Isopeptide Ligation Catalyzed by Quintessential Sortase A

MECHANISTIC CUES FROM CYCLIC AND BRANCHED OLIGOMERS OF INDOLICIDIN

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The housekeeping transpeptidase sortase A (SrtA) from Staphylococcus aureus catalyzes the covalent anchoring of surface proteins to the cell wall by linking the threonyl carboxylate of the LPXTG recognition motif to the amino group of the pentaglycine cross-bridge of the peptidoglycan. SrtA-catalyzed ligation of an LPXTG containing polypeptide with an aminoglycine-terminated moiety occurs efficiently in vitro and has inspired the use of this enzyme as a synthetic tool in biological chemistry. Here we demonstrate the propensity of SrtA to catalyze “isopeptide” ligation. Using model peptide sequences, we show that SrtA can transfer LPXTG peptide substrates to the ε-amino of specific Lys residues and form cyclized and/or a gamut of branched oligomers. Our results provide insights about principles governing isopeptide ligation reactions catalyzed by SrtA and suggest that although cyclization is guided by distance relationship between Lys (ε-amino) and Thr (α-carboxyl) residues, facile branched oligomerization requires the presence of a stable and long-lived acyl-enzyme intermediate.

Sortases are cysteine transpeptidases that catalyze covalent anchoring of surface proteins to the cell wall in Gram-positive bacteria. Catalytic action of sortase entails a peptide ligation reaction that occurs in two steps and involves an acyl-enzyme intermediate (1–4). The prototypical sortase (SrtA) of Staphylococcus aureus recognizes a pentapeptide LPXTG motif present near the carboxyl terminus of surface proteins, cleaves the scissile T-G peptide bond, and captures the protein as an acylenzyme intermediate with the active site cysteine covalently linked to a Thr residue by a thioester bond (5–8). This acyl-enzyme intermediate is resolved by nucleophilic attack of the α-amino group of a pentaglycine sequence present in peptidoglycan leading to anchoring of proteins to the cell wall (Fig. 1A).

Whereas the housekeeping sortases, e.g. SrtA of S. aureus, are responsible for cell wall anchoring of surface proteins, other members of the sortase family, referred to as pilin sortases, carry out covalent assembly of long protein fibers termed pilus (9, 10). The pilus structure is assembled from pilin subunits. These subunits consist of an LPXTG motif at the C terminus, a pilin motif containing a conserved lysyl residue (WXXXV-VYPKN) at the N terminus, and an E box motif (YXLXETXAPXGY) harboring a conserved glutamyl residue located in the central region of the protein (11, 12). Sortase-mediated pilin polymerization also proceeds via the intermediary of a thioester acyl-enzyme intermediate (Fig. 1B), which is resolved through nucleophilic attack of the ε-amino group of the lysyl residue (cf. α-amino of pentaglycine for cell wall anchoring) in the pilin motif of another subunit (13–15). The reaction is facilitated by head to tail arrangement of subunits wherein the N-terminal domain of one subunit is abutted against the C-terminal domain of another allowing correct disposition of the critical lysine of the pilin motif in close proximity to the C-terminal LPXTG motif (16–18). Thus sequence specificity as well as proximity of the reacting ends appears to be an important feature of the “isopeptide” ligation reaction catalyzed by pilin sortases.

SrtA-mediated peptide ligation reaction can be replicated efficiently in vitro with short LPXTG peptides and oligoglycine (8). The propensity of SrtA to recognize a simple pentapeptide consensus sequence and a triglycine has inspired the use of this enzyme as a synthetic tool in biological chemistry (19). Several examples including introduction of novel functionality (viz. fluorescent labels, lipids, glycosylphosphatidylinositol anchors etc.) into proteins, generation of circular proteins, and surface immobilization of proteins have emerged in recent years (20–32). In contrast, “isopeptide ligation” activity of pilin sortases has not been so far applied to the synthesis of branched polypeptides. This is presumably because recombinant pilin sortases exhibit poor catalytic efficiency in vitro (33). The synthetic utility of pilin sortases is further marred by restrictions imposed by stereochemical aspects of the ligation reaction alluded to above. Given the limited synthetic utility of pilin sortases, we turned our attention toward SrtA of S. aureus and explored if this enzyme could accept ε-amino group of Lys residue in the transpeptidation reaction. We were motivated by the fact that SrtA displays rather relaxed specificity for the amine nucleophile and can accept a variety of molecules, terminating in aminomethyl (H₂NCH₂-) or aminoethyl (H₂NCH₂CH₂-) moieties, as substitutes for oligoglycines (28, 31, 34).

