limiting adjustments.[61–63] For β-lactam antibiotics, the production of β-lactamases is the most common mechanism through which bacteria reach resistance.[38,64] Specific resistance to β-lactamase inhibitors used as drugs in an antibiotics cocktail may in principle occur through as many pathways. However, as most β-lactamase inhibitors are themselves β-lactams, we will focus on mutation of the β-lactamase to resist inhibition. Mutations in β-lactamases that render them resistant to inhibition have been observed in both active site residues and residues that are far away from the active site. Overall, the effect of mutations has been observed to be highly context-dependent (e.g. [38,65–72]). In BlaC, a directed evolution strategy was employed by Feiler *et al.*, who found the I105F substitution to confer slightly decreased ampicillin-clavulanate sensitivity to *E. coli* cells due to an increased enzymatic activity. The magnitude of the inhibition itself, however, remained similar.[30] Kurz *et al.* performed site-directed mutagenesis targeted at creating a clavulanate-resistant BlaC variant, based on sequence variations conferring such resistance in homologues enzymes from other species. This approach yielded variants displaying *in vitro* clavulanate resistance due to having a significantly decreased affinity. *In vivo*, however, the slow generation time of Mtb allowed inhibition anyway.[29] This approach was continued by Egesborg *et al.*, who successfully used combinatorial mutations to increase the *in vitro* clavulanate resistance of BlaC beyond the generation time of Mtb, which is 24 hours.[73] Soroka *et al.*, on the other hand, based their approach on structural information from resistant homologues and found that the G132N substitution, which did not affect hydrolysis of other β-lactams, allowed for efficient hydrolysis of clavulanate by BlaC.[74] Though these substitutions were not tested *in vivo*, these results do suggest that β-lactam-clavulanate combinations are at risk for the development of resistance. However, the

same group later found that the increased clavulanate resistance of G132N is paired with decreased avibactam resistance.[75]

These results suggest that multiple pathways may exist through which BlaC can gain resistance to clavulanic acid. However, the extent to which resistance is reached varies considerably and there appear to be functional trade-offs. Furthermore, little is known about epistasis between these mutations, or the functionality that may be reached through the combination of multiple mutations in general. Moreover, it remains unclear to what extent these pathways, mostly found via human design, represent the most efficient routes towards resistance that are available for BlaC. Lastly, it is currently unknown if the apparent functional trade-offs can potentially be exploited through better inhibitor design.

Research questions

The ultimate goal of the research line in the Ubbink group is to design β-lactamase inhibitors for combination therapy in which BlaC resistance against inhibitors does not occur. This is an ambitious goal, for which a number of other questions need to be answered first. One requirement is to identify which evolutionary pathways by BlaC adaptation are actually available to reach inhibitor resistance. If such pathways are found, we need to understand the molecular causes of the resistance. Consequently, the first objective is to broaden and deepen our understanding of the wild type enzyme. While structures with atomic detail are available and BlaC catalytic activity has been characterized, little is known about the dynamic properties of the enzyme and also a molecular understanding of the interaction with substrates and inhibitors, in particular clavulanic acid, is lacking. Once comprehensive knowledge of the wild type enzyme has been obtained, it will be possible to generate, characterize and understand mutants that confer inhibitor resistance upon BlaC. The research questions that are addressed in this thesis are:

1. Is BlaC inhibition by clavulanic acid reversible? If so, why have there been conflicting results on this topic? (Chapter 2)
2. What is the dynamic behaviour of BlaC in solution in the resting state and in complex with clavulanic acid? (Chapter 3)
3. Is it possible for BlaC to gain resistance to clavulanic acid inhibition through one or a few amino acid mutations? If so, what are the involved mechanisms? Do they involve conformational dynamics? (Chapter 4)

Methodology

Protein dynamics

1

To date, 49 crystal structures have been published of BlaC, giving a high-resolution look at the structural features of the enzyme, either in free form or bound to various substrate and inhibitor adducts. However, in order to fully understand the function of an enzyme, not only the static structural features are required, but also structural changes as a function of time. Proteins do not exist in a single conformation but rather as a dynamic ensemble of conformations. The sampled conformational landscape may have any number of local energy minima, each with different populations. Exchange between the different conformations occurs on a wide range of time scales. Moreover, a large body of evidence illustrates the importance of this flexibility in various biological processes, including catalysis.[76–83]

The method of choice to measure the dynamics behaviour of proteins is nuclear magnetic resonance (NMR) spectroscopy. In NMR spectroscopy, the magnetic resonances of individual nuclei in a protein are measured. The frequency at which they resonate depends on their chemical environment. Therefore, changes in the chemical environment of individual nuclei can be detected. If the protein is dynamic, some atoms will experience several chemical environments. This phenomenon is called chemical exchange and can be probed using NMR spectroscopy.

