Mechanism of the *Escherichia coli* MltE lytic transglycosylase, the cell-wall-penetrating enzyme for Type VI secretion system assembly

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Lytic transglycosylases (LTs) catalyze the non-hydrolytic cleavage of the bacterial cell wall by an intramolecular transacetalization reaction. This reaction is critically and broadly important in modifications of the bacterial cell wall in the course of its biosynthesis, recycling, manifestation of virulence, insertion of structural entities such as the flagellum and the pili, among others. The first QM/MM analysis of the mechanism of reaction of an LT, that for the *Escherichia coli* MltE, is undertaken. The study reveals a conformational itinerary consistent with an oxocarbenium-like transition state, characterized by a pivotal role for the active-site glutamic acid in proton transfer. Notably, an oxazolinium intermediate, as a potential intermediate, is absent. Rather, substrate-assisted catalysis is observed through a favorable dipole provided by the \(N\)-acetyl carbonyl group of MurNAc saccharide. This interaction stabilizes the incipient positive charge development in the transition state. This mechanism coincides with near-synchronous acetal cleavage and acetal formation.

The lysozyme family of the glycoside hydrolases (GHs) catalyzes the cleavage of the \(\beta\)-1→4-glycosidic linkage connecting the \(N\)-acetylmuramic acid (MurNAc) and \(N\)-acetyl-\(d\)-glucosamine (GlcNAc) saccharides of the \((\text{MurNAc-}\text{GlcNAc})_n\) polymer (the peptidoglycan) of the cell wall of bacteria. While the non-bacterial lysozymes themselves are hydrolytic catalysts, the lytic transglycosylase (LT) sub-families of the GHs are not.\(^1,2\) The LTs act on the same MurNAc-\(\beta\)-1→4-GlcNAc glycosidic linkage of the peptidoglycan to accomplish a non-hydrolytic scission so as to create two daughter strands having (respectively) 1,6-anhydroMurNAc and GlcNAc termini (Fig. 1).\(^3-5\) LT catalysis is used by bacteria for a host of functional transformations, including peptidoglycan biosynthesis, remodeling, recycling, and excavation for insertion of secretion systems and of flagella and pili. Evidence also correlates LT dysregulation to the bactericidal mechanism of the \(\beta\)-lactam antibiotics.\(^6\) The range of cell-wall processes that the LT reactions enable is impressive. This first QM/MM analysis of the LT reaction was undertaken to shed light on this unique transformation, critical for homeostasis of the bacterial cell wall.

The stereochemistry of LT catalysis is overall retention with respect to the anomeric carbon of the MurNAc saccharide. While the origin of the anhydroMurNAc product is that of an intramolecular interception of an oxocarbenium entity, the steps leading to this event are uncertain. One proposed mechanism uses substrate-assistance by forming an oxazolinium intermediate. In this mechanism, the MurNAc amide functional group acts first as a nucleophile and then as a nucleofuge (Fig. 1).\(^7\) Oxazolinium intermediates in GlcNAc glycosyl transfer are well recognized,\(^8\) and invoking this intermediate would account for the retention of configuration by the LTs through a sequence of two half-reactions, each requiring inversion.\(^9\) This proposed intermediate was also suggested to account for dispersing the charge developed at the anomeric carbon during bond breaking.\(^10\)

Within the LT sub-family, a glutamic (or aspartic) acid has the pivotal catalytic role. We elected to use QM/MM analysis to elucidate the LT reaction mechanism, using the membrane-bound lytic transglycosylase E (MltE) enzyme of *Escherichia coli* as our example. The small mass of this protein (approximately 21 kDa), the absence of peripheral domains that may impart influence, and the availability of quality crystal structures for MltE made it

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the appropriate choice for this study. MltE is a lipoprotein catalyst involved in the late stages of type VI secretion system assembly. MltE is also the primary endolytic LT (i.e., cleavage in the middle of a peptidoglycan strand) of E. coli. Its QM/MM study was anticipated to provide the first insights into the enigmatic mechanism of the LT enzymes.

We analyzed the molecular-dynamics production-phase trajectory of the complex and selected several snapshots that had suitable distances \(d_3, d_4, \) and \(d_5\) for the proton transfer events (Fig. 2 and Supplementary Fig. 1). One of the snapshots with appropriate distance parameters after MM optimizations was selected for the QM/MM calculations (see Supplementary Computational Methods for details). The selection of residues for the QM layer (Fig. 2) was made with attention to a comparative sequence analysis of the LT family and available X-ray structures (Supplementary Figs 2–5). Residue E64 (the catalytic glutamic acid); the side chains of S73, S75, and Y192; and the two active-site water molecules (Wat1 and Wat2) were carefully selected.