Here we show that SrtA of S. aureus is indeed capable of catalyzing ligations involving ε-amino groups of Lys residues. Our results provide insights about principles governing isopeptide ligation reactions catalyzed by SrtA to produce cyclic and/or branched peptides and suggest that the presence of a long-lived highly stable acyl-enzyme intermediate may be obligatory for facile branching.

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Abbreviations used: SrtA, sortase A; ES-MS, electrospray mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography.

**Expression and Purification of Recombinant Sortase A (SrtA)**—SrtA22 sequence corresponding to amino acids 60 to 204 was expressed and purified as described previously (34). Briefly, Escherichia coli BL21(DE3) cells containing the srtA plasmid were grown in LB broth at 37 °C until the optical density at 600 nm reached 0.6 and then the cells were induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside at 30 °C for 3 h. The cells were then harvested, suspended in buffer A (10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM 2-mercaptoethanol), and lysed by sonication. The extract was centrifuged at 12,857 × g for 30 min and the supernatant was subjected to affinity chromatography on a nickel-nitrilotriacetic acid column pre-equilibrated with buffer A. After washing with buffer B (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM imidazole, 1 mM 2-mercaptoethanol), the protein was eluted in buffer C (10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 250 mM imidazole, 1 mM 2-mercaptoethanol). The excess imidazole from the purified protein was removed using a desalting column.

**Synthesis and Purification of Peptides**—The peptides were synthesized using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy and a standard protocol for automated solid-phase synthesis (433A, Applied Biosystems). As the starting material, Wang resin pre-loaded with the desired amino acid or Rink-amine AM resin were used. Peptide cleavage and side chain deprotections were performed in a mixture containing trifluoroacetic acid (TFA), triisopropylsilane, and water (95:2.5:2.5, v/v). After filtration of the resin, the crude peptides were precipitated in cold diethyl ether and subsequently extracted in water. The crude peptides were purified by RP-HPLC using a C-18 preparative column (100 Å, 10 μ, 30 × 250 mm, Phenomenex, gradient: 8–72% acetonitrile in 0.1% TFA over a period of 130 min, flow rate: 30 ml/min) and the purity was verified using a C-18 analytical column (100 Å, 5 μ, 4.6 × 250 mm, Phenomenex, gradient: 4–72% acetonitrile in 0.1% TFA over a period of 130 min, flow rate: 1 ml/min).

**Mass Spectrometry**—The RP-HPLC-purified peptides were dissolved in 50% acetonitrile containing 0.1% formic acid and analyzed by positive-ion mode ES-MS (Table 1). The ES-MS spectra were recorded using a Micromass LCT mass spectrometer. Data acquisition and processing were performed using MassLynx version 4.0 software. For MALDI-TOF, peptide samples were mixed with α-cyano-4-hydroxycinnamic acid matrix and analyzed on a Applied Biosystems 4800 MALDI TOF/TOF analyzer. Specific samples were further analyzed by tandem mass spectrometry.

**Amino Acid Sequencing**—The N-terminal sequencing by Edman degradation was carried out using the automated amino acid sequencer (Procise 491 HT, Applied Biosystems) supported by an on-line phenylthiohydantoin analyzer.

**Sortase-catalyzed Ligation Reaction**—The transpeptidation reaction was carried out in a buffer containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM CaCl2, 2 mM 2-mercaptoethanol (pH 7.5). The assay was carried out in a reaction volume of 0.1 ml, wherein 0.2 mM LPXTG peptide was incubated with 0.1 mM sortase and/or 0.4 mM of the extraneous nucleophile for 12 h at 37 °C and quenched by the addition of 10-fold excess of chilled 0.1% TFA. The reaction products were analyzed by analytical RP-HPLC and characterized using ES-MS.

**Trypsin Digestion**—Trypsin digestion was carried out in 50 mM ammonium bicarbonate buffer (pH 8.0) with tosylphenylalanyl chloromethyl ketone-treated trypsin. About 100 μg of peptide was incubated with 5 μg of trypsin in a reaction volume of 0.05 ml at 37 °C for 2 h after which it was quenched by addition of 10-fold excess of chilled 0.1% TFA. The trypsin digest was analyzed by analytical RP-HPLC and characterized using ES-MS or MALDI-TOF.

**V8 Protease Digestion**—100 μg of the peptide was digested with 5 μg of V8 protease in ammonium bicarbonate (50 mM, pH 8.0). The assay was performed in a reaction volume of 0.05 ml for 2 h at 37 °C and quenched by addition of 1 μl of TFA. The reaction products were analyzed by MALDI-TOF mass spectrometry.

