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Structure of *Escherichia coli* Lytic Transglycosylase MltA with Bound Chitohexaose

**IMPLICATIONS FOR PEPTIDOGLYCAN BINDING AND CLEAVAGE**

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Karin E. van Straaten,1 Thomas R. M. Barends,2 Bauke W. Dijkstra, and Andy-Mark W. H. Thunnissen

From the Laboratory of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Crystal structures of an inactive mutant (D308A) of the lytic transglycosylase MltA from *Escherichia coli* have been determined in two different apo-forms, as well as in complex with the substrate analogue chitohexaose. The chitohexaose binds with all six saccharide residues in the active site groove, with an intact glycosidic bond at the bond cleavage center. Its binding induces a large reorientation of the two structural domains in MltA, narrowing the active site groove and allowing tight interactions of the oligosaccharide with residues from both domains. The structures identify residues in MltA with key roles in the binding and recognition of peptidoglycan and confirm that Asp-308 is the single catalytic residue, acting as a general acid/base. Moreover, the structures suggest that catalysis involves a high energy conformation of the scissile glycosidic linkage and that the putative oxocarbenium ion intermediate is stabilized by the dipole moment of a nearby α-helix.

Lytic transglycosylases (LTs) are bacterial muramidases that cleave the bacterial cell wall heteropolymer peptidoglycan (murain) for turnover and recycling to facilitate cell growth and division, as well as to allow local cell wall opening without loss of integrity (1, 2). The lytic activity of these enzymes is directed toward the β-1,4-glycosidic bonds between N-acetylmuramic acid (MurNAc) and GlcNAc residues, the two sugar units that make up the glycan strands of peptidoglycan. Concomitant to bond cleavage, they catalyze an intramolecular transglycosylation reaction resulting in the formation of muropeptides terminated with a non-reducing 1,6-anhydromuramidic acid residue.

LTs are found in a wide variety of bacteria, as well as in a few eukaryotic species, and are often present as several distinct enzymes within the same species. For instance, in *Escherichia coli*, at least six different LTs have been identified: one soluble (Slt70) and five that are outer membrane-anchored (MltA-MltD, EmtA) (1, 3, 4). A comparison of genes encoding LTs allowed their classification into four families based on the identification of different sets of consensus motifs (5). LT family 1 includes proteins with amino acid sequence similarity to Slt70, MltC, MltD, and EmtA, whereas MltA and MltB are representatives of families 2 and 3, respectively. The fourth LT family comprises mostly enzymes encoded by *A* bacteriophages.

Studies of the LTs have focused on understanding their precise cellular roles, as well as on explaining their reaction mechanism. Concerning the latter interest, important insights have been obtained from the study of crystal structures of these enzymes, both in unliganded forms and in complex with various murein-derived compounds (6–11). These investigations showed that members from LT families 1, 3, and 4 have a catalytic domain that resembles the fold of goose-type lysozyme (12). The reaction catalyzed by these enzymes most likely takes place via a general acid/base mechanism that requires participation of a single catalytic residue, i.e., an invariant glutamic acid residue located at the C-terminal end of an α-helix. The reaction mechanism of the LTs thus differs from that of hen egg white lysozyme and from those of most other glycoside hydrolases, which require the participation of two catalytic carbohydrate groups in the active site (13). Based on inhibition studies and crystallographic analysis, it has been proposed that the lysozyme-like LTs may utilize the N-acetyl group of the muramic acid residues in the peptidoglycan polymer to provide anchimeric assistance in catalysis (7, 10, 14) similar to the reaction mechanisms described for various chitinases, N-acetyl-β-hexosaminidases, and hyaluronidases (15–18).

