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## Structure and mechanism of action of two bacterial enzymes

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# *Chapter 5*

**Summary, General Discussion and Future Perspectives**



X-ray crystallography is the most powerful technique to determine the three-dimensional (3D) structures of proteins at high resolution. The 3D structure information of a protein is essential for understanding its biochemical and biological function. However, to fully understand how a protein works, also requires biophysical and biochemical data describing protein dynamics and the binding of substrates, substrate analogs or macromolecular partners (proteins or DNA). In this thesis, protein X-ray crystallography and several biochemical methods, i.e., site-directed mutagenesis, activity assays, kinetics studies and limited proteolysis, have been employed to elucidate the mechanism of action of two different enzymes: the membrane-bound lytic transglycosylase MltE from *E. coli* (Chapter 2) and the aspartate ammonia lyase AspB from *Bacillus* sp YM55-1 (Chapter 3). In addition, preliminary structural studies are described for two transcriptional regulators from *Bacillus cereus*, which are members of the structurally and functionally ill-characterized PadR protein family (Chapter 4).

### **Membrane-bound lytic transglycosylase MltE**

Lytic transglycosylases are enzymes which play a role in the metabolism and remodelling of peptidoglycan (PG), the main constituent of the bacterial cell wall (1). These enzymes are considered unique since they combine two activities: (i) they cleave the glycosidic bond between an N-acetylmuramic acid and N-acetylglucosamine residue in the glycan strands of peptidoglycan, and (ii) they form a new 1,6-anhydro bond in the MurNAc residue concomitant to cleavage. In *E. coli*, seven lytic transglycosylases have been studied, i.e., MltA, MltB, MltC, MltD, MltE, MltF and Slt70 (2-10). All LTs are located in the periplasmic space, either as soluble proteins (Slt70) or as membrane-associated proteins, attached to the outer-membrane via an N-terminal lipoyl anchor or via a single transmembrane spanning helix (MltA-F). Most of the LTs are exo-enzymes, which degrade PG starting from the terminal 1,6-anhydro-MurNAc residues of the glycan strands, producing 1,6-anhydromuropeptides containing a single disaccharide unit (i.e., GlcNAc-1,6-anhydroMurNAc). MltE is the only endo-acting enzyme, which produces 1,6-anhydromuropeptides containing multiple disaccharide units (i.e. (GlcNAc-MurNAc)<sub>n</sub>-GlcNAc-1,6-anhydroMurNAc with n=1,2, or more) (4). A previously published crystal structure of MltE revealed that it has a lysozyme-like fold, similar to the catalytic LT

domains of Slt70 and MltB (8, 11), consisting mainly of  $\alpha$ -helices and having a bilobal shape with an elongated deep binding groove running across the surface (12). The groove was predicted to contain six or eight subsites, numbered -4 to +2 or -4 to +4, respectively, for binding to the sugar residues of a glycan chain. The predicted catalytic residue, Glu64, is located at the center of the binding groove, between subsites -1 and +1, similar to Slt70 and MltB (13, 14). Different from Slt70 and MltB, the binding groove of MltE lacks a steric obstruction near the +2 subsite, which would limit the accessibility for an incoming glycan strand, explaining the endo-specific activity of this enzyme. A number of questions about the function of MltE were not answered by the crystal structure of MltE. Why is the enzyme only able to produce longer 1,6-anhydromuropeptides, which implies that it cannot cleave the glycosidic bond connecting the terminal GlcNAc-1,6-anhydroMurNAc disaccharide unit to the preceding MurNAc residue in a glycan chain? And why is it only active against isolated *E. coli* glycan strands from which the peptides have been removed?

