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The Crystal Structure Analysis of Group B Streptococcus Sortase C1: A Model for the 'lid' Movement upon Substrate Binding

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Abstract

A unique feature of the class-C-type sortases, enzymes essential for Gram-positive pilus biogenesis, is the presence of a flexible 'lid' anchored in the active site. However, the mechanistic details of the 'lid' displacement, suggested to be a critical prelude for enzyme catalysis, are not yet known. This is partly due to the absence of enzyme-substrate and enzyme-inhibitor complex crystal structures. We have recently described the crystal structures of the *Streptococcus agalactiae* SAG2603 V/R sortase SrtC1 in two space groups (type II and type III) and that of its 'lid' mutant and proposed a role of the 'lid' as a protector of the active site hydrophobic environment. Here, we report the crystal structures of SAG2603 V/R sortase C1 in a different space group (type I) and that of its complex with a small-molecule cysteine protease inhibitor. We observe that the catalytic Cys residue is covalently linked to the small molecule inhibitor without lid displacement. However, the type I structure provides a view of the sortase SrtC1 lid displacement, while having structural elements similar to a substrate-sorting motif suitably positioned in the active site. We propose that these major conformational changes, seen in the presence of a substrate mimic in the active site, may represent universal features of class C sortase substrate recognition and enzyme activation.

Introduction

Pathogenic bacteria employ multiple strategies to ensure their survival, proliferation and persistence in a hostile host environment. Many Gram-positive pathogens express fine fibers called pili on their bacterial cell surface.^{1; 2; 3} These filaments are virulence factors that participate in many interactions, such as adherence to host epithelia, colonization and

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invasion of host tissues, biofilm formation, and stimulation of the host immune responses.^{4; 5; 6; 7} Gram-positive pili are typically composed of two to three structural pilus proteins or pilins^{1; 8; 9} and require sortase enzymes for pilus assembly and anchoring.^{1; 10; 11} A typical pilus is made of a shaft of covalently connected major pilins, with one minor pilin at the tip of the shaft and the other minor pilin at its base; a few exceptions occur in some pathogens.^{12; 13; 14} The pilus biogenesis mechanism, initially proposed for *C. diphtheriae* SpaABC pili^{10; 11; 15; 16; 17} and subsequently investigated in other streptococcal pilus systems such as *S. agalactiae*,¹⁸ consists of cross-linking the major pilins, a process catalyzed by the class-C-type sortases or the pilus-specific sortases;^{18; 19} termination of pilin cross-linking; and anchoring of the pilus on the bacterial cell wall by the class-A-type sortase enzyme,^{20; 21} which is generally known as the housekeeping sortase. Deletion of either the major pilins or the associated class C sortases results in the abrogation of the pilus assembly.^{15; 18} In many pathogens, deletion of the adhesive pilin components or the corresponding sortase enzyme decreases bacterial adhesion to cells, as well as to tissues in animal models.^{20; 22; 23} Apiliated phenotype in some pathogens is associated with a decrease in virulence.⁷

Streptococcus agalactiae or Group B Streptococcus (GBS) is a major cause of bacterial infections in neonates within the United States and worldwide.^{24; 25} The most commonly occurring GBS infections in neonates are septicemia, pneumonia, and meningitis. GBS disease can also manifest as a range of complications in pregnant adults, such as symptomatic and asymptomatic bacteriuria, endometritis, amnionitis, meningitis, and pyelonephritis.²⁶ In recent years, invasive GBS disease has also emerged in the non-pregnant, elderly population.^{27; 28; 29} Due to the absence of commercial vaccines and the problem of increased antibiotic resistance, a fundamental understanding of the mechanisms of bacterial adherence and invasion of host epithelial barriers is required for developing alternative treatments for GBS disease.^{30; 31; 32; 33}

GBS pili are typically encoded by the pathogenicity islands I (PI-1) and 2 (PI-2).⁹ The GBS strain SAG2603 V/R (serotype V) expresses two pili clusters, PI-1 and PI-2a, the latter of which is one of the two variants of PI-2.^{9; 18} The PI-1 cluster, also known as the *srtC1-srtC2* locus, consists of six genes that encode three pilin proteins (GBS80, GBS52 and GBS104), two pilus-specific sortase enzymes (SrtC1 and SrtC2) and a transcription factor belonging to the *araC* family of regulators. The locus for the *srtA* gene, coding for the housekeeping sortase A, is far from the *srtC1-C2* locus.¹⁸ SrtC1 catalyzes the polymerization of the GBS80 subunits (with the sorting motif IPNTG) to form the pilus backbone, and in this respect, SrtC1 shares functional redundancy with SrtC2.

The incorporation of the minor pilins GBS52 and GBS104 into the pilus specifically requires SrtC1 and SrtC2, respectively; however, the precise sequence of events involved in this process is not known. In the next step, the Lys residue of the pilin-like motif of GBS52, which is bound to SrtA, resolves the SrtC1-pilus polymer complex.³⁴ This leads to the release of SrtC1, termination of the pilus assembly, and anchoring of the assembled pili into the growing peptidoglycan cell wall.²¹ The sorting motifs of the minor pilins differ only in the first amino acid residue, i.e., IPKTG and FPKTG in GBS52 and GBS104, respectively. Both SrtA and SrtC1 can recognize the IPKTG of GBS52.