MltAs (LT family 2, also classified as glycoside hydrolase family 102 in the CAZy database) occupy a special place among the LTs. They are characterized by six conserved amino acid sequence motifs (5), none of which bears any resemblance to those present in the other LTs. Moreover, their three-dimensional structure, as revealed by recent crystal structure determinations of the enzymes from *E. coli* and *Neisseria gonorrhoeae* (11, 19), is completely different from that of lysozyme. It comprises two main domains, one of which has a double-β-barrel fold resembling the catalytic domain of endoglucanase V, a cellulytic enzyme from *Humicola insolens* (20). The two
domains are separated by a long groove, which, in analogy to the catalytic domain of endoglucanase V, most likely serves to bind and cleave a glycan strand. An invariant aspartate residue in MltA, Asp-308 in the *E. coli* enzyme, has a position equivalent to that of the catalytic acid of endoglucanase V, Asp-121, whereas there is no second carboxylate group in MltA at a position equivalent to Asp-10, the catalytic base of endoglucanase V. Furthermore, mutation of Asp-308 to an alanine residue in *E. coli* MltA resulted in a completely inactive enzyme, whereas similar mutations of two other invariant aspartate residues (Asp-261 and Asp-297) had less dramatic or minor effects on catalytic activity, strongly suggesting that Asp-308 in MltA, aspartate residue in glycan strand. An invariant aspartate residue the catalytic domain of endoglucanase V, most likely serves to bind and cleave a glycan strand. An invariant aspartate residue in MltA, Asp-308 in the *E. coli* enzyme, has a position equivalent to that of the catalytic acid of endoglucanase V, Asp-121, whereas there is no second carboxylate group in MltA at a position equivalent to Asp-10, the catalytic base of endoglucanase V. Furthermore, mutation of Asp-308 to an alanine residue in *E. coli* MltA resulted in a completely inactive enzyme, whereas similar mutations of two other invariant aspartate residues (Asp-261 and Asp-297) had less dramatic or minor effects on catalytic activity, strongly suggesting that Asp-308 in *E. coli* MltA functions as the single general acid/base catalyst in the lytic transglycosylase reaction mechanism of MltA (11). Previous proposals concerning the function of MltA were made on the basis of analysis of unliganded native structures and by studying the effects of site-directed mutagenesis. Important questions concerning the precise role of amino acid residues in substrate binding and cleavage and whether anchimeric assistance in catalysis by the N-acetyl group of the substrate plays a role in the catalytic mechanism of MltA could not be adequately addressed in the absence of structural information on sugar-bound enzyme complexes. Here we describe the structure of the inactive *E. coli* MltA-D308A mutant in two different apo-conformations, as well as in complex with the hexasaccharide chitohexaose ([(GlcNAc)₆]). This ensemble of structures provides evidence for a remarkable conformational flexibility of MltA, allowing it to accommodate large differences in the relative spatial disposition of its two structural domains. Although in the apo-forms of the enzyme the two domains are positioned such that they form a similarly wide, although differently shaped, groove in the chitohexaose-bound form of MltA, the domains have reoriented such as to cause a substantial narrowing and reshaping of the groove, optimizing the fit to the oligosaccharide. The MltA-chitohexaose structure further provides detailed information on the oligosaccharide binding interactions and the likely mechanism of catalysis of MltA, possibly involving a novel mode of stabilization of the oxocarbonyl intermediate state by the dipole moment of a nearby α-helix.

**EXPERIMENTAL PROCEDURES**

**Structure Determination of apo-sMltA-D308A**—Expression and purification of sMltA-D308A were carried out as described previously (21). Crystallization of apo-1 sMltA-D308A was performed at conditions similar to those used for native sMltA (15 mg/ml protein in 250–350 mM NaCl, 10 mM MgCl₂, 100 mM sodium acetate buffer, pH 4.2), yielding isomorphous crystals (space group P₃₁). Prior to data collection, the apo-1 crystals were transferred to a stabilizing solution containing 8% polyethylene glycol 8000, 375 mM NaCl, 10 mM MgCl₂, 7.5% isopropyl alcohol, and 100 mM sodium acetate buffer, pH 4.2, allowing a small, intact crystal fragment to be rescued and frozen for data collection using glycerol as cryoprotectant. Soaking of the apo-1 crystals in solutions containing chitohexaose (Sigma) generally resulted in extensive crystal deformation and cracking. However, in one particular case, a short soak of 20 min in a solution containing 20 mM chitohexaose, 250 mM NaCl, 10 mM MgCl₂, 7.5% isopropyl alcohol, and 100 mM sodium acetate buffer, pH 4.2, allowed a small, intact crystal fragment to be rescued and frozen for data collection using glycerol as cryoprotectant. Analysis of the x-ray data revealed a dramatic change in unit cell dimensions, without a change in space group, as compared with the apo-1 crystals (see Table 1). Subsequent structure determination, however, did not reveal any bound chitohexaose but allowed a large conformational change to be observed in the sMltA-D308A protein, explaining the change in crystal packing and identifying a second crystal form of apo-sMltA-D308A (apo-2). Data sets for the two apo-forms of sMltA-D308A were collected at beam line ID14-1 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. All data were collected at 100 K, processed with
DENZO, and merged with SCALEPACK (22). The data collection statistics are shown in Table 1.