To obtain structural insights into the PG binding mode and mechanism of action of MltE, new crystal structures were determined of this enzyme in a ternary complex with the muropeptide GlcNAc-MurNAc-L-Ala-D-Glu and the glycopeptide inhibitor bulgecin A and in a binary complex with chitopentaose ((GlcNAc)<sub>5</sub>) (**Chapter 2**). MltE was crystallized in a different space group ( $P2_12_12_1$  with five protein molecules per asymmetric unit) as compared to the previously reported ligand-free structure ( $C222_1$  with 2 protein molecules per asymmetric unit) (12). The binary complex was crystallized using an inactive MltE mutant (MltE-E64Q).

The ternary complex structure of MltE was determined at 2.3 Å resolution. The structure reveals how the two ligands bind simultaneously at non-overlapping subsites in the binding groove of MltE, with bulgecin A occupying subsites -2 and -1, whereas the muropeptide occupies subsites +1 and +2. The binding modes of the sugar residues at the -2 and +1 subsites explain why these two subsites prefer to bind a GlcNAc residue rather than a MurNAc residue. The C3-hydroxyl groups of the GlcNAc residues are buried in the protein-saccharide interface at the back of the binding groove, leaving no space for replacement by the larger C3-lactyl groups of the MurNAc residues. The binding modes of the L-proline moiety of bulgecin A at subsite -1 and the GlcNAc residue at subsite +1, and their interactions with the catalytic residue Glu64 residue further suggest that the ternary

complex structure is valuable as a transition state mimic of the  $\beta$ -1,4-glycosidic bond cleavage reaction. Furthermore, the hydrogen bonding interactions between bulgecin A and residues Ser73 and Gln188 are indicative of a substrate-assisted mechanism with the N-acetyl group of the -1 MurNAc residue being important for stabilizing the oxocarbenium ion intermediate, like previously proposed for Slt70 and MltB (13, 14). The substrate binding interactions at subsite +2 of MltE are distinct compared to those observed in Slt70 and MltB. Substrate interactions at subsite +2 of MltE are formed only with the saccharide residue, in the other two LTs substrate interactions are formed predominantly with the peptide moiety that is linked to the MurNAc residue (13, 15).

The binary complex structure of MltE-E64Q was determined at 1.9 Å and 2.5 Å resolution. Chitopentaose occupies subsites -4 to +1. All five GlcNAc residues adopt low energy chair conformations and the conformations of the glycosidic bonds interconnecting the -4, -3, -2 and -1 sugar residues are similar to those observed in chitin. The glycosidic bond, which connects the -1 and +1 GlcNAc residues, however, has significantly different bond dihedrals, causing a rotation of the +1 GlcNAc residue away from Gln64. As a consequence the +1 GlcNAc residue of chitopentaose binds less deeply in the PG binding groove as compared to the +1 GlcNAc residue of the muredipeptide in the ternary complex structure of MltE. The binding interactions of -2 GlcNAc residue of chitopentaose are identical to the -2 GlcNAc residue of bulgecin, while the binding mode of the -1 GlcNAc residue of chitopentaose is very similar to the L-proline moiety of bulgecin.

The crystal structures of saccharide-bound complexes of MltE allowed a detailed mapping of the protein residues and the interactions, which are responsible for substrate binding at subsites -4 to +2. Furthermore, the structures confirm the proposed reaction mechanism of MltE (Chapter 2) and provide a structural basis for explaining the similarities and differences in catalytic function between MltE and the other LTs. The amino acid residues at subsites -2 to +1 of MltE are mostly conserved in Slt70 and MltB, but not the residues in subsites -4, -3 and +2. Based on the two saccharide-bound structures of MltE, a model of PG-bound MltE could be constructed. The modeled PG glycan strand contained eight saccharide residues, bound to subsites -4 to +4, thus indicating the locations of the additional subsites +3 and +4 and their putative saccharide-binding interactions. Subsite +4 is largely solvent exposed and can accept both a “regular”