If the exchange between states occurs on a time scale that is slower than the difference in resonance frequencies between the states, it is called slow exchange and all states with a population above the detection limit can be measured separately. If the chemical exchange process occurs on a similar time scale as the difference in resonance frequencies, it is referred to as intermediate exchange. In this case, significant line broadening will be observed, leading to a decrease in peak intensity. If the exchange process is much faster than the resonance frequency difference, it is called fast exchange and only an average state is measured. Nevertheless, also in these cases, NMR spectroscopy offers various possibilities to explore some of the characteristics of the process underlying the exchange.

Protein motions cause fluctuations in the local magnetic fields surrounding nuclei through changes in dipole-dipole orientations and due to chemical shift anisotropy. The frequency distribution of these fluctuations is called the spectral density $J(\omega)$. When the frequency of a magnetic field fluctuation matches the energy difference between nuclear spin states, it can cause spin transitions. Spin energy transitions, in turn, can be detected by NMR spectroscopy in the form of relaxation. A variety of NMR experiments has therefore been designed that probe various types of relaxation. In particular, the longitudinal (T_1) relaxation, transverse (T_2) relaxation and steady-state heteronuclear Overhauser effect

(NOE), based on the phenomenon of cross-relaxation, of each backbone amide are often measured. The magnitudes of these types of relaxation are each defined by the spectral density at a combination of frequencies.[84] The spectral density itself, in turn, can be described in terms of motion parameters.[85,86] This allows the use of motion parameters to perform a least-squares fit of the spectral density function to the measured relaxation rates. This approach is called Lipari-Szabo formalism and the theory behind it has been explained extensively in the seminal papers by Lipari and Szabo and by others,[85–90] so we will not go into much detail here. The goal is to extract meaningful parameters of the motion, which can be interpreted in structural terms. Typically, fast internal motion of the measured amide bonds can be described with just two motion parameters per amide bond, S^2 and τ_e, using a least-squared fit of the spectral density function as described in Equation 1.1[85,86,88] to the combined data. S^2 is the order parameter, which represents the spatial restriction of the internal bond motion. It has a value between 0 and 1, 1 meaning no local motion. τ_e is the effective correlation time of the individual amide bond vector, which represents the rate of internal motion. τ_c is the rotational correlation time of the molecule, a measure for the tumbling rate of the protein. Overall tumbling of the protein itself is an important contributor to the magnetic field fluctuation and thus to the relaxation. Unlike S^2 and τ_e, however, τ_c is a global parameter which is not fitted separately per measured amide bond but rather only once for the entire dataset.

Equation 1.1

$$J(\omega) = \frac{2}{5} * \tau_c * \left[\frac{S^2}{(1 + (\tau_c * \omega)^2)} + \frac{(1 - S^2) * (\tau_c + \tau_e) * \tau_e}{(\tau_c + \tau_e)^2 + (\omega * \tau_c * \tau_e)^2} \right]$$

If the tumbling and diffusion of the molecule are not isotropic, they will affect amides that are oriented in different orientations in the molecule differently. In this case, instead of a single value τ_c, a global anisotropic diffusion tensor must be fitted based on the molecular structure of the protein.[91] Furthermore, although Equation 1.1 does not assume any particular type of internal motion, it does assume that the dynamic behaviour can be described with just one frequency of internal motion. In practice, proteins may move at a large range of frequencies. A single amide bond might therefore experience multiple types of movement. In these cases, the measured NOE values tend to be sensitive to the fastest detectable movement, typically in the ps-ns range, while the observed R_2 tends to be affected by all movement. The observed R_2 then cannot be explained by Equation 1.1 alone, so for these amides, an extra contribution to the R_2 is added, R_{ex}. While τ_e and S^2 represent the fast local movement of the amide bond relative to the rest of the protein, R_{ex} then reports on a slower movement, typically indicating movement of a larger protein segment. This slow chemical exchange forms an additional individual parameter in a fitting process that already has many parameters, which often leads to over fitting. It is therefore usually difficult to interpret the R_{ex} beyond the indication that µs-ms motions may be

present. However, for many proteins, this represents the motion with the most relevant biological significance. Fortunately, the contribution of chemical exchange to the apparent transverse relaxation can also be measured directly, using the relaxation dispersion experiment.