GBS sortase C1 (GBSSrtC1) and GBS sortase A (GBSSrtA) exhibit the typical sortase fold.^{35; 36} The putative active sites of these enzymes are formed by three β -strands, with the conserved TLXTC motif harbored on the central strand of the active site. In GBSSrtC1, the binding pocket is a long and narrow groove, whereas a shallow and wider pocket is observed for GBSSrtA. The conformations of the catalytic residues are also subtly different between the two GBS sortases. The cysteine sulfhydryl of the GBSSrtA points toward the $\beta 7/\beta 8$ loop,

whereas that of GBSSrtC1 is directed toward the elongated groove. In addition, GBSSrtC1 contains a 'lid' region positioned in its putative active site, which is absent in that of GBSSrtA. This 'lid', considered a universal feature of class C sortases, was also observed in the crystal structures of pilus-specific sortases from GBS PI-2a of strain 515, *Streptococcus pneumoniae* *rlrA* pathogenicity island, *Streptococcus pyogenes* (serotype M1 strain 370) FCT island and *Actinomyces oris* strain T14V type I fimbriae.^{37; 38; 39; 40}

Site-directed mutagenesis and enzyme kinetic studies of class C sortases established the essential requirement for a catalytic Cys residue for the transpeptidation reaction and confirmed the role of the neighboring Arg and His residues, both for the polymerization of major pilins as well as for incorporation of the assembled pili into the bacterial cell wall.^{41; 42} Manzano et al. (2009) demonstrated that the lid of class C sortases is secured in the active site by a three-residue anchor motif DP(Y/W/F) and suggested that the charge interactions between the Asp of this motif and the active site catalytic Arg dictate the enzyme stability.⁴¹ In addition, we have recently identified a fourth essential residue (HB-lid), a leucine in GBSSrtC1, preceding the anchor motif of the lid region and positioned just above the hydrophobic pocket of the active site. The HB-lid residue is seen spatially conserved across all class C sortases of known structure.³⁶ Another universal feature of the 'lid' region is its high flexibility; however, both the flexibility and the conformation of the 'lid' vary between class C sortases. The GBSSrtC1 'lid' is unique in its length and in the high flexibility of the hinge regions at both ends. Site-directed mutagenesis and thermal stability studies on pneumococcal, pilus-specific sortases suggest that the active site with the lid represents an inactive state of the enzyme, and it is intuitive that the lid displacement is essential for enzyme activation and function.^{37; 41}

The inefficient and unstable anchor motif mutant of *S. pneumoniae* SrtC1 was shown to regain stability upon the addition of substrate-like peptide, suggesting that the 'lid' displacement and substrate binding in the active site could happen concomitantly.⁴¹ Like the displacement of the blocking loop in penicillin-binding proteins, another class of transeptidases with occluded active sites that become occupied when incubated with peptide-like substrates *in vitro*,⁴³ the lid displacement in pilus-specific sortases could result from recognition of the appropriate sorting motif. Because GBSSrtC1 can recognize both GBS80 and GBS52 (sorting signals IPNTG and IPKTG, respectively) and because the housekeeping SrtA can only recognize GBS52, one could suggest that specific structural features, in addition to the sorting motifs that dictate reaction specificity, may possibly present on both participants of the transpeptidation reaction. Understanding the structural correlates that dictate such narrow substrate specificity of sortases is of high interest. Substrate recognition by pilus-specific sortases, the mechanics of the 'lid' region displacement and its post-displacement status are interesting phenomena to visualize. In addressing some of these questions, we report the crystal structures of GBSSrtC1 in a space group different from that of the previously reported structures and of GBSSrtC1 in complex with MTSET (2-(trimethyl-ammonium)-ethyl-methanethiosulfonate bromide), a potent cysteine protease inhibitor. Fortuitously, the apo-GBSSrtC1 exhibited significant and interesting conformational changes upon interactions with a loop from the neighboring molecule, while the intruding loop bears remarkable resemblance to the substrate sorting motifs. Furthermore, we show that while displacement of the lid may be essential for promoting substrate accessibility to the active site, the catalytic Cys is available for interactions even in the presence of the lid, as observed in the GBSSrtC1-inhibitor complex.

Results

The crystal structure of apo-GBSSrtC1-I

The packing of GBSSrtC1-I is different from that in the previous GBSSrtC1 structures—

The GBSSrtC1 full-length recombinant protein (2–219, using the numbering scheme of *S. aureus* SrtA) is crystallized in the *C2* space group (hereafter referred to as GBSSrtC1-I for type I GBSSrtC1 crystals) with a monomer in the asymmetric subunit,⁴⁴ unlike the previously reported type II and type III crystals of the same recombinant protein in the *P2₁2₁2₁* and *P3₁2* space groups, both with two molecules in the asymmetric subunit related by a non-crystallographic two-fold axis. The GBSSrtC1-I structure contains a core with the sortase fold³⁵ and two N-terminal helices (Figure 1). Apart from the loop connecting helix 2 (H2) to strand 1 (β 1), this structure exhibits an average rmsd of 0.72 Å for 165 C α atoms with type II and III GBSSrtC1 crystal structures. The monomer (**A**) present in the asymmetric unit of GBSSrtC1-I crystals is juxtaposed with three symmetry-related molecules (**B**, **C**, and **D**) (Figure 2a). The first symmetry mate (**C**) forms a dimer interface involving the respective β 6 strands, an association previously observed for the two non-crystallographic 2-fold related monomers of both type II and type III GBSSrtC1 structures.³⁶ The junction of H1-H2 forms the crystallographic 2-fold related packing interface with the same region of the second symmetry mate (**D**); the side chain of Arg18 of one molecule forms a hydrogen bond with Asn22 of the neighboring molecule and vice-versa. The last and most significant interaction consists of two loops (β 4/ β 5 and β 7/ β 8) from a symmetry mate (**B**) facing the putative active site of the monomer in the asymmetric unit (Figure 2a and 2b).