The structure of native MltA was used as a starting point for the refinement of the apo-1 sMltA-D308A model. First, all solvent molecules were removed from the native sMltA model. Then, the model was refined against the apo-1 diffraction data using CNS (23), first by employing rigid body refinement and followed by simulated annealing using torsion angle dynamics. Inspection of $2mFo - DFc$ electron density maps confirmed the presence of the D308A mutation in the model. The apo-1 model was refined to convergence through iterative cycles of positional refinement using CNS and manual rebuilding with XFIT (24). Progress of the refinement was monitored by following $R_{free}$. Water molecules were placed automatically by XFIT in peaks >3.0σ in $Fo - DFc$ maps and within hydrogen-bonding distance of nitrogen or oxygen atoms; water molecules without electron density in $2mFo - DFc$ maps contoured at 1σ and B factors above 60 Å² were removed from the model.

Phases for the apo-2 sMltA mutant were determined by molecular replacement with the program MOLREP (25) using the two domains of the apo-1 model as independent search

![Diagram of MltA with Bound Chitohexaose](image)
models. Refinement of the apo-2 model was carried out as for the apo-1 model, except that REFMAC5 (26) was used as the refinement program. The final apo-1 and apo-2 models (see Table 1 for refinement statistics) extend from residue 3 to 337 and contain 227 and 153 water molecules, respectively.

Structure Determination of Chitohexaose-bound sMltA-D308A—Crystals of chitohexaose-bound sMltA-D308A were obtained by co-crystallization. A rare case of tetrahedral twinning prohibited a standard structure determination of the sugar-bound sMltA-D308A complex. Instead, it required the use of selenomethionine-substituted protein to allow multiwavelength anomalous dispersion data collection and the application of special twin-adapted refinement protocols. A detailed description of the procedure followed to determine the structure of this complex has been published elsewhere (27). The statistics of the final refined model are given in Table 1. The model displays good geometry. Terminal residues 1–2 and 338–345 were omitted due to unclear electron density. Electron density for the hexasaccharide GlcNAc residues is well defined, except for the reducing GlcNAc residue at subsite $+2$, which shows disordered binding (see the supplemental data).

Structural Analysis—All models were validated using MOLPROBITY (28) and the ADIT validation server (at the Protein Data Bank). For structural analyses, the following programs were used: domain rotation by DYN- DOM (29), superpositions by LSQ- MAN (30), and enzyme-ligand hydrogen bond analysis by CON- TACT (25). The figures were prepared with POVSCRIPT (31), POV- RAY, and PYMOL.

Construction of Glycan-bound sMltA Models—A $(\text{GlcNAc-MurNAc})_3$-bound sMltA model was constructed by manually replacing the C3-OH groups of the $-3$, $-1$, and $+2$ sugars in the chitohexaose-bound structure with D-lactyl groups. A short run with CNS was performed to remove bad contacts,
restraining the positions of the sugar rings and restricting protein atom movement to side chains with atoms within a 5 Å cut-off distance from the glycan hexamer. To assess the possibility of induced substrate distortion at subsite −1, an extra run was performed with CNS, enforcing a sofa conformation for the −1 MurNAc residue by applying appropriate dihedral restraints.