Line broadening due to chemical exchange occurs when the exchange rate is around the same frequency as the chemical shift difference, i.e. there is intermediate exchange. In this case, it is possible to refocus the line broadening by application of 180˚ pulses with a higher frequency. The rate of the motion and chemical shift difference can therefore be estimated by measuring the transverse relaxation as a function of 180˚ pulse frequency. This can be done by applying a Carr-Purcell-Meiboom-Gill (CPMG) pulse train with a varied pulse frequency within a fixed relaxation time.[92–94] Exchange parameters are calculated by fitting the Bloch-McConnell equations[95,96], which describe the relation between exchange and peak broadening, to the relaxation data.[97] Modern NMR spectrometers can perform this experiment with pulse frequencies in the range of 10^1 – 10^3 s^{-1}, making this experiment ideally suited to analyse exchange processes in that frequency range. To access faster motions, a continuous spin-lock field can be used instead of a 180˚ pulse train. In this case, the power of the lock field is varied rather than the pulse frequency. This type of experiment is called $T_{1\rho}$ and is used to investigate motions in the 10^3 – 10^6 s^{-1} frequency range.[98,99]

Another experiment that can be applied to elucidate slow protein dynamics is Chemical Exchange Saturation Transfer (CEST).[100] As mentioned before, nuclei will likely experience different chemical environments in different protein conformations. In practice, there is often one ground state with a high population and one or more excited states that are too sparsely populated to be observed directly. However, if there is exchange between the states in a frequency range of 10^0 – 10^2 s^{-1}, saturation transfer methods can be employed to change the spin population of one state by irradiating the other. The CEST experiment uses this principle by applying a saturating B_1 field at a large series of frequencies covering the entire spectrum. The effects on the resonance peak of the main state are measured. If there is no exchange, the intensity of the peak will diminish only if the frequency of the B_1 field is at or very near its own frequency. However, if there is exchange with some other state at another frequency, saturation of that minor state will be transferred through exchange to the main state. As a result, the peak intensity will also diminish upon saturation of the secondary state. This effect not only demonstrates the presence of chemical exchange, but also allows the position(s) of (a) minor state resonance(s) to be determined, which may hold a clue as to what the minor state could structurally look like. Furthermore, when the power of the B_1 field is varied, the exchange rate and population of the minor state can also be determined.[100]

Dynamics of β-lactamases

Nothing is known about the presence or role of dynamics in BlaC. However, considering the conservation of structure and function, it seems prudent to review what is known about dynamic behaviour of related β-lactamases.

A combination of NMR backbone dynamics studies and molecular dynamics (MD) simulations of class A β-lactamase TEM-1 has revealed that it is very rigid on the pico- to nanosecond time scale, showing almost no local, fast motion. Micro- to millisecond time scale motions were observed in the omega-loop and the vicinity of the active site,[101,102] leading to the proposal of a slow, cavity-filling motion of the Ω-loop. Point mutations of TEM-1 residue 105, which acts as a lid on the active site, resulted in an alteration of the motions in the active site, which could be correlated to the alterations in the catalytic efficiency of the mutants, implying that the dynamics might be involved in catalysis.[103]

Backbone dynamics studies on PSE-4, another class A β-lactamase, yielded similar results. PSE-4 is very rigid in the pico-nanosecond time scale, but shows some micro-millisecond dynamics for several residues near the active site. However, significant dynamics differences with TEM-1 were found for several important residues, emphasising the importance of comparing various β-lactamase variants.[104]

The proposal of Ω-loop motion was corroborated by MD simulations comparing free states to substrate-bound states of both TEM-1 and PSE-4. These simulations revealed a marked flexibility increase of the Ω-loop upon substrate binding.[105,106] A synthetic chimera protein consisting of a TEM-1 enzyme with its Ω-loop replaced by that of PSE-4 was found to display increased slow motions relative to either of its parental enzymes.[107] A reconstruction of an ancient β-lactamase, proposed to be the most likely common ancestor of all gram-negative β-lactamases, was shown to combine a broader substrate profile with an active site with potentially more slow dynamics than modern enzymes.[108–110] This likely reflects the idea that specialised enzymes employ conformational pre-organisation to fit their preferred substrate, whereas generalists use flexibility to adapt to various substrates.