The Michaelis complex (Fig. 3a and Supplementary Fig. 6) was obtained following two-layer ONIOM QM/MM energy minimization method. The dominant features of the Michaelis complex are the hydrogen-bonding pattern of E64 and the \(\text{E}^{-1}\) conformation of the \(-1\) MurNAc saccharide (see Supplementary Information for details: the hexose conformers are described as boat (B), chair (C), envelope (E, previously “sofa”), half-chair (H, sometimes called half-boat or twist), and skew (S, sometimes called twist-boat) conformations (Fig. 4), according to the Cremer and Pople nomenclature). The competence of this Michaelis complex was tested for its ability to traverse the full reaction coordinate across a 1D potential-energy surface (PES) scan (see Supplementary Information for details). Coordinates obtained from the 1D PES provided the requisite starting points for two subsequent 2D-PES calculations of the key reaction steps. The first 2D-PES scan corresponds to the formation of the local energy minimum (\(\Pi_{00}\)), and the second scan to the formation of 1,6-anhydroMurNAc in a \(B_{32}\) conformation (\(\Pi_{10}\)) (Fig. 3).

The first 2D-PES scan starts from the Michaelis complex (I) and uses the glycosidic bond \(d_2\) scanned at 0.10 Å intervals from 1.40 to 2.20 Å and the distance between the glycosidic oxygen (O1) and the O\(^{-2}\) hydrogen...
of E64 (d3, scanned at –0.10 Å intervals from 2.50 to 1.00 Å) as the reaction coordinates. The lengthening of the C–O glycosidic bond is accompanied by the approach of hydrogen atom from E64 to O1. In this progression, the MurNAc undergoes an E1 → [E1]† → 2.5B conformational path. The locations of the Michaelis complex I (Fig. 3a and Supplementary Fig. 6: d2 = 1.40 Å, d3 = 2.50 Å) and the local minimum II3D (Fig. 3c and

Figure 3. MltE transformation. (a) Stereo representation of the Michaelis complex I. (b) The potential-energy surface with respect to the d2 and d3 reaction coordinates. (c) Intermediate II2D. (d) Transition species Ts22D between II2D and III2D. (e) The reaction path (orange arrows) from II2D through Ts22D to III2D. (f,g) 1,6-anhydroMurNAc in the B3,O conformation (III2D and IIIQM). (h) The QM potential-energy surface for B3,O to 1C4 conformational change. (i) 1,6-anhydroMurNAc in the 1C4 conformation (IVQM). Hydrogen bonds and the d3 are shown as blue and red dashed lines, respectively.

Figure 4. Conformational itinerary of −1 MurNAc along MltE transformation. I denotes the Michaelis complex; Ts12D, the transition species between I and II2D; II2D, the intermediate; Ts22D, the transition species between II2D and III2D; III2D and IIIQM, the 1,6-anhydroMurNAc in the B3,O conformation; Ts3QM, the transition species between III2D and IVQM; IVQM, the 1,6-anhydroMurNAc in the 1C4 conformation. A −1 MurNAc adopts boat (B), chair (C), envelope (E), and skew (S) conformations. The B3,O to 1C4 transformation is off-enzyme reaction.
Species II_D progresses toward the transition species Ts2_D, having the fully broken (2.90 Å) glycosidic bond in our second 2D-PES calculation. Continued progression results in the formation of 1,6-anhydroMurNAc in a B_{1,O} conformation (species III_{2D}), as shown in Fig. 3. Proton transfer mediated by the E64 carboxylate acting as a general base directs intramolecular bond formation between the O6 and the anomeric C1 of MurNAc. The reaction coordinates d_1 (distance between O6 and C1 of MurNAc, corresponding to bond formation) and d_5 (O6–H bond of MurNAc, corresponding to proton transfer) define the path to formation of species III_{2D} (Fig. 2). These distances were scanned at 0.10 Å intervals (d_1 from 3.29 to 1.39 Å, and d_5 from 0.90 to 2.00 Å). The resulting PES (Fig. 3e) shows the second transition species in a 2,5_S conformation (species III_{2D}). The 1,6-anhydroMurNAc in a B_{1,O} conformation (Fig. 3f: species III_{2D} at d_1 = 1.49 Å and d_5 = 1.80 Å). Progression along this path (arrows) to species III_{2D} at ~3.91 kcal·mol^{-1} is exothermic with respect to the Michaelis complex. The transition species Ts2_D, between species II_D and III_D, is 10.39 kcal·mol^{-1} higher in potential energy than II_D.