The type I GBSSrtC1 'lid' is displaced from the putative active site

A characteristic feature of all pilus-specific sortases, including GBSSrtC1, is the presence of a lid region positioned along the putative active site.^{37; 38} As detailed previously,³⁶ the hinge region on both ends of the lid, that is, the region between H2 and the 'lid' on its N-terminal side (residues 37–42) and the linker between the lid and the β 1 strand (residues 53–69), are disordered in GBSSrtC1 structures of type II and type III. The reported crystal structure of the 'lid' DP(Y/W/F) motif mutant (PDB code: 3RBJ) helped us to conclude that the minimal region of the lid (residues 43–52), specifically Leu47, is positioned to protect a hydrophobic pocket in the enzyme active site.³⁶ However, residues 38–48 in GBSSrtC1-I, which also include part of the 'lid' (residues 43–48) and the N-terminal hinge (residues 37–42) reported for type II and type III GBSSrtC1 structures, are disordered.

The 20-residue long β 4/ β 5 linker that intrudes into the active site of the neighboring GBSSrtC1 molecule hosts a beta-turn and a short helical segment. The central residues Leu125-Pro126-Thr127-Ala128, which constitute a beta-turn, form the protruding end of the loop (hereafter called 'sym-loop' for symmetry loop; Figure 3a). Significantly, the superposition of GBSSrtC1-I and its active-site-bound sym-loop on type II or type III chain A or B structures (PDB Code: 3RBK and 3RBI) reveals that Pro126 of the sym-loop occupies the exact same space and superimposes perfectly with Leu47, the HB-lid residue suggested to be the fourth essential anchoring residue of the lid region in class C sortases (Figures 3b, 3c and 4).³⁶ In addition, the Thr127 of this sym-loop is positioned close to the Asp49 of the DP(Y/W/F) motif of GBSSrtC1-II/III structures (Figure 3c), resulting in polar interactions between sym-loop Thr127 and catalytic Arg193 (~ 3.08 Å). Because of the interactions between the intruding LPXTG-like sequence of the sym-loop and the putative catalytic site, the naturally occurring 'lid' is displaced from its location, providing us a glimpse of possible substrate-induced interactions.

Part of the displaced 'lid' and the C-terminal hinge (residues 49–69) form a five-turn α -helix (H3) that lies roughly parallel with the β 4 strand, at approximately 60° with respect to H2. H3 is held in this orientation by multiple interactions with the barrel core and the H2 helix. The hydroxyl of the Tyr51 side chain present at the N-terminal end of H3 forms a hydrogen bond with Glu26 and the hydroxyl of Tyr63. The latter lies almost midway down H3 facing the barrel core and forms a hydrogen bond with His102 ($d_{\text{N}\delta\text{1-O}} = 2.95 \text{ \AA}$) on the β 3 strand of the barrel core. The aromatic ring of Tyr63 also forms a π -stacking interaction with Tyr31 on the H2 helix in an off-center parallel-displaced orientation ($d \sim 4.5\text{--}4.7 \text{ \AA}$).⁴⁵

The loop (residues 69–72) between H3 and the β 1 strand is well ordered and structured, with an average B-factor of $39.44 \text{ \AA}^2$ (mean overall $B = 39.1 \text{ \AA}^2$). The outside face of the H3 helix hosts Arg65 and Glu62, which, along with two Arg residues on the H2 helix and a Glu between H3 and the β 1 strand, forms a highly polar region. The Arg24 on H2 hydrogen bonds with Glu71 on the H3- β 1 link ($d = 2.96 \text{ \AA}$); the interaction between Asn5 from the H1 helix and Gln72 on the H3- β 1 link further stabilizes the H3.

The crystal structure of the GBSSrtC1-MTSET complex

The crystals of the complex of GBSSrtC1 and MTSET belong to the $C2$ space group, with six molecules in the asymmetric subunit arranged in three dimers. The dimer interface is identical to that seen in the type II and type III apo-GBSSrtC1 structures (Figure 5a). All six molecules possess the sortase barrel core, but the length and conformations of the N-terminal helical regions are variable, indicating a certain degree of plasticity in these sections. For instance, an atypical four-turn H2 and a three-turn H1 α helix is observed only in molecule A.