Interestingly, all the NMR spectroscopy studies on class A β-lactamases have yielded high-quality spectra in which only a few of the non-proline residues could not be assigned a resonance peak. In all cases, these residues were located in the active site and included serine 70. This implies that those resonances were broadened beyond detection by some intermediate exchange process specific to the active site. The first direct measurement of motion in the ms time scale (~50-3000 s^{-1}) by CPMG relaxation dispersion measurements reported only very restricted motions for both TEM-1 and PSE-4. Interestingly, the TEM-1 – PSE-4 chimera mentioned before showed strikingly increased dynamics in this time scale, occurring in a broad region around the active site.[111] The authors speculate that

what they observed may have been a shift in time scale, with the underlying dynamics in the wild type enzymes being too fast for the CPMG experiments to probe. A similar hypothesis was put forth recently to explain markedly increased dynamics in a BlaR1 β-lactamase sensing domain upon binding with a ligand.[112] In that case, the β-lactamase inducer 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP) shifted the time scale of the otherwise hidden active site dynamics to the slow exchange regime, where it could be identified using NMR. Although BlaR1 is not a β-lactamase, similar mechanisms may well apply to class A β-lactamases. Furthermore, a novel approach based on millisecond hydrogen–deuterium exchange mass spectrometry was used recently to measure solvent accessibility of TEM-1 regions upon engaging in reaction with β-lactams that were either efficiently, slowly or not hydrolysed.[113] Although the resolution of this approach is limited in both space and frequency compared to its NMR-based counterparts, the authors identify several regions in which the dynamics modes associated with the various catalytic processes are clearly distinct, making a valuable contribution to the search for dynamics-based inhibitors.

The relevance of the subject, the elusiveness of β-lactamase intermediate/slow dynamics and their correlation with activity and the possibility of time scale shift upon ligand binding, both in β-lactamase simulations and measurements on related proteins, make it all the more striking that no NMR dynamics measurements on class A β-lactamase / inhibitor complexes have been reported to date. The need for such measurements has been recognised for years.[104] The absence of such data may be due to technical difficulties, as a typical NMR dynamics experiment length of several days restricts this option to extremely stable complexes. This experimental limitation, in turn, takes us back to the need to properly understand such enzyme/inhibitor complexes, the involved mechanisms and the (ir)reversibility of inhibition.

Simulated evolution

To identify mutations that confer resistance, one can look at mechanisms in other β-lactamases and try to rationally extrapolate those to BlaC. As discussed before, however, it can be difficult to predict the effects in a different enzyme due to epistatic mutations.[114] Furthermore, such rational inference only provides a very limited number of possible approaches compared to the available sequence space. Importantly, these approaches tend to be blind to all mechanisms except those that are already known. For these and other reasons, several other techniques have been developed to simulate evolution and identify evolutionary pathways.

Ideally, all possible mutations would be tested. For single mutations, methods have been developed and applied that enable this approach.[115–118] Such saturation mutagenesis was performed for the full TEM-1 protein and combined with functional essays such as Minimal Inhibitory Concentration (MIC) measurements of antibiotics.[119–121] This systematic

approach yields a wealth of information about the effects of single mutations in the studied landscapes. However, without combinations of mutations, no information about epistasis is obtained. This was addressed by Steinberg and Ostermeier,[122] who applied the combination of saturation mutagenesis and functional essays to the TEM-17, TEM-19, and TEM-15 β-lactamase alleles to determine how the different epistatic landscapes affect the effect of single mutations. The comparison of the various landscapes is extremely valuable as it has not only yielded vast amounts of information about these enzymes but also helped to improve our understanding of epistasis in general. However, despite the large investment of time and resources that are required, this approach still yields no data on the feasibility of reaching new functions via combined mutations in the same landscape.