RESULTS

Structure Determination—E. coli MltA (hereafter referred to as sMltA) was expressed and purified as a soluble protein, having its N-terminal 20-residue lipoprotein signal sequence and following lipoyl-bearing cysteine residue replaced by a single methionine residue. Saccharide binding to sMltA was probed by co-crystallization of the inactive D308A mutant and chitohexaose ((GlcNAc)₆). In addition, two crystal structures of D308A sMltA without sugars were obtained (apo-1 and apo-2; for details, see Table 1 and “Experimental Procedures”). In contrast to the two apo-forms, structure determination of the chitohexaose-bound form of sMltA-D308A was not straightforward due to a rare case of tetartohedral twinning, as described elsewhere (27). Although in the apo-crystal forms, the proteins are packed as monomers, in the chitohexaose-bound sMltA-D308A crystal asymmetric unit, they are arranged as tetraters. Each of the four protein molecules in these tetraters has a chitohexaose bound, as based on analysis of Fₒ - DF, Fourier maps calculated early in the refinement. The four protein molecules are well superimposable (overall root mean square difference (r.m.s.d.) of 0.69 Å for 330 equivalent Cα atoms, see the supplemental data), with structural differences being largely the result of small rigid body-like reorientations of the two domains. Significant local differences in backbone conformation are observed only at the two termini of the polypeptide chains and at a number of flexible turns and loops (e.g. residues 12–20, 28–32, 70–75, 88–95, and 192–222).

Overall Structures and Conformational Flexibility—The chitohexaose-bound structure of sMltA-D308A reveals large conformational differences with respect to the apo-1 and apo-2 structures (Fig. 1). As described previously (11), E. coli sMltA comprises two structural domains (A and B (11)). The larger domain A (residues 3–104 and 244–337) has a double-ϕβ-barrel fold. Domain B (residues 105–243) also has a β-barrel structure, but it is different from that of domain A. It is present as an insertion between the first (β3) and second (β10) strand of the double-ϕβ-barrel. A small helical subdomain, comprised of helices α6–α8, is inserted between the last two strands of the β-barrel of domain B. The overall structure of apo-1 sMltA-D308A is nearly identical to that of the native enzyme determined previously (overall r.m.s.d. for all Cα atoms is ~0.2 Å). It shows a conformation in which the small α-helical subdomain (Bo) of domain B participates in interdomain contacts with the loop between helix α9 and strand β14 of domain A. A wide groove, blocked at one end by the Bo subdomain, runs at the interface between domains A and B. In the apo-2 structure, the domains have separated due to an ~30° rigid body-like rotation of domain B away from domain A with the two interdomain linker segments, i.e. residues 104–107 and 238–242, functioning as flexible hinges. As a result, a very wide and open groove is formed at the domain interface. In contrast, the chitohexaose-bound structure of sMltA-D308A reveals that, upon binding of the hexasaccharide, domain B undergoes an ~37° rotation (relative to the apo-1 structure) toward domain A, bringing the two domains closer together, and causes a relocation of the Bo-subdomain relative to the α9-β14 loop. As a consequence, the active site groove has become narrower and deeper, and the blockage by the Bo-subdomain has been released, allowing the tight embrace of the bound chitohexaose.

Binding of Chitohexaose in sMltA-D308A—The chitohexaose binds in the active site groove from subsite −4 (non-reducing end) to subsite +2 (reducing end) (Fig. 1B; for subsite nomenclature, see Davies et al. (32). The hexasaccharide has an extended conformation, with all GlcNAc residues in full Cα chair conformations and with all N-acetyl groups pointing away from their corresponding sugar rings. Most significantly, it has an intact glycosidic bond at the site of bond cleavage between subsites −1 and +1, the first time this has been observed for a lytic transglycosylase. In contrast to various chitinase structures with bound chitin fragments (16), the sMltA-D308A-chitohexaose complex provides no evidence for distortion of the −1 sugar ring nor for a possible participation in catalysis of the N-acetyl group of the −1 GlcNAc residue. However, the −1 to +1 glycosidic linkage has rotated away from its energetically most favorable conformation (Fig. 2 and supplemental data), and the O3 hydroxyl group of the +1 GlcNAc has lost its stabilizing hydrogen-bonding interaction with the −1 O5 ring oxygen atom. Instead, the O3 hydroxyl group is positioned under the C1 carbon of the −1 GlcNAc at a close distance of ~3 Å.