The typical C-type-sortase-specific 'lid' is present in all six monomers, with temperature factors up to twice the mean B value of the respective full molecule. The flexibility of the lid is further emphasized by the variable conformation of residues beyond Y51 of the anchor motif (Figure 5b). Electron density for the covalently linked inhibitor molecule (Figure 5c) is seen for all six molecules with no significant changes in the conformations of the residues in and around the catalytic site. The orientation of the Cys184 side chain is toward the center of the active site groove, while the inhibitor molecule linked to the Cys184 $S\gamma$ atom is oriented away from the catalytic His122 and packed between the β 7/ β 8 loop on one side and Tyr51 of the anchor motif on the other. The presence of the lid and the inhibitor-bound Cys in the active-site groove shows that the catalytic residue is accessible to small-molecule inhibitors but does not disturb the anchor motif.

Discussion

Class C sortases are critical for the covalent cross-linking of major pilin proteins, a characteristic feature of Gram-positive pilus biogenesis. A detailed, mechanistic understanding of the sortases' role and the structural features that dictate their substrate specificities is still evolving. Efforts toward a clear understanding are impeded by a 'lid' region in the enzyme active site, which represents an inactive state of the enzyme.⁴¹ The enzyme's substrate specificity and the substrate's structural requirements for the enzyme 'lid' displacement may be two intertwined factors, and deciphering either one will provide insights to the other. Toward this understanding, we present two crystal structures of GBSSrtC1, one in complex with an inhibitor and the other of the apo-enzyme, in which the 'lid' is displaced from the active site. While the 'lid' is intact in the inhibitor complex, the apo-enzyme crystal structure exhibits an extra 5-turn α -helix, which is formed by the displaced 'lid' and its flexible C-terminal hinge region, compared to the other apo-enzyme structures of GBSSrtC1 presented in an earlier report.³⁶ The vacated space in the active site of GBSSrtC1-I is occupied by a substrate mimic, compensating for the hydrophobic and

polar interactions normally provided by the 'lid'. The present crystal structures provide the first glimpse of a pilus-specific class-C-type sortase bound to an inhibitor and a substrate mimic.

The GBSSrtC1 protein was incubated with MTSET for co-crystallization, and diffraction-quality crystals emerged out of the initial poorly formed crystalline material over a period of about two years. The six molecules present in the asymmetric unit are arranged as three dimers in which the NCS 2-fold related partners have an interface similar to that observed for the apo-structure dimers in type II and III crystal packing, which confirms that the dominant dimer association is unlike that seen for most other pilus-specific sortases. Despite much higher B factors for the minimal parts of the 'lid' in the active site compared to the corresponding regions in the type II and type III apo-GBSSrtC1 structures, the conformations of the critical Leu47, Asp49, Pro50 and Tyr51 residues of the lid, the catalytic residues, and their neighbors in the active site remain almost the same. The binding of the small-molecule irreversible cysteine protease inhibitors MTSET and E64 in solution was confirmed by mass spectrometry and NMR, respectively (results not shown). Because the network of interactions between the 'lid' and the active site residues controls the lid's position, its displacement and the subsequent binding of the LPXTG sorting motif, we can suggest that the GBSSrtC1 catalytic Cys residue is spatially available for binding in solution. However, we could not crystallize E64-bound GBSSrtC1, possibly due to the steric interactions between E64 and the 'lid' region around the active site Cys. It is also likely that only a fraction of the protein binds E64, hindering crystallization of the complex.

In comparison, the apo-GBSSrtC1-I crystal structure shows two interesting features in the enzyme active site: (1) an intruding, active-site-docked, substrate-like loop from the neighboring molecule and (2) the displaced 'lid', which is transformed into a five-turn α -helix. Manzano et al. (2009) demonstrated that mutants of *S. pneumoniae* sortase SrtC-1 anchor motif (Asp58Gly) and the catalytic residue (Arg202Glu) are much less stable than the wild-type protein.⁴¹ Thermal stability measurements on anchor motif mutants showed a drastic decrease in enzyme stability, possibly due to the lack of key 'lid' interactions originating from the DP(Y/W/F) anchor motif; stability was restored by the addition of substrates that host a conserved motif containing proline and threonine residues.⁴¹ However, in our previous report we showed that the crystal structure of an anchor motif mutant of GBSSrtC1 still has the 'lid' in the active site, mainly due to the hydrophobic interactions between the Leu47 (the hydrophobic 'lid' residue, HB-lid) and the hydrophobic residues present at the bottom and surrounding walls of the active site (the hydrophobic pocket). Therefore, we concluded that more than the anchoring DP(Y/W/F) motif, the conserved and preceding HB-lid residue, which is present in all pilus-specific sortases, is critical for positioning the 'lid' in the active site and is, therefore, important for enzyme stability.

It is important to note that the stabilizing YPRTG (sorting motif of minor pilin) and IPQTG (major pilin sorting motif) peptides, which are recognized by *S. pneumoniae* SrtC-1, have the conserved Proline, Threonine and Glycine residues in same positions; this is also the case with the sorting motifs of GBS major and minor pilins, GBS80 and GBS52, which host IPNTG and IPKTG, respectively. Interestingly, the intruding loop with the LPTA sequence (a sequence similar to the generic sorting motif) observed in the GBSSrtC1-I structure is a tight β -turn and is contained in a 20-residue long loop that connects the β 4 and β 5 strands. Unfortunately, the crystal structures of full-length GBS pilin proteins (GBS80, GBS52 and GBS104) up to their C-terminal ends have not been determined, making it difficult to speculate on the secondary structural elements around the sorting motifs. Similarly, despite our best efforts, we (and many others) have not been able to generate structures of full-length substrate protein bound sortase complex structures that could reveal the mode of