The progress from II_D to Ts2_D coincides with an increased positive charge on C1 (from 0.451 to 0.522 e), as calculated by natural population atomic charges (Supplementary Table 1). During this progress, the distance between the N-acetyl carbonyl oxygen of MurNAc (O_NAc) and the C1 shortens in order to stabilize the incipient positive charge on C1. As d_1 increases from 1.80 to 2.90 Å, d_5 (the distance between C1 and O_NAc) decreases from 3.14 to 2.83 Å. The increase in d_1 provides the necessary space between C1 and O1 for the H1 (the hydrogen on the C1) to assume the planar arrangement for the oxocarbenium species in Ts2_D. The MurNAc of Ts2_D shows a dihedral angle of C5–O5–C1–C2 (37.4°) and an out-of-plane angle of θ_NAc (10.9°). Additionally, the distance between C1–O5 shortens from 1.35 to 1.28 Å. These changes reflect the oxocarbenium character of species Ts2_D.

![Diagram of transition](image-url)

We note the importance of solvation within the active site for catalysis. In the Ts2_D species, Wat1 bridges between the H^+ of S75 and the O_NAc via hydrogen bonds (1.83 Å for both, Fig. 3d). In addition, Wat1 forms another hydrogen bond (1.89 Å) with the oxygen of C3 hydroxyl group of GlcNAc. These hydrogen bonds are maintained throughout the transition from species II_D to Ts2_D. The location of Wat1 prevents oxazolinium formation. Interestingly, a water molecule poised in a similar location is observed in the X-ray co-crystal structures (PDB IDs: 4HJZ, 1QTE, 1QUT, 1D0K, 5AO7, 3D3D, and 1D9U) of LT enzymes MltE, Slt70, Slt35, SltB3, SltB4, and SltB5.

The absence of the oxazolinium species from the two PESs likely is linked with the lack of a second carboxylate group. Indeed, the favorable energy change of this transformation might be a critical driving force for product release. To provide some degree of inhibition of MltE. At concentrations as high as 1 mM, we observed no inhibition of MltE.
gain insight into the energy barrier for this boat-to-chair transformation, a QM 2D-PES scan was conducted for the 1,6-anhydroMurNAc outside of the active site. Two dihedral angles were scanned in this calculation; dihedral C2–C3–C4–C5 (from 63.9° to −46.1° with −5.0° intervals) and dihedral H2–C2–N′–C′ (from −180.0° to 150.0° with 30.0° intervals). The 2D PES shows a potential energy for the B3,O boat that is +2.00 kcal·mol−1 above the 1C4 chair (Fig. 3h). The transition point (Supplementary Fig. 12: species Ts3,QM at C2–C3–C4–C5 = −31.1° and H2–C2–N′–C′ = −150.0°) adopts an E1O conformation that is +5.50 kcal·mol−1 higher than the B3,O conformation.

Interestingly, concomitant rotation occurs about the C2–N′ single bond, with the boat-to-chair transition. This rotation can be attributed to a relieving of the electrostatic repulsions in the 1C4 conformation among the O5, C4 oxygen, and N-acetyl carbonyl oxygen.