Binding of the chitohexaose involves a large number of van der Waals interactions as well as several sugar-protein hydrogen bonds (Fig. 2 and supplemental data). The tightest binding occurs at subsites −2, −1, and +1, where the sugar residues are cut off almost entirely from solvent. Almost all protein residues participating in sugar binding are part of the conserved amino acid sequence motifs of MltA (5). Binding of the −2 GlcNAc residue involves a strong aromatic stacking interaction between the sugar ring and the phenyl ring of Tyr-180, as well as several

![Structure of MltA with Bound Chitohexaose](image)

**FIGURE 2. Interactions of sMltA-D308A with Chitohexaose.** A, schematic drawing showing the hydrogen bond interactions (dashed lines) of chitohexaose with protein residues in the active site groove. Protein residues from domain A and B are shaded blue and yellow, respectively. Hydrogen bonds occurring in only two or three of the protein molecules of the crystallographic tetramer are indicated by blue dashed lines. B and C, stereo views of the sugar-protein interactions at the six sugar binding sites. The polypeptide chain is drawn as a green ribbon, whereas the residues participating in sugar binding are shown as balls-and-sticks, with the carbon atoms colored in green. The GlcNAc residues of the hexasaccharide are shown as balls-and-sticks with the carbon atoms colored in yellow. Nitrogen atoms are colored in blue, oxygen is colored in red, and phosphorous is colored in orange. Hydrogen bonds are shown as dashed lines. D, stereo view showing a superposition of the chitohexaose-bound mutant structure and the native sMltA structure (11) focusing on subsites −1 and +1 and the position of the catalytic Asp-308. Colors are as in B and C with protein carbon atoms of chitohexaose-bound sMltA-D308A colored in green. Residues Asp-308 and Thr-99 are from the superimposed native sMltA structure, with carbon atoms colored magenta.
interactions with the N-acetyl group, which is completely buried in the protein-saccharide interface at the back of subsite \(-1\). One of the interactions with the N-acetyl group involves a hydrogen bond between the side chain of Asp-297 and the N-acetyl amide, explaining the significant decrease in activity observed previously for a D297A sMltA mutant (11). Similar sugar binding interactions occur at subsite \(+1\), with the side chain of Val-161 making stabilizing van der Waals contacts with the sugar ring, whereas at the back, the N-acetyl group of the \(-1\) GlcNAc residue forms a hydrogen bond with the main chain carbonyl oxygen of Gly-100. The GlcNAc residue bound at subsite \(-1\) is tightly sandwiched between the \(H1102\)-loop connecting strands \(H12\) and \(H13\) in domain A (residues 298–306) and the C terminus of \(alpha\)-helix 5 from domain B (residues 161–164). The \(psi\)-loop forms a lid covering the \(beta\)-face of the \(-1\) sugar ring, whereas the C-terminal end of helix \(alpha5\) covers the \(alpha\)-face, to Asp-308 and the C1 atom so that it can serve its role as catalytic nucleophile.

Recognition of (Peptido)Glycan—The natural substrate of MltA is peptidoglycan, which differs from chitin in having every other GlcNAc residue replaced by a peptide-carrying MurNAc residue. The activity of MltA, however, does not depend on the presence of the peptides since the enzyme is able to cleave peptidoglycan chains from which the peptide moieties have been removed (11, 34). However, the enzyme cannot readily cleave chitin-based oligosaccharides such as chitohexaose,5 showing that the MurNAc C3-lactyl groups are essential for its activity. A model of a (GlcNAc-MurNAc)3 hexasaccharide, constructed on the basis of our chitohexaose-sMltA-D308A structure, shows