binding and visualize the structural elements of both partners. Two complex crystal structures of *Staphylococcus aureus* sortase A, one with a substrate sorting motif peptide⁴⁶ and the other containing an engineered link to a substrate-like peptide,⁴⁷ are available, but both structures suffer from structural limitations to explain substrate-enzyme interactions. Interestingly, Yamamura et al. (2011)⁴⁸ recently suggested that the introduction of a beta-hairpin prior to the sorting motif enhanced the sortase enzyme mediated ligation of proteins with a C-terminal sorting motif to a protein that acts as a nucleophile to resolve the acyl enzyme intermediate, analogous to the pilin-pilin linking during pilus polymerization. This introduced beta-hairpin, similar to the conformation of the substrate-mimic LPTA in GBSSrtC1-I, may provide the required rigidity for the scissile bond and its surroundings.

The intruding loop from the symmetry mate, a result of crystallization and packing arrangements within the crystal, surprisingly provides a unique insight into the possible mechanism of substrate binding for GBSSrtC1. The third (X) and to some extent the first (Leu) residue of the sorting motifs (LPXTG) are variable among most Gram-positive surface proteins, while sortases always recognize and cleave the peptide bond between the Thr and Gly residues. Because the intruding loop hosts the LPTA sequence, which contains residues that are critical for binding the sortase active site,⁴¹ we suggest that the LPTA sequence is a mimic of the canonical LPXTG sorting motif and is therefore able to dislodge the 'lid' from the active site of GBSSrtC1. It is all the more significant that the proline residue of this substrate-mimic loop superimposes exactly with the position of the highly conserved HB-lid (Leu47 in GBSSrtC1-II and III crystal structures) residue and occupies the vacated hydrophobic space in the GBSSrtC1-I structure (Figure 4). One of the perceived roles of the Pro residue, an invariant feature of the sorting motif, is precise positioning of the scissile bond, i.e., the peptide bond between Thr and Gly residues, which is susceptible for nucleophilic attack by the catalytic Cys residue. The correct orientation of this bond is especially critical in sortases, in which the active site Cys and its catalytic partners are positioned on rigid β -strands rather than on flexible loops as observed in other cysteine proteases such as papain and calpain. Our modeling studies confirm the role of Pro, facilitated by the hydrophobic residues of the active site, in positioning the Thr residue closer to the catalytic Arg residue. The distance between the Thr of LPTA and the catalytic Arg guanidino group (presently ~ 3.0 Å) will be shorter in the substrate LPXTG-bound structure, as the introduced third variable residue X (N or K in GBS PI-1 pilins) would shift the side chain of the third residue closer to the flexible catalytic Arg193 side chain.^{49; 50} It is also possible that the third residue occupies the position of the Asp of the anchor motif, having hydrogen bond interactions with the catalytic Arg. These possibilities are consistent with the conservation of residues as well as the importance of the anchor motif and HB-lid positions. Furthermore, the ensuing interactions (hydrogen bond formation with the catalytic Arg and hydrophobic interactions of the enzyme with the Leu-Pro segment of the sorting motif) have been suggested earlier by Bentley et al. (2008)⁵¹ as essential for *Staphylococcus aureus* SrtA function. We propose that given the nature of the hydrophobic pocket and the HB-lid residue, these interactions are especially important for class C sortases.

The hinge between the anchor motif and the $\beta 1$ strand, which was disordered to varying extents in GBSSrtC1-II and -III crystal structures, forms a five-turn alpha helix (H3) in the GBSSrtC1-I structure (Figure 6a and 6b). The minimal parts of the lid (residues 44–54) observed in the active sites of GBSSrtC1-II and -III crystal structures are mostly disordered in GBSSrtC1-I; residues 50–72, which include Asp49 and Pro50 of the DP(Y/W/F) motif, form the helix H3 and the C-terminal hinge. The propensity of this segment to form a helix was also predicted by PSI-PRED software.⁵² The N-terminus of the H3 helix begins after Pro50 of the DP(Y/W/F) motif, a residue that moves as far as 30 Å (C α positions) from its anchored position observed in GBSSrtC1-II and -III crystal structures (Figure 6b). In

comparison, the region corresponding to the H3 helix in the crystal structure of the DP(Y/W/F) mutant (PDB code 3RBJ) was mostly disordered and not seen in the electron density, while residues 60–72 remain as a loop. The latter was also observed in native GBSSrtC1-II and -III and in the pneumococcal SrtC1 and SrtC2 crystal structures.

Hence, based on the GBSSrtC1-I crystal structure, we suggest that the substrate sorting motif, present as part of a loop region, is able to bind to the GBSSrtC1 active site by displacing the ‘lid’ region. This displacement is facilitated by the hydrophobic interactions between the Pro residue of the substrate LPXTG motif and the hydrophobic residues of the enzyme active site. The accompanying conformational changes manifest as an α -helix that moves out of the way, facilitating the binding of bulky substrates like major pilins in the sortase active site. Instead of a 23-residue flexible loop (residues 50–72), a semi-rigid and compact 5-turn α -helix would occupy limited space and could be transformed and transported back into the active site after exit of the reaction product.