MurNAc residue and a terminal 1,6-anhydroMurNAc residue. Modeling further indicates that subsite +2 is highly specific for a regular MurNAc residue: a 1,6-anhydroMurNAc residue in subsite +2 would not be able to make sufficient binding interactions. This is different from the situation in Slt70 and MltB, in which subsite +2 can accept both a regular MurNAc and a 1,6-anhydroMurNAc residue. However, since Slt70 and MltB contain steric obstructions preventing PG binding beyond the +2 subsite, productive binding of a natural glycan strand by these enzymes is always associated with binding of a terminal 1,6-anhydroMurNAc residue in subsite +2. Thus, glycosidic bond cleavage in natural PG by Slt70 and MltB requires binding of a glycan chain of minimally three disaccharide units, (GlcNAc-MurNAc)<sub>2</sub>-GlcNAc-1,6-anhydroMurNAc, occupying subsites -4 to +2 with the terminal 1,6-anhydroMurNAc residue bound to subsite +2. “*In-vitro*”, MltB is also able to cleave a PG fragment of two disaccharide units, (GlcNAc-MurNAc)<sub>2</sub>, occupying subsites -2 to +2 (6). Cleavage by MltE, on the other hand, requires binding of a glycan chain of minimally four disaccharide units, (GlcNAc-MurNAc)<sub>3</sub>-GlcNAc-1,6-anhydroMurNAc, with the terminal 1,6-anhydroMurNAc residue occupying subsite +4 (longer glycan strands may bind with a regular MurNAc residue in subsite +4). Since cleavage always occurs at subsite -1, this explains why the products of MltE never consist of a single disaccharide unit (GlcNAc-1,6-anhydroMurNAc), but minimally have two disaccharide units (GlcNAc-MurNAc)<sub>n</sub>-GlcNAc-1,6-anhydroMurNAc, with n=1 or more) (4). The endospecific activity of MltE is therefore enhanced by the strong binding preference of subsite +2 towards a regular MurNAc residue. The modeling studies further indicate that productive binding of a glycan chain in the PG binding groove of MltE is associated with a conformational change of the -1 MurNAc residue from a chair to a sofa, thus confirming the role of substrate distortion as an important aspect of the lytic transglycosylase reaction mechanism. Substrate distortion of the -1 sugar helps to stabilize the formation of the oxocarbenium ion intermediate.

An interesting finding of our studies is that MltE from *E. coli* shows cleavage activity against intact peptidoglycan from *Micrococcus luteus* cells. This is intriguing as previous studies demonstrated that MltE cannot cleave *E. coli* peptidoglycan “*in-vitro*”, unless the peptides are first enzymatically removed from the MurNAc residues (4). Also, overproduction of MltE in the periplasm of *E. coli* does not result in rapid bacteriolysis, indicating that also “*in-vivo*” the activity of MltE is highly restricted. Our studies further

show that MltE is completely inactive against chitin, confirming that the presence of the C3-lactyl groups on the MurNAc residues is essential for productive binding and glycosidic bond cleavage. The low activity of MltE against intact *E. coli* PG seem to suggest that the peptides in *E. coli* PG form a steric obstruction preventing productive PG binding. However, this does not explain why *E. coli* MltE is active against *M. luteus* PG, which also contains many peptide cross-bridges, albeit with a different amino acid composition. A more likely explanation for the species-specific cleavage behavior of MltE is that the peptide cross-bridges in *E. coli* PG inhibit catalysis via a mechanism involving their binding to peptide-specific binding sites. Peptide binding may lock the substrate in a non-productive binding mode and/or prevent release of PG after cleavage, causing the enzyme to stall. Due to their different amino acid composition, the peptide cross-bridges of *M. luteus* PG are probably not recognized by MltE and therefore do not affect PG binding and cleavage. Unfortunately, the crystal structures of the MltE complexes do not allow the identification of peptide binding sites. The +2 MurNAc residue in the ternary MltE complex has a short peptide stem, but lack of electron density indicates that it is highly disordered.