GHs bind their carbohydrate substrates in the non-ground-state conformation that optimally positions the exocyclic moiety at the anomeric carbon for departure as a leaving group. Understanding this conformational distortion is recognized as having widespread value for the development of GH inhibitors as antibiotics or potentiators of clinical antibiotics33. Our calculations support existence of such a substrate distortion in MltE. The conformation of the MurNAc in the peptidoglycan in solution is 1C4. On binding to MltE, overall turnover chemistry uses an E1→[E1]3→[E3]2→[II2D]→[II2D]3→[II2D]2→[II2D]1→[II2D]0 conformational itinerary (Figs 4 and 6). The initial E1 conformation imposed by MltE on its MurNAc substrate enables access to the transition point 1 (Ts1,2D) through least motion of the nuclei33. Formation of the intermediate II2D, by lengthening of the C–O glycosidic bond in response to hydrogen bonding by E64 (activation barrier of 17.09 kcal·mol−1) is overall endothermic by 6.55 kcal·mol−1. Subsequent further glycosidic-bond lengthening and proton transfer gives a free-base E64 poised to activate the C6 hydroxyl for interception of the oxocarbenium (Ts2,2D). The II2D conformation for Ts2,2D provides a favorable in-line approach of the nucleophilic O6 oxygen. Intramolecular bond formation between O6 and C1 gives the 1,6-anhydroMurNAc product in a B3,O conformation (species III2D). The transition species Ts2,2D is 10.39 kcal·mol−1 higher in potential energy relative to II2D, and formation of III2D is exothermic by −3.91 kcal·mol−1. There does not appear to be a unique rate-limiting step, as the two transition-step species are essentially of equal energy (Fig. 6). Relaxation of the B3,O boat to the 1C4 1,6-anhydroMurNAc chair is concurrent with, or subsequent to, product release. Our calculations are consistent with a mechanism of near-synchronous bond formation and bond cleavage, enabled by complementary conformational and electrostatic stabilization.
In this particular aspect, the reaction of MltE is an example of the mechanistically challenging front-face retaining glycoside-transferase enzymes\(^6\), exhibiting a near-synchronous pathway involving a short-lived oxocarbenium-like species\(^8\).

The LT family is implicated in a host of transformations preserving the function and integrity of the bacterial cell wall. This study establishes a mechanistic framework for further interrogation of the critical roles this family has in the biosynthesis, maturation and turnover of this important biopolymer.

**Methods**

**Calculations.** Molecular dynamics (MD) simulations used the AMBER 11 suite\(^5\). AMBER FF99 and GAFF provided simulation parameters. A snapshot was selected as the starting point, chosen by monitoring the distances and angles from the production phase trajectory, for the design of the QM/MM calculation. The two-layer version of the ONIOM\(^4\) method implemented in Gaussian 09\(^6\) was used. In a two-layer ONIOM method, the total energy of the system is obtained from three independent calculations: \(E_{\text{ONIOM}} = E_{\text{real,MM}} + E_{\text{model,MM}} - E_{\text{model,QM}}\) where ‘real’ refer to the whole system and ‘model’ refers to the chemically important part of the system (QM layer). The real system is calculated at MM level. MM method cannot describe bond breaking or formation. The model system is treated with more accurate, but considerably expensive QM method. The QM layer used the B3LYP/6-31G(d,p)//B3LYP/6-31G(d) level of theory while the MM layer used the AMBER FF99 force field. The QM layer included 123 atoms: the MurNac-GlcNac substrate; the catalytic residue E64; the side chains of S73, S75, and Y192; and the two active-site water molecules (Wat1 and Wat2). Potential-energy points in the QM/MM calculations were generated over a two-dimensional grid of two direct coordinates. All the stationary points (i.e. species I, T1D, T2D, T3D, T4D, T5D, T3Q, T3QM, and IVQM) were fully optimized with no reaction coordinate constraints before characterization by frequency calculations. Frequency calculations were performed with scale factors of 0.873 and 0.944 at B3LYP and M06-2X levels of theory, respectively, at 25°C and 1 atm. For more details, see Supplementary Computational Methods.

\((2R)-2-[(3aR,5R,6S,7R,7aR)-3a,6,7,7a-tetrahydro-6-hydroxy-5-(hydroxymethyl)-2-methyl-5H-pyran-3,2-d]thiazol-7-yl]oxy\)propanamide (1). Compound 6 (0.10 g, 0.32 mmol, see Supplementary Information) was dissolved in methanol (4 mL) and 7N ammonia in methanol was added (3 mL, 21 mmol). The reaction mixture was stirred overnight at RT, similar to a previously reported method\(^37\). The reaction mixture was filtered through a cotton plug and concentrated by rotary evaporation. The titled compound was obtained as a white solid (97 mg, 99%) after vacuum drying; TLC (1:9 MeOH:CH₂Cl₂): \(R_f = 0.17\).

\(^1\)H NMR (400 MHz, CD₃OD) \(δ = 1.43 (d, J = 6.9 Hz, 3 H), 2.27 (d, J = 2.0 Hz, 3 H) 3.37 (dd, J = 11.3, 5.6, 2.7 Hz, 1 H), 3.63-3.69 (m, 1 H), 3.69-3.73 (m, 1 H), 3.73-3.80 (m, 1 H), 3.91 (t, \(J = 4.8\) Hz, 1 H), 4.31 (q, \(J = 6.9\) Hz, 1 H), 4.48 (ddd, \(J = 7.1, 5.1, 1.0, 2.0\) Hz, 1 H), 6.38 (d, \(J = 7.1\) Hz, 1 H); \(^13\)C NMR (101 MHz, CD₃OD) \(δ = 118.16, 19.38, 61.51, 68.45, 74.95, 75.76, 77.34, 80.44, 89.48, 169.55, 177.90\). MS (\(m/z\)) \([\text{M} + \text{H}]^+\), calcd for C₁₁H₁₉N₂O₅S, 291.1009; found, 291.1023.

