Summary

Most bacteria possess a cell wall composed of peptidoglycan, which protects them from hazardous influences from the environment in which they live. Peptidoglycan is a heteropolymer built up of linear glycan chains of alternating β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, cross-linked by short peptides attached to the *D*-lactyl groups of the MurNAc residues. In *E. coli* these peptides contain *L*-alanine-*D*-glutamyl-*m*-diaminopimelyl-*D*-alanine, of which 40-50% are cross-linked by peptide bridges. The peptidoglycan forms a bag-shaped structure, the murein sacculus, which completely encloses the bacterial cell. The murein sacculus maintains the shape and size of the cell, provides mechanical support, and prevents the bacterium from osmotic lysis. The importance of peptidoglycan is highlighted by the fact that several efficacious antibiotics inhibit its biosynthesis. By inhibiting the enzymes involved in cell wall biosynthesis, the cell wall gets severely damaged, and the cell lyses because of the high internal osmotic pressure. Penicillin and related β-lactams are examples of such very effective and widely applied antibacterials that interfere with the murein biosynthesis, causing cell lysis and death. However, the rapid spread of resistance against β-lactam and other antibiotics is causing a serious health problem, creating an urgent need for alternative antibiotics and new antibiotic targets. Such potential new targets could be the β-lactam insensitive lytic transglycosylases (LTs), which act on the glycosidic bonds in peptidoglycan, and which participate in peptidoglycan maintenance and processing as, for example, required during cell septation and division. Specifically, these enzymes catalyse the cleavage of the β-(1,4)-glycosidic bond between MurNAc and GlcNAc residues in peptidoglycan. Although similar in action to lysozymes, the lytic transglycosylases generate different reaction products. While lysozymes cleave the β-1,4-glycosidic bond *via* a hydrolytic reaction producing muramyl residues with a reducing end, LTs perform an intramolecular glycosyltransferase reaction between the C1 and O6 atoms of the MurNac residue, resulting in the formation of non-reducing 1,6 anhydromuramyl residues. The molecular basis of this difference in product specificity is however not known. More specific questions are how in the lytic transglycosylases water is excluded from the active site, how the oxo-carbenium ion is stabilized, and how the -1 carbohydrate residue is brought into a conformation that facilitates attack of its C1 atom by the O6 atom. Furthermore, the transglycosylase reaction is unique to

bacteria and to some bacteriophages and inhibition of lytic transglycosylases appears to enhance the efficacy of β-lactam antibiotics. Therefore, the lytic transglycosylases seem interesting targets for novel structure-based drug design.

This thesis describes the determination and analysis of the 3D-structure of the lytic transglycosylase MltA from *Escherichia coli* by X-ray crystallography. This work aims to further increase our knowledge of the molecular details of the cleaving mechanism and the typical 1,6-anhydromuropeptide production by the lytic transglycosylases from *Escherichia coli*.

Chapter 1 gives a general overview of the present knowledge of the bacterial cell wall structure, antibiotic resistance and the lytic transglycosylases. Furthermore, the X-ray structures of known lytic transglycosylases are summarized together which their proposed catalytic mechanism. In addition, the reaction mechanisms are discussed of other structurally or functionally related enzymes that cleave β-1,4-glycosidic bonds.

Chapter 2 describes the preliminary work involved in the structure determination of MltA. A soluble form of the lytic transglycosylase MltA (sMltA) with its membrane anchor and signal sequence deleted was over-expressed in *Escherichia coli* and purified to homogeneity by means of cation-exchange chromatography. Rod-shaped protein crystals were obtained, using the hanging drop method, which diffracted to 2.2 Å resolution. The crystals belong to space group $P3_121$ or $P3_221$ with unit cell parameters $a = b = 103.70$ Å, $c = 109.84$ Å with one molecule in the asymmetric unit. In addition, since MltA shows no sequence homology with other lytic transglycosylases, a selenomethionine substituted sMltA derivative of the enzyme was prepared, purified and crystallized to obtain experimental phases by the multi-wavelength anomalous diffraction (MAD) approach in order to elucidate the crystal structure.