What is the precise functional role of MltE in PG metabolism? Deletion of the MltE-encoding gene indicates that the protein is not essential for cell survival (Haigh and Williams, personal communication in (4)). However, it is possible that the function of MltE in the *mltE*-deletion mutant is taken over by one of the other lytic transglycosylases. It has been suggested that MltE, being an endo-specific lytic transglycosylase, is an ideal candidate for trimming the glycan chains of PG to their proper lengths, following polymerization of PG by PG-synthases. It would require MltE to work in close collaboration with an amidase, though, to deal with the peptide obstructions. No protein partner has yet been identified for MltE, although experimental data for other lytic transglycosylases supports the hypothesis that these enzymes work in multi-protein complexes (16). Furthermore, it is unclear how MltE is able to reach the PG layer, being a relatively small protein and anchored to the outer membrane. The outer PG layers have to be close enough to MltE in order to be degraded which can be achieved by kinks or wrinkles in the PG network (17) or due to dynamic movements of the outer membrane (18).



It is evident that further studies are required to identify and understand the precise function of MltE. Pull-down assays may perhaps identify protein partners of MltE (e.g. the proposed amidase). Advanced microscopic techniques are needed to visualize the exact location of MltE in the outer-membrane of the *E. coli* cell and how this protein connects to the PG layer. Such techniques are being developed (e.g. cell tomography using confocal or electron microscopy), but currently the resolution of the generated 3D images is insufficient to allow identification and localization of small proteins like MltE.

### **Aspartate ammonia lyase AspB**

Aspartate ammonia lyases (or aspartases) are enzymes that catalyze the reversible deamination of L-aspartate to yield fumarate and ammonia. Aspartases are members of the aspartase/fumarase superfamily, which also includes enzymes such as fumarase (19), argininosuccinate lyase (20), adenylosuccinate lyase(21),  $\delta$ 1-crystallin (22) and 3-carboxy-cis,cis-muconate lactonizing enzyme (CMLE) (23). Although sequence similarities can be as low as 15%, these enzymes have highly similar ternary structures and all function as homotetramers with three conserved regions in each protomer forming four composite active sites. One of the conserved regions at the active sites is a flexible loop that contains the sequence motif GSSxxPxKxN. This so-called SS-loop forms a flexible lid, which regulates access to the active site and has an important role in substrate binding and catalysis. Members of the aspartase/fumarase superfamily process common succinyl-containing substrates and all produce fumarate as one of their products (except CMLE which produces a lactone). Structure determination of fumarase, argininosuccinate lyase, adenylosuccinate lyase, and  $\delta$ 1-crystallin in complex with substrates or substrate analogs already suggested that these enzymes use a common catalytic mechanism (21, 24-26). Crystal structures have also been determined for a number of aspartases, e.g. for the enzyme from *E. coli* (AspA) (27) and *Bacillus* sp YM55-1 (AspB) (28). However, prior to the studies described in this thesis, no experimentally determined structure was available of an aspartase with bound substrate or substrate-analogue, preventing a detailed assessment of the catalytic mechanism of this particular class of aspartase/fumarase enzymes.

Thus, as described in **Chapter 3** of this thesis, crystal structures were elucidated of ligand-free AspB and of an AspB complex bound with L-aspartate at 2.4 Å and 2.6 Å resolution, respectively. The enzyme was crystallized at a different condition and in a different space group (*P1* with eight protein molecules per asymmetric unit) than previously published (space group *P2<sub>1</sub>2<sub>1</sub>2* with two protein molecules per asymmetric unit,(28)). The structure of L-Asp-bound AspB allowed us to pinpoint the residues responsible for substrate binding and catalysis. Binding of L-aspartate at the active site forces the substrate to adopt a high energy, enediolate-like conformation, with a concurrent change in electronic structure of the β-carboxylate oxygens, which are stabilized via an extensive hydrogen-bonding network involving a cluster of conserved serine and threonine residues. Furthermore, substrate binding induces a closure of the SS-loop, which moves towards the substrate and positions the highly conserved Ser318 residue in close vicinity of the Cβ atom of L-aspartate. This position would allow Ser318 to act as catalytic base in the first step of the catalytic reaction mechanism, abstracting the Cβ-proton of L-aspartate. Site-directed mutagenesis of the SS-loop, in combination with kinetic studies, confirms the important functional role of SS-loop.