5 K. E. van Straaten, T. R. M. Barends, B. W. Dijkstra, and A.-M. W. H. Thunnissen, unpublished results.
that subsites \(-2\) and \(+1\) are specific for GlcNAc since there is not sufficient space at these subsites to accommodate the C3-lactyl group of a MurNAc residue. As a consequence, the MurNAc residues will bind in subsites \(-3\), \(-1\), and \(+2\) with their lactyl groups pointing outwards from the groove (Fig. 3A). This predicted binding mode is in agreement with the finding that 1,6-anhydro-MurNAc, but not 1,6-anhydro-GlcNAc, products are formed (34), implying that the scissile glycosidic bond connects a MurNAc and a GlcNAc residue at subsites \(-1\) and \(+1\), respectively. Indeed, a well defined pocket for binding the lactyl-group of a MurNAc residue can be discerned at subsite \(-1\) (Fig. 3B). It is lined by residue Gly-163 and the side chains of Tyr-180 and Val-234, residues of conserved sequence motifs I, II, and IV (5), respectively, which form a hydrophobic surface suitable for binding the lactyl methyl group. Furthermore, the hydroxyl group of Tyr-180 is ideally positioned to form a hydrogen bond with the carboxylate group of the lactyl moiety, which, also considering the interactions of this residue with the GlcNAc residue at subsite \(-2\), points to a crucial role of Tyr-180 in peptidoglycan binding. Interestingly, the second tyrosine residue from conserved sequence motif IV, Tyr-188, may have a similar function in binding the lactyl group of the \(+2\) sugar. Unfortunately, subsite \(+2\) in the structure of the chitohexaose-sMltA mutant complex shows considerable disorder, prohibiting a reliable analysis of the sugar-protein interactions. The \(-3\) MurNAc lactyl group is largely solvent-exposed and appears not to make contacts with protein atoms. Finally, in agreement with the non-essentiality of the murein peptide moieties for activity of MltA, no well defined sites or pockets lined with conserved residues could be identified on the protein surface, where the peptides might be accommodated.

**DISCUSSION**

Our analysis of the chitohexaose-bound sMltA-D308A structure provides the first experimental evidence of how an oligosaccharide binds in the active site of a family 2 lytic transglycosylase. It reveals that the conserved amino acid residues characterizing this family all have prominent roles in substrate binding and cleavage. In particular, Asp-308 is in a position suitable for a
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function as general acid/base. As in other lytic transglycosylases, there is no active site residue that can fulfill a role as nucleophile.

The implications of the large conformational flexibility observed in sMltA are unclear. In their natural environment inside the periplasm, lytic transglycosylases are believed to always stay associated with the peptidoglycan polymer, thus suggesting that the two apo-conformations observed for sMltA are not physiologically relevant. On the other hand, the observed conformational flexibility may well play a role in the regulation of the lytic activity of MltA, which is potentially detrimental to the cell. In that respect, it is interesting to note that MltA has been proposed to function as a component of a large murein-synthesizing complex in which its lytic activity is strictly coordinated to the synthetic activity of other components (35). Such coordination may benefit from a large conformational flexibility, allowing allosteric control, as observed in many other molecular machines.

The structural details of chitohexaose-bound sMltA-D308A allow us to propose a possible mechanism for the mode of action of MltA (Fig. 4A). In the first step of the reaction, Asp-308 acts as an acid, donating a proton to the oxygen of the scissile glycosidic bond. Proton transfer is facilitated by the interactions of the enzyme with the substrate at subsites −2, −1, and +1, enforcing a geometry of the −1 to +1 glycosidic linkage in which one of the lone pair orbitals of the glycosidic oxygen is optimally oriented for proton acceptance from Asp-308 (Fig. 4B). Cleavage of the glycosidic bond leads to the formation of a positively charged MurNAc oxocarbenium ion intermediate at subsite −1. In the second step of the reaction, Asp-308, in its ionized form, can act as a base, abstracting a proton from the C6 hydroxyl group of the −1 residue, thus activating O6 for an intraresidue nucleophilic attack on the C1 atom of the oxocarbenium ion. This will complete the reaction and result in the formation of the 1,6-anhydroMurNAc product.

This general course of the catalyzed reaction is similar to what has been proposed for the mode of action of the other lytic transglycosylases (7, 10). Two aspects of the lytic transglycosylase reaction mechanism are worth noting. Firstly, distortion of the −1 sugar into a “half-chair” or “sofa” conformation, as proposed for lysozyme (36, 37), may be one of the driving forces in catalysis by MltA. The proposed interactions of the C3-lactyl group at subsite −1 of MltA may be crucial for stabilizing this distortion by providing additional necessary binding energy. This would explain why MltA is unable to cleave chitin and why no distortion of the −1 GlcNAc sugar is observed in the chitohexaose-bound sMltA-D308 complex. The distortion of the −1 sugar toward a half-chair conformation is further proposed to be influenced by interactions of the −1 exocyclic C5-hydroxymethyl group, in particular with the highly conserved Tyr-101, Ala-259, and the catalytic Asp-308. Modeling studies indicate that the C5-hydroxymethyl group adopts a more favorable semiaxial position when the −1 MurNAc residue is in a sofa conformation rather than in a chair conformation (Fig. 4B).