The presence of a displaceable ‘lid’ that blocks substrate accessibility in the enzyme active site and thereby acts as a gateway for substrate recognition and enzyme activity has been observed in a number of other enzymes and proteins.^{53; 54; 55} However, the mechanisms of lid displacement and subsequent stabilization of the displaced lid vary in complexity and magnitude. Conformational changes in enzymes after substrate binding, some of which are propagated a few tens of angstroms away, are also well recorded and studied.^{50,56} An exquisite example of an intruding loop from the symmetry-related neighboring molecule that bears similarities to the substrate primary sequence, producing significant conformational changes in the host macromolecule, is the case of the integrin I domain.^{57,58} The coordination of the Mg^{2+} ion in this structure is completed by a glutamate side chain of a symmetry mate, and the resulting interactions that mimic the naturally occurring integrin-ligand association contributed to a 10 Å shift of the C-terminal helix, which is a prelude for integrin-mediated inside-out/outside-in signaling across the cell membrane. We propose here that the observed shift of DP(Y/W/F) motif by ~30 Å and formation of a five-turn α -helix, which results from interactions between a LPXTG-like (symmetry-related) loop and the enzyme active site, presents a snapshot of substrate-induced ‘lid’ displacement that is essential for pilus polymerization by pilus-specific sortases.

Despite our best efforts, we were unable to develop a fluorescence resonance energy transfer (FRET)-based enzyme activity assay for GBSSrtC1, possibly due to the ‘lid’ blocking the active site and/or the absence of the C-terminal membrane-anchoring domain in the recombinant proteins being tested. The membrane-anchoring domain is essential for efficient *in vivo* pilus polymerization, as was shown in *Corynebacterium diphtheriae*,⁴² and is also needed for testing the enzyme activity by the FRET assay, as reported for GBS strain 515 SrtC1.⁴⁰ Cozzi et al. (2011) revealed that the C-terminal transmembrane region of the GBS515 SrtC1 recombinant protein, including the positively charged tail, is required for enzyme functionality; however, the same full-length recombinant of SrtC1 of strain 515 is not amenable to crystallization.⁴⁰ The full-length construct of GBS SAG2603 V/R strain SrtC1 is difficult to express (unpublished results). While the expression and purification of full-length constructs of sortases and pilin proteins have been problematic so far, it is vital to determine the structures of these proteins, as well as that of a complex of the two, to determine the precise function and mechanism of pilus-specific sortases.

Materials and Methods

Protein expression, purification and crystallization

Recombinant SAG2603 V/R GBSSrtC1 (residues 43–260) was expressed and purified as described previously.⁴⁴ The hanging-drop vapor-diffusion method was used for

crystallization of both the apo-protein and the complex with MTSET. The apo-protein, at a concentration of 24 mg/ml or higher ($\epsilon = 14200 \text{ M}^{-1} \text{ cm}^{-1}$ as obtained from *DNASStar* software, DNASStar Inc., Madison, Wisconsin, USA) was crystallized with 15–25% (w/v) PEG 3350 and 0.2 M Tris buffer (pH 8.0–8.8) and 0.2 M ammonium acetate at 4°C. Two microliter drops containing equal volumes of the protein and the reservoir solution were equilibrated against a 1 ml solution in the reservoir. GBSSrC1 type I crystals with a plate-like morphology were obtained within 3–7 days. The production of Se-Met GBSSrC1₄₃₋₂₆₀ was based on a standard protocol using a methionine auxotrophic bacterial strain.⁵⁹ Briefly, B834(DE3) cells were transformed with the GBSSrC1 plasmid pSrtC1_{S43-Q260}⁴⁴ and plated on agar overnight at 37°C. A single colony was grown overnight at 37°C in 20 ml of Luria Bertani medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. 20X M9 medium (100 ml) was used to prepare the final 1 L of growth medium, which contained 1 M MgSO₄ (2 ml), FeSO₄ (2 ml at 12.5 mg/ml), 40% glucose (10 ml), amino acid mix (20 ml at 4 mg/ml), vitamins (1 ml at 1 mg/ml) and seleno-L-methionine (4 ml at 10 mg/ml). The growth medium was supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. Bacteria were grown at 37°C until the optical density at 600 nm reached 0.5. Protein expression was induced with 0.4 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), and bacteria were grown overnight at 37°C thereafter. The cells were harvested, and the protein was subsequently purified according to the protocol used for the native GBSSrC1.⁴⁴ The purified protein was concentrated to 60–100 mg/ml as determined by UV spectroscopy and was subsequently crystallized under the same conditions as the native protein, also resulting in plate-like crystals. For the co-crystallization of GBSSrC1 and the inhibitor, the cysteine protease inhibitor MTSET (2-(trimethylammonium)-ethyl-methanethiosulfonate bromide) and the recombinant SrtC1₄₃₋₂₆₀ at 36.2 mg/ml concentration were incubated overnight with a five-fold excess of the inhibitor. One microliter of inhibitor-protein complex solution was mixed with an equal volume of the reservoir solution and equilibrated against a 1-ml solution (1.6 M ammonium sulfate, 5% PEG3350 and sodium cacodylate, pH 6.5) at 4°C. Poorly formed leaf-shaped crystals, unsuitable for x-ray diffraction, were obtained after a week. Sharp-edged, diffraction-quality crystals emerged out of the initial crystals after approximately two years and these enzyme-inhibitor complex crystals gave satisfactory diffraction.