The crystal structure of the AspB/L-Asp complex, combined with site-directed mutagenesis data, strongly indicates Ser318 as the base catalyst in the reaction mechanism. A role as catalytic base for a serine residue is quite unusual considering the low stability of a serine oxyanion at neutral pH (the pK<sub>a</sub> of a free serine residue is ~15 ). Stabilization of the Ser318 oxyanion may be achieved via interactions with the backbone amides of adjacent SS-loop residues (Ile320 and Met321). Furthermore, activation of Ser318 towards its oxyanion form may involve the β-carboxylate group of L-Asp, acting as a base abstracting the proton of the serine hydroxyl group, as has previously been suggested for other aspartases. The  $k_{cat}/K_m$  pH profiles of AspB, however, indicate that the pK<sub>a</sub> of Ser318 is already substantially lowered in the free enzyme, challenging the hypothesis of a mechanism of substrate-assisted catalysis (29). Unfortunately, the current experimental data presented in Chapter 3 cannot explain the precise mechanism by which Ser318 is activated. Other techniques, like NMR or mass spectrometry, may perhaps provide the necessary data to address this interesting question.

Intriguingly, the crystal structure of the AspB/L-Asp complex also indicates a possible functional role for the so-called “C-terminal small domain” of AspB (residues 407-466). Upon substrate binding it was observed that this domain becomes relatively disordered. To further analyse the role of the C-terminal small domain, we performed experiments combining limited proteolysis, activity assays, mass spectrometry and X-ray crystallography. An inactive, but stable proteolytic fragment of AspB, missing the C-terminal subdomain, could be crystallized and its structure was determined to a resolution of 3.0 Å. The crystal structure revealed that upon deletion of the C-terminal small domain the SS-loop becomes highly disordered. Other residues in the active site with a role in substrate binding and catalysis are not affected by the C-terminal domain deletion, though. The lack of catalytic activity of the AspB fragment therefore strongly indicates that the C-terminal domain has a role in controlling the conformation of SS-loop, and thereby regulating the catalytic activity.

The experimental evidence presented in Chapter 3 supports the notion that members of the aspartase/fumarase superfamily use a common catalytic mechanism involving general base-catalyzed formation of a stabilized enediolate intermediate. However, it should be noted that so far only a few superfamily members have been investigated by structural and functional studies, and many others still await determination of their structures and catalytic mechanism. An interesting example is ethylenediamine-N,N'-disuccinic acid lyase (EDDS lyase). This enzyme catalyzes two sequential 1,2-elimination reactions to convert EDDS into ethylenediamine and fumarate (30). This ability makes EDDS lyase a promising biocatalyst for the preparation of biodegradable metal chelators such as EDDS and its derivatives. The presence of an additional reactive amino group in the substrate suggests EDDS may use a different reaction mechanism. Another example is 3-carboxy-cis,cis-muconate lactonizing enzyme, where the catalytic mechanism of the lactonization reaction is still undiscovered.

### **PadR-like transcription regulators**

PadR-like transcription regulators are bacterial proteins, which share a common fold and regulate the expression of genes related to multidrug resistance, virulence and