In a sofa conformation, the O6 hydroxyl may release its close contact with Ala-259 and form a hydrogen bond with Asp-308. In this way, the O6 hydroxyl would get primed for its role as nucleophile in the second step of the reaction. In addition, the unfavorable close contact between the −1 C1 atom and the +1 O3 hydroxyl group, as observed in the chitohexaose-sMltA-D308A complex, would be removed in the sofa conformation. Finally, the sofa conformation would allow an even tighter fit of the −1 sugar in between the ψ-loop and helix α5, completely shielding the oxocarbenium ion intermediate from solvent, thus preventing a water molecule from attacking this highly reactive intermediate.

Secondly, the proposed mechanism of MltA implies the occurrence of an oxocarbenium ion reaction intermediate. Oxocarbenium ions are highly unstable, and in the general acid/base catalytic mechanisms of glycosyl hydrolases, they only occur as transition states (38). Lytic transglycosylases are retaining β-glycosidases, and therefore, cleavage of the β-1,4-glycosidic bond and formation of the 1,6-anhydro bond must take place in two consecutive steps. The reaction mechanism of retaining β-glycosidases usually involves a glycosyl-enzyme intermediate (38), but the lack of a second carboxylate group in the active site of MltA, suitable to act as a nucleophile in the first reaction step, prohibits this possibility. An alternative possibility is that the N-acetyl group of the −1 MurNAc residue acts as a nucleophile in the first reaction step via a mechanism of substrate-assisted catalysis, as has been proposed for the other lytic transglycosylases (7, 10). In the chitohexaose-bound sMltA-D308A structure, however, the orientation of the N-acetyl group of the −1 sugar is incompatible with such an assisting role in catalysis, i.e. a reorientation should occur to bring the carbonyl oxygen of the acetamido near the C1 carbon. Considering the large structural flexibility observed for MltA, and in the absence of an experimental structure with bound peptidoglycan, the possibility of such a reorientation cannot be completely excluded. Our modeling studies indicate, however, that a reorientation of the N-acetyl group of the −1 MurNAc is highly unfavorable as it would disrupt stabilizing hydrogen bonds with the protein and cause steric hindrance with the side chain of Val-161, as well as with the O3 hydroxyl of the +1 sugar. Another alternative mechanism for the lytic activity of MltA may be proposed in which the C3-hydroxyl of the +1 sugar residue acts as a nucleophile in the first reaction step, attacking the anomeric center from the α-face, thereby forming a reaction intermediate with an α-1,3-glycosidic bond. Although, again, at present, there is no experimental evidence to rule out such a mechanism, it seems unlikely as the intermediate would not be inherently active, and consequently, the reaction would still require a second catalytic residue in the active site. Thus, in the absence of a suitable candidate to act as a nucleophile in the first step of the reaction, we favor the reaction mechanism of MltA in which an oxocarbenium ion intermediate is formed, and we propose that this intermediate is stabilized by the helix dipole of helix α5. The −1 sugar is positioned just above the C terminus of this helix, at about 4 Å distance, and thus, the negatively charged side of the α5 helix dipole could be a significant contributor to the stabilization of the positively charged oxocarbenium ion intermediate. In this respect, MltA differs completely from the other structurally characterized lytic transglycosylases, for which no involvement...
of an α-helix dipole in stabilization of the oxocarbenium intermediate has been observed.