Chapter 3 gives a detailed description of the 2.0 Å resolution crystal structure of sMltA from *E. coli*. The structure, obtained using MAD, reveals a novel lytic transglycosylase fold. sMltA contains two domains, separated by a large groove. Domain A is built up of two discontinuous amino acid sequence segments (residues 3-104 and 244-337) and consists of an N-terminal part (residues 3-90) and a β-barrel core. The N-terminal part contains two antiparallel β-strands (β1 and β2) connected by three helices (α1-α3). The β-barrel core is the main feature of domain A and consists of a mixed parallel/antiparallel six-stranded closed β-barrel made of β-strands β3 and β10-β14, with one small α-helix (α9). It has a double-psi β-

barrel fold topology, similar to that of endoglucanase V (EGV) from *Humicola insolens*. Domain B (residues 105-243) is inserted between strands β3 and β10 of domain A of sMltA. It also contains a small mixed parallel/antiparallel six-stranded β-barrel (β4-β9), and a small helical sub-domain (α5-α8). The β-barrel has a different topology compared to the β-barrel topology of domain A, but is weakly related to the RNA binding domain of ribosomal proteins L25 and TL5.

Comparison of sMltA and the catalytic domain of EGV, combined with molecular modeling studies and site directed mutagenesis experiments, allowed us to identify structural regions and important residues involved in substrate binding and catalysis of MltA. Importantly, Asp308, which is in an equivalent position to the catalytic acid of the *H. insolens* endoglucanase, was assigned as the single acid/base catalyst in the lytic transglycosylase mechanism. Mutation of Asp308 into an alanine residue yielded a completely inactive enzyme in support of a catalytic role of Asp308. At the end of this chapter a general reaction mechanism is proposed.

To obtain a detailed description of the MltA-peptidoglycan interactions and the cleavage mechanism the D308A-sMltA mutant was co-crystallized with the substrate analogue chitohexaose. **Chapter 4** and **chapter 5** deal with the co-crystallization, the refinement procedure and the structure elucidation of the D308A-sMltA mutant complex.

Because phasing with molecular replacement using the native structure failed, selenium-MAD was used to obtain initial phases. However, structure determination was hampered due to a rare case of tetartohedral twinning. Therefore, a special refinement protocol was designed to take tetartohedral twinning into account, which was successfully applied to refine the complex structure. This refinement protocol is described in **chapter 4**. The resulting structure explains the success of using MAD in phasing the data, as well as the reasons for tetartohedral twinning.

Chapter 5 gives a detailed description of the crystal structure of the D308A-sMltA mutant in complex with chitohexaose at 2.25 Å resolution. The chitohexaose binds to subsites -4 to +2 and spans the -1/+1 cleavage site. All six GlcNAc residues have a full $^4C_1$ chair conformation. Interestingly, the complex structure reveals a large conformational change upon binding the chitohexaose, which results in closure of the active site and tight interactions of both domains with the chitohexaose. This conformational change is probably necessary for regulation of the enzyme activity. Furthermore, these results allowed us to give a more

detailed comparison of the MltA cleavage site compared to endoglucanase V. A more detailed catalytic mechanism is proposed, involving Asp308 as the single catalytic acid/base. The partial negative charge due to the helix dipole moment of α-helix 5 at the -1 α-face may stabilize the proposed oxo-carbenium ion intermediate. Furthermore, the C-terminus of α-helix 5 may shield the intermediate from the solvent to prevent hydrolysis. No catalytic nucleophile is present, in line with other lytic transglycosylases that have a different fold. Furthermore, in contrast to other LTs, there is no evidence for sMltA that it employs substrate assisted catalysis using the *N*-acetamido group.