Twenty percent ethylene glycol in the reservoir solution was used as the cryo-protectant for both crystals during diffraction data collection.

Data collection, structure solution and refinement

Diffraction data from the Se-Met type I GBSSrC1 crystals were collected on the SER-CAT 22-BM beamline at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Chicago. Diffraction data sets were processed with HKL2000⁶⁰ (Table 1), and SOLVE/RESOLVE from the SGXPRO software⁶¹ was used to find the two Se sites, perform density modification and generate the electron density maps. Model building was done using COOT.⁶² Diffraction data from the GBSSrC1-MTSET complex crystals were collected at the in-house facility using an R-AXIS IV detector and a RIGAKU rotating anode X-ray generator operating at 100 mA. D*trek⁶³ was used to process the diffraction data (Table 1) collected at a 1.541 Å wavelength, and the structure was solved by molecular replacement using the β -barrel core of type I GBSSrC1 as the search model. Molecular replacement was performed using PHASER, available in the CCP4 software suite.⁶⁴ The model was built using the COOT graphics software, and iterative cycles of model building and refinement were performed using CNS⁶⁵ and REFMAC from the CCP4 suite of software.⁶⁴ Table 2 presents the refinement statistics for both structures described in this manuscript.

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Figure 1. The eight-stranded β -barrel fold of GBSSrtC1-I

The central β -barrel is flanked by three helices- H1, H2 and H3. Also seen are the β -strands (4, 7 and 8) that form a long cleft with the three catalytic residues, H122, C184 and R193 (shown as sticks) residing on one end of this cleft. Missing residues, linking helices H2 and H3, are represented by a dashed red line.

Figure 2. Crystal packing interfaces of GBSSrC1-I

2a. One GBSSrC1 molecule, represented in dark blue (**A**), present in the asymmetric subunit exhibits three unique interfaces with symmetry-related molecules shown in cyan (**B**), green (**C**) and magenta (**D**), respectively. Molecules **A** and **C** are related by a crystallographic 2-fold axis, an association similar to the dimeric arrangement reported earlier for the crystal structures of GBSSrC1.³⁶ The $\beta 4/\beta 5$ linker of symmetry related molecule **B** is projected into the active site of **A**, whose catalytic residues (Cys184, H122 and R193) are represented in yellow sticks.

2b. A surface representation of the GBSSrC1 molecule in the asymmetric unit (basic residues in blue, acidic residues in red, and hydrophobics are in gray) with a symmetry mate (pale blue) projecting its $\beta 4$ – $\beta 5$ loop into the long cleft of the former that constitutes the active site of GBSSrC1.

Figure 3. Superposition of loops in the active site

3a. Superposition of the crystal structures of GBSSrC1-I (cyan) and a single monomer of GBSSrC1-II (PDB Code: 3RBK) (represented in yellow color) reveals that the $\beta 4/\beta 5$ loop (highlighted in red) of the GBSSrC1-I symmetry-mate (represented in pale blue), overlaps with the 'lid' of the GBSSrC1-II monomer.

3b. A closer and stereo-view of superposition as detailed in 3(a). Residues from the $\beta 4/\beta 5$ loop of GBSSrC1-I symmetry-mate (the red loop from the pale blue molecule in Figure 3a) and those from the lid region of GBSSrC1-II (represented in yellow) overlap such that the $^{125}\text{LPTA}^{128}$ region of $\beta 4/\beta 5$ loop is positioned in the active site of GBSSrC1-I and its P126 residue superposes precisely with Leu47 of the GBSSrC1-II lid region.

3c. A close-up view of the $^{125}\text{LPTA}^{128}$ loop residues (in red) from the symmetry mate superposed on the $^{45}\text{PALKDPY}^{51}$ residues of GBSSrC1-II 'lid' region. While P126 of the

substrate mimic occupies the position of the critical 'lid' residue L47, the T127 is positioned closer to the catalytic R193 residue (distance of ~ 3.0 Å), a possible arrangement for the actual LPXTG substrate-bound sortase during enzyme reaction.

Figure 4. A stereo view of the sym-loop above the hydrophobic pocket of GBSSrtC1-I
 The residues comprising the hydrophobic pocket of GBSSrtC1-I (in cyan color) are shown in sticks. The ¹²⁶Pro-Thr¹²⁷ part of the sym-loop is shown in red. For comparison, the lid region of GBSSrtC1-II monomer (PDB Code: 3RBK) and its corresponding active site region (shown in yellow) is superposed.

Figure 5. Crystal structure of the complex of GBSSrtC1 with the inhibitor, MTSET

5a. One of the three dimers present in the asymmetric subunit of GBSSrtC1-MTSET complex crystal structure is shown. The dimeric association is similar to that seen in GBSSrtC1-II and III crystal forms (PDB codes: 3RBK and 3RBI) and also similar to the crystal packing arrangement detailed in Figure 2a, between GBSSrtC1-I molecules **A** and **C**. Covalently attached MTSET inhibitors are labeled and the missing regions for the 'lid' and its hinge segments are shown as dashed red lines for one monomer.