\((2R)-2-[(3aR,5R,6S,7R,7aR)-3a,6,7,7a-tetrahydro-6-hydroxy-5-(hydroxymethyl)-2-methyl-5H-pyran-3,2-d]thiazol-7-yl]oxy\)propanoic acid (2). Compound 6 (0.10 g, 0.32 mmol) was dissolved in 1:1 THF:water (2 mL). Solid LiOH monohydrate (15 mg, 0.36 mmol) was added. The mixture was stirred for 2 h at RT. The solution was filtered using a cotton plug and concentrated. The solid was vacuum dried to give (100 mg, 99%) of an off-white solid: \(^1\)H NMR (400 MHz, CD₃OD) \(δ = 1.35 (d, J = 6.9 Hz, 3 H), 2.25 (d, J = 2.5 Hz, 3 H), 3.08-3.21 (m, 1 H), 3.54 (dd, \(J = 12.0, 6.4\) Hz, 1 H), 3.63-3.76 (m, 2 H), 4.04-4.19 (m, 2 H), 4.67 (ddd, \(J = 7.1, 5.1, 1.0, 2.0\) Hz, 1 H), 6.34 (d, \(J = 7.1\) Hz, 1 H); \(^13\)C NMR (101 MHz, CD₃OD) \(δ = 18.61, 18.80, 62.49, 68.16, 73.98, 76.12, 77.76, 78.80, 88.84, 168.73, 180.25\). MS (\(m/z\)) \([\text{M} + \text{H}]^+\), calcd for C₁₁H₁₉LiNO₅S, 298.0931; found, 298.0915.

**Cloning and purification of MltE wild-type.** The cloning and purification of MltE from E. coli K12 substrain MG1655 was previously reported by our lab\(^36\). MltE wild-type was cloned into pET-24a(+) vector (Novagen) using restriction enzyme NdeI to XhoI. The gene encodes for residues 1–18 of MltE. The wild-type MltE was expressed and purified as previously reported. The final concentration of the MltE wild-type was determined by a BCA (Bicinchoninic Acid) Protein Assay kit (Fierce). The final yield of the purification was approximately 56 mg of protein per 0.5 L of liquid culture. The proteins were stored at –80°C and after thawing on ice, no precipitate formed.

**Lytic transglycosylase activity assay.** The E. coli MltE fluorescence activity assays were conducted using a EnzChek® Lysozyme Assay Kit (Invitrogen). The kit includes fluorescein-labeled sacculus (cell wall) from the Gram-positive bacteria Micrococcus lysodeikticus. Sacculi of Micrococcus species is commonly used in the analysis of LT activity, as it is commercially available and provides a high-level of reactivity with LT\(^1⁶\). MltE reactions (100 μL) were prepared by incubation of 50 μL of sacculus (substrate at a 1X dilution in 100 μL, as described in the kit) and 50 μL of MltE (final protein concentration 8 μM). Immediately after mixing, the change in fluorescent intensity was monitored for 30 minutes at room temperature on a Cary Eclipse Fluorescence Spectrophotometer (Agilent). Prior to the experiment, the protein was buffer exchanged into 100 mM NaPO₄, pH 7.5 supplemented with 100 mM NaCl using a Zeba Desalting Column (Thermo Fisher Scientific). Reactions containing compound 1 or 2 were incubated on ice for 20 min in the presence of MltE, prior to incubation with the sacculus at the start of the reaction. Fluorescence readings were obtained at an excitation wavelength of 485 nm and an emission wavelength of 516 nm. The results are displayed in Supplementary Fig. 11.
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Author Contributions
B.B., K.V.M. and M.K. performed the calculations. D.R.M. and E.S. synthesized the compounds 1 and 2 and carried out activity assay. D.A.D., J.F.F. and J.A.H. participated in mechanistic discussions. B.B., J.F.F. and S.M. wrote the paper. All authors edited the manuscript.

Additional Information
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