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REFERENCES

1. Höltje, J. V. (1995) Arch. Microbiol. 164, 243–254
2. Koraimann, G. (2003) CMLS Cell. Mol. Life Sci. 60, 2371–2388
3. Kraft, A. R., Templin, M. F., and Höltje, J. V. (1998) J. Bacteriol. 180, 3441–3447
4. Romeis, T., Vollmer, W., and Höltje, J. V. (1993) FEMS Microbiol. Lett. 111, 141–146
5. Blackburn, N. T., and Clarke, A. J. (2001) Biochemistry 36, 3441–3447
6. Thunnissen, A. M. W. H., Dijkstra, A. J., Kalk, K. H., Rozeboom, H. J., Engel, H., Keck, W., and Dijkstra, B. W. (1997) J. Bacteriol. 179, 6726–6734
7. Thunnissen, A. M. W. H., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1995) Biochemistry 34, 12729–12737
8. van Asselt, E. J., Thunnissen, A. M. W. H., Dijkstra, A. J., Kalk, K. H., Takacs, B., Keck, W., and Dijkstra, B. W. (1999) J. Mol. Biol. 291, 877–898
9. van Asselt, E. J., Kalk, K. H., and Dijkstra, B. W. (2000) Biochemistry 39, 1924–1934
10. van Straaten, K. E., Dijkstra, B. W., Vollmer, W., and Thunnissen, A. M. (2005) J. Mol. Biol. 352, 1068–1080
11. Thunnissen, A. M. W. H., Isaacs, N. W., and Dijkstra, B. W. (1995) Proteins 22, 245–258
12. Davies, G., and Henrissat, B. (1995) Structure (Lond.) 3, 853–859
13. Reid, C. W., Blackburn, N. T., Legaree, B. A., Auzanneau, F. I., and Clarke, A. J. (2004) FEBS Lett. 574, 73–79
14. Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., and Dijkstra, B. W. (1995) Biochemistry 34, 15619–15623
15. Tews, I., Terwisscha van Scheltinga, A. C., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997) J. Am. Chem. Soc. 119, 7954–7959
16. Williams, S. J., Mark, B. L., Vocadlo, D. J., James, M. N., and Withers, S. G. (2002) J. Biol. Chem. 277, 40055–40065
17. Markovic-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. J., Muller, U., and Schirmer, T. (2000) Structure (Camb.) 8, 1025–1035
18. Markovic-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. J., Muller, U., and Schirmer, T. (2000) Structure (Camb.) 8, 1025–1035
19. Powell, A. J., Liu, Z. J., Nicholas, R. A., and Davies, C. (2006) J. Mol. Biol. 359, 122–136
20. Davies, G. J., Dodson, G. G., Hubbard, R. E., Tolley, S. P., Dauter, Z., Wilson, K. S., Hjort, C., Mikkelsen, J. M., Rasmussen, G., and Schülein, M. (1993) Nature 365, 362–364
21. van Straaten, K. E., Dijkstra, B. W., and Thunnissen, A. M. W. H. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 758–760
22. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
23. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
24. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
25. Collaborative Computational Project, Number 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
26. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
27. Barends, T. R. M., de Jong, R. M., van Straaten, K. E., Thunnissen, A. M. W. H., and Dijkstra, B. W. (2005) Acta Crystallogr. Sect. D Biol. Crystallogr. 61, 613–621
28. Davis, I. W., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2004) Nucleic Acids Res. 32, 615–619
29. Hayward, S., and Berendsen, H. J. C. (1998) Proteins: Struct. Funct. Genet. 30, 144–154
30. Kleywegt, G. J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 1878–1894
31. Fenn, T. D., Ringo, D., and Petsko, G. A. (2003) J. Appl. Crystallogr. 36, 944–947
32. Davies, G. J., Wilson, K. S., and Henrissat, B. (1997) Biochem. J. 321, 557–559
33. Hol, W. G. (1985) Prog. Biophys. Mol. Biol. 45, 149–195
34. Ursinus, A., and Höltje, J. V. (1994) J. Bacteriol. 176, 338–343
35. Vollmer, W., von Rechenberg, M., and Höltje, J. V. (1999) J. Biol. Chem. 274, 6726–6734
36. Kuroki, R., Weaver, L. H., and Matthews, B. W. (1993) Science 262, 2030–2033
37. Strynadka, N. C., and James, M. N. (1991) J. Mol. Biol. 220, 401–424
38. McCarver, J. D., and Withers, S. G. (1994) Curr. Opin. Struct. Biol. 4, 885–892

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