The application of a comprehensive scan to combinatorial mutations would complicate the problem exponentially. In the single amino acid replacement saturation mutagenesis of TEM-1 such as performed by Stiffler *et al*., the fitness effect of an impressive 4997 mutants (263 positions * 19 mutations) were tested.[121] Should we want to increase the number of mutations from 1 to 2 per mutant, each of the 4997 mutations should be combined with 262 * 19 / 2 = 2489 other mutations, bringing the total number of mutants to be created and tested to 12.4 million. Likewise, at three mutations per mutant this number becomes 21 billion and at 4 mutations the number of mutants to test rises to 25 trillion. Clearly, the mapping of all possible combinations of mutations would be beyond challenging. Fortunately, another technique exists that is considerably less daunting.

The synthesis of gene products by polymerases in the polymerase chain reaction (PCR) can be disturbed by the addition of extra magnesium, manganese and/or skewed nucleotide concentrations. This technique is called error-prone PCR and typically produces a large number of gene products with several single nucleotide mutations each. This approach is also sometimes referred to as random mutagenesis, though in truth, the mutations are not quite random. Firstly, the polymerase has a bias under these conditions for A/T to C/G mutations.[123,124] Secondly, mutations that occur early in the PCR reaction are duplicated in successive steps and therefore overpopulated in the final reaction mixture. Thirdly, certain amino acid mutations on the protein level may be reached through a single base pair mutation on the DNA level, whereas other amino acid mutations require two or three base pair mutations and are thus much less frequent. Nevertheless, error-prone PCR allows the comparatively easy synthesis of extremely large numbers of gene products harbouring semi-random combinations of mutations. Also, it is worth noting that the biases in substitution rates that arise from the error-prone PCR approach actually reflect, to some extent, the same biases that occur in natural evolution, which were shown to suppress deleterious mutations and enrich for adaptive mutations.[125] The obtained gene products can be cloned into bacterial cells to produce the encoded protein. Functional β-lactamases will provide their host cells with resistance to β-lactam antibiotics and can thus be selected *in vivo*. This approach has been applied frequently, yielding useful insights into

both enzymatic function and evolution (e.g. [126–129]). It was applied to BlaC by Feiler *et al.* to identify mutations that increase activity with ampicillin.[30] Here, we will use a double selection pressure, applying both ampicillin and the β-lactamase inhibitor clavulanic acid. The ampicillin will kill our host *Escherichia coli* unless it possesses a functional β-lactamase, while the clavulanic acid will make BlaC dysfunctional unless it acquired mutations that enable it to resist inhibition. This way, we can screen large numbers of BlaC mutant proteins and select only those that have mutated to somehow resist inhibition, while maintaining enough functionality and stability to effectively hydrolyse ampicillin. As we are interested in the evolutionary routes that are available for wild type BlaC, more so than in the pinnacle of resistance that could be reached with BlaC as starting point, we will not apply multiple rounds of directed evolution. Rather, we will apply one round of mutagenesis followed by stringent selection. By sequencing individual mutants, we expect to find the mutations that have recently been reported to increase clavulanic acid resistance and hope to find novel mutations and combinations there-of. Subsequently, we will characterise the identified mutants and identify evolutionary pathways that confer resistance. Ultimately, understanding of these pathways will prove vital in the design of inhibitors with improved resistance to evolution.

Thesis outline

Chapter 1 of this thesis gives a general introduction to BlaC, the questions that were probed in this study and the methodologies that were used. **Chapter 2** shows that unlike what was commonly held previously, clavulanic acid does not irreversibly inhibit BlaC. Instead, the enzyme is able to recover from inhibition after a period of time that we found to depend strongly on the conditions. In particular, we found that the presence of phosphate catalyses the recovery more than 20-fold. **Chapter 3** expands upon the understanding of BlaC with the first description of its internal motions. NMR spectroscopy was used to get an impression of what conformational dynamics are present in BlaC and how they change upon binding by clavulanic acid. It shows that BlaC, like related β-lactamases, is very rigid on the pico-nanosecond time scale, while displaying important millisecond dynamics that are localised specifically in the active site. Furthermore, it is revealed that binding of the inhibitor clavulanic acid causes a major increase of the dynamics on all time scales. In **chapter 4**, random mutagenesis coupled with selective functional screening is applied to identify mutations that increase the ability of BlaC to resist inhibition by clavulanic acid. One inhibitor-resistant mutant is found to display increased slow motions in the active site, while the millisecond motions in another have severely decreased, indicating that similar phenotypic function can be reached through various evolutionary routes. **Chapter 5** comprises a general discussion of the work in this thesis in the perspective of the overarching research questions and applications.