5b. A single monomer from GBSSrtC1-MTSET structure is shown in grey with the lids and MTSET-bound catalytic Cys of six molecules in the asymmetric unit highlighted in different colors. While the position of Y51 of the DP(Y/W/F) anchor motif is conserved among six chains, the conformation of the lid beyond this residue is different in three monomers (blue, cyan and green) and disordered in other three. The conformation of the Cys linked MTSET molecule is varied among the six GBSSrtC1-MTSET molecules.

5c. The 2Fo-Fc electron density map (contoured at 2.0σ and shown in cyan for the L182-P186 region) is superposed with Fo-Fc difference map (contoured at 2.5σ and colored in red for the Cys184-MTSET region for chain B) and both maps are calculated using phases

having no contribution of the inhibitor. For this, the MTSET linked Cys184 is replaced with Ala184 and the structure factors and phases are calculated after few cycles of REFMAC. The 2Fo-Fc map clearly shows density for the cis-peptide link between Thr185 and Pro186 residues and Fo-Fc map reveals the position of the bound inhibitor.

Figure 6. Movement of the lid from the active site to form a five-turn α -helix (H3)

6a. The N-termini and the lid including the C-terminal hinge region observed in GBSSrC1-I (cyan), GBSSrC1-II (yellow) and the *S. pneumoniae* SrtC1 (SPNSrC1; magenta) are superposed. With the fully-ordered SPNSrC1 lid as a reference, the different conformations, yet similar positions, of the observed minimal parts of the GBSSrC1-II lid, which is disordered on either end, can be seen. This lid is absent in GBSSrC1-I where the entire C-terminal hinge is involved in helix formation (H3) and the N-terminal part is disordered. The helix H3 is absent in SPNSrC1 and GBSSrC1-II structures as their lids are positioned in the respective active sites.

6b. The extent of the conformational change in the lid region between GBSSrC1-I (cyan) and GBSSrC1-II (yellow) is evident from the position of Pro50, which is present as part of the anchor motif in the lid in GBSSrC1-II (yellow) and at the end of helix H3 in GBSSrC1-I (cyan). The translated Pro50 in GBSSrC1-I, due to the helix H3 formation, from the observed position in GBSSrC1-II lid anchor is pointed by an arrow.

Table 1

Data collection statistics for Se-Met GBSSrtC1-I and GBSSrtC1-MTSET complex

GBS Sortase	Se-Met GBSSrtC1-I (Peak)	GBSSrtC1-MTSET complex
No of crystals	1	1
Beamline	SER-CAT 22-BM	R-AXIS IV generator
Wavelength (Å)	0.9748	1.5418
Detector	MAR 225 CCD	R-AXIS Image Plate
Crystal to Detector distance (mm)	200	150
Rotation range per image (°)	0.5	0.5
Total rotation range (°)	0–180	0–180
Exposure time per image (s)	7.9	300
Resolution range (Å)	50.00–2.50 (2.54–2.50)	27.71–2.85 (2.95–2.85)
Space group	C2	C2
Unit cell parameters (Å/°)	81.6, 49.6, 71.1, 90.0, 114.0, 90.0	122.4, 70.6, 194.4, 90.0, 90.4, 90.0
Mosaicity (°)	0.22	1.45
Total number of measured reflections	34827 (1737)	134525 (13976)
Unique reflections	7138 (378)	38964 (3895)
Redundancy	4.8 (4.5)	3.4 (3.5)
Mean I/σI	40.6 (10.5)	11.1 (3.0)
Completeness (%)	78.1 (82.5)	99.6 (100.0)
χ^2	3.5 (1.0)	1.0 (1.2)
* R _{merge} (%)	6.1 (16.2)	6.5 (33.1)
Overall B factor from Wilson plot	54.0	88.4
Number of molecules in asymmetric subunit	1	6

* $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ are the intensities of symmetry-related reflections and $\langle I(hkl) \rangle$ is the average intensity over all observations.

Numbers in the parenthesis represent values for the last resolution shell.

Table 2

Refinement Statistics

GBS Sortase	GBSSrtC1-I	GBSSrtC1-MTSET complex
Resolution range (Å)	50.0–2.45	27.7–2.85
Reflections used in refinement	15818	36974
Number of non-H protein atoms	1478	8832
R _{work} (%)	24.4	25.0
R _{free} (%)	30.2	29.6
Mean B values (Å ²)	39.2	51.9
Type (and number) of ligands/ions	-	ETM (6), SO4(5), Cl(1)
Number of waters	30	108
B factor ligand/ion	-	104
B factor water	24.0	36.8
R.m.s.d in bond lengths (Å)	0.02	0.01
R.m.s.d. in bond angles (°)	2.13	1.61
Number of residues in allowed region (%)	94.6	95.4
Number of residues in disallowed region (%)	0.5	0.45
PDB Code	3TB7	3TBE

Access to the active site of pilus-specific sortases is blocked by the presence of a 'lid' loop, whose displacement was suggested to be essential for the pilus-specific sortase function. With the help of an inhibitor and substrate-mimic complex crystal structures of group B streptococcus sortase SrtC1, we suggest that the active site catalytic Cys residue is functional even in the presence of 'lid' and we present a model for the 'lid' displacement upon the substrate LPXTG sorting motif recognition and binding to the SrtC1 active site.