detoxification. Their structures consist of two domains, an N-terminal winged helix-turn-helix domain associated with DNA binding and a variable C-terminal domain of one or more  $\alpha$ -helices required for dimerization. The PadR-like protein family is divided into two subfamilies: subfamily 1 (PadR-s1) members have a relatively large C-terminal domain containing multiple  $\alpha$ -helices, while subfamily 2 (PadR-s2) members have a small C-terminal domain containing a single  $\alpha$ -helix. Only a few PadR-s1 proteins and one PadR-s2 protein have been structurally and functionally investigated. The characterized PadR-s2 protein is LmrR, which regulates the expression of genes encoding a multi-drug ABC efflux pump in *L. lactis* via a mechanism involving multidrug binding and induction (31, 32). Multidrug binding is achieved in a hydrophobic pore at the dimer interface, and involves a pair of dimer-related tryptophan residues (Trp96 and Trp'96), which bind the planar, lipophilic drugs via stacking interactions. The tryptophan residue in the C-terminal helix of LmrR is highly conserved in the PadR-s2 protein family. It is currently unknown whether the conserved C-terminal tryptophans in other PadR-s2 proteins also have a role in multidrug binding. A few structures of PadR-s2 members are available in the Protein Data Bank, in which the equivalent tryptophan residues are buried in a completely closed dimer interface. As these proteins lack any functional characterization, the biological significance of this structural difference is unclear.

The genomes of *B. cereus* strains ATCC14579 and ATCC 10987 contain a number of *padR-s2* genes. One of these genes, addressed as locus BC4206 in *B. cereus* strain ATCC14579, was recently shown to become significantly up-regulated upon exposure of *B. cereus* to the enterocin AS-48 (33). Interestingly, the BC4206 gene is co-upregulated with an adjacent gene, locus BC4207, which codes for a putative membrane protein. This suggests that the product of BC4206 (which we named *bcPadR1*) regulates transcription of BC4207, which in turn results in enterocin resistance. Another homolog of LmrR was found in *B. cereus* ATCC 10987 as the product of gene locus BCE3449 (which we named *bcPadR2*). BCE3449 is part of a putative operon that shows similarities to the operon containing the *lmrR* gene, and contains two genes encoding a putative antibiotic ABC efflux pump. Considering their operon organizations, and the presence of the conserved tryptophan residue in their C-terminal region, it seems possible that *bcPadR1* and *bcPadR2* proteins, like LmrR, act as drug-binding transcriptional regulators. To investigate this possibility structures of *bcPadR1* and *bcPadR2* were determined by X-ray crystallography.

The structures of *bcPadR1* and *bcPadR2* were determined to 2.5 Å and 2.2 Å resolution, respectively (**Chapter 4**). As predicted, the two structures contain an N-terminal winged helix-turn-helix domain and a single C-terminal  $\alpha$ -helix. Unlike LmrR, they show a completely closed dimer interface and the conserved dimer-related tryptophan residues in the C-terminal helices are completely buried. The *bcPadR1* and *bcPadR2* proteins are structurally highly similar to RTP, a replication terminator protein from *Bacillus subtilis* (34), although they lack significant sequence homology. To identify residues with a role in DNA-binding, *bcPadR1* and *bcPadR2* were superimposed on the structures of DNA-bound homologs. The comparison indicates that *bcPadR1* and *bcPadR2* have a functional DNA binding domain, but the limited sequence conservation of their DNA-binding regions prohibits an accurate assessment of the putative DNA binding interactions. Identification of the target genes regulated by *bcPadR1* and *bcPadR2*, and the location and sequence of their operator DNA sites, requires additional functional studies and DNA foot-printing analysis.

The structures of *bcPadR1* and *bcPadR2* presented in this thesis show no evidence for a drug binding pore or a role of the conserved C-terminal tryptophan residues in multidrug binding. Most probably, DNA binding by these two PadR-like proteins is not allosterically modulated by ligands, and their transcriptional regulatory activities do not follow a drug-induction mechanism as shown by LmrR. It may be possible that transcriptional regulation by the *bcPadR* proteins follows an indirect induction mechanism involving another protein. Such an indirect induction mechanism has recently been proposed to play a role in the phenolic stress response in *Lactobacillus plantarum*, where a PadR protein acts together with a member of the universal stress protein family (35). It will be interesting to study whether the *bcPadR* proteins form part of a similar regulatory system.

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