**Kinetic Measurements**—YALPETGK was used as a model sortase substrate for assaying hydrolysis or transpeptidation reactions involving triglycine or agmatine. The assays were performed in Tris-HCl buffer (50 mM, pH 7.5) containing 150 mM NaCl, 5 mM CaCl2, and 2 mM 2-mercaptoethanol. The reactions were quenched by the addition of 10-fold excess of chilled 0.1% TFA and monitored by analytical RP-HPLC at 280 nm. The product was quantified by integrating the area under the peak. The identity of the respective product was confirmed by ES-MS. The reaction velocities obtained from the progress curves at various substrate concentrations were fit to the equation, \( v = \frac{K_{cat}}{S + K_m} \), and kinetic parameters were determined.

**Western Blotting**—Samples were boiled in SDS sample buffer and separated on a 15% polyacrylamide gel. The gels were electro-transferred onto a nitrocellulose membrane and subjected to
immunoblotting with monoclonal anti-polystyrene antibodies (1:2,000 dilution) at room temperature for 1 h. The membrane was washed three times with 0.05% Tween in PBS and incubated with secondary antisera (goat anti-mouse HRP-conjugated antibodies: 1:10,000 dilution) at room temperature for 1 h. Finally, the membrane was developed by chemiluminescence after washing with PBS-T.

Circular Dichroism (CD) Measurements—CD spectra were recorded on a Jasco-710 spectropolarimeter. The measurements were performed in 10 mM potassium phosphate buffer (pH 7.5). The spectral data recorded using a 2-mm cell have been presented as mean residue ellipticity (MRE) expressed in degree cm$^2$ dmol$^{-1}$.

RESULTS

SrtA Catalyzes Intermolecular Transfer of a Model LPXTG Peptide to a Pilin-specific Lys Residue in a Short Peptide Sequence

The process of covalent pilus assembly in Gram-positive bacteria projects a complex mechanism. However, general principles governing the assembly process have emerged in the past couple of years, particularly, from detailed mechanistic studies of pilus formation in Corynebacterium diphtheriae and Streptococcus pneumoniae. The importance of the tripartite motif (a pilin motif, an E-box motif, and an LPXTG like sortase-recognition motif) and the identity of specific pilin sortases (distinct from a housekeeping sortase) have been established. It is abundantly clear that pilin sortases cleave the scissile T-G peptide bond in the recognition motif and ligate the threonyl carboxylate to an e-amino of a lysyl residue in the pilin motif of another subunit. Some evidence has been provided for the involvement of a Glu residue located in the E-box motif of the pilin motif in pilus assembly (9–12, 33, 35–39).

Table 1 lists the ES-MS analysis of the peptides used in the study. The transpeptidation product (Peak 1A) was further characterized to delineate the site of peptide ligation. MALDI-TOF spectrum of the V8 protease digest of the transpeptidation product yielded a peptide fragment of m/z 1410.45 (supplemental Fig. S2A), which corresponded to the m/z of the peptide adduct YALPNTGK. That this peptide adduct was branched and involved linkage between the e-amino of the LPXTG motif and Thr carboxylate of the YALPNTGK sequence was ascertained from MS/MS analysis of the m/z 1410.45 fragment (supplemental Fig. S2B and supplemental Table S1). Formation of the branched peptide product, albeit in small amounts, highlights the intrinsic propensity of the YPK sequence to undergo transpeptidation.

SrtA Catalyzed the Intramolecular Cyclization through Isopeptide Ligation in a Pilin Motif Sequence Nested with the LPXTG Motif

Next a single peptide containing both the pilin motif and sortase-recognition sequence was designed to explore the possibility of oligomerization through isopeptide ligation at Lys$^{183}$ (183) conserving Lys residue in the pilin motif of RrgB. Accordingly, a 24-mer peptide, VDAHYYPKNTAEKPAGGLNLK (designated P2), was synthesized in which residues 176–190 of RrgB representing the pilin sequence was nested with an LPXTG sequence at the C terminus separated by a triglycine linker. Incubation of P2 with SrtA followed by RP-HPLC (supplemental Fig. S3) revealed the formation of two new peaks (3A and 3B), which corresponded to the mass of a hydrolyzed peptide (YALPNT, calculated mass: 677.34 Da). Taken together the data suggested that peaks 1 and 2 were transpeptidation and hydrolyzed products, respectively.

The transpeptidation product (Peak 1A) was further characterized to delineate the site of peptide ligation. MALDI-TOF spectrum of the V8 protease digest of the transpeptidation product yielded a peptide fragment of m/z 1410.45 (supplemental Fig. S2A), which corresponded to the m/z of the peptide adduct YALPNTGK. That this peptide adduct was branched and involved linkage between the e-amino of the LPXTG motif and Thr carboxylate of the YALPNTGK sequence was ascertained from MS/MS analysis of the m/z 1410.45 fragment (supplemental Fig. S2B and supplemental Table S1). Formation of the branched peptide product, albeit in small amounts, highlights the intrinsic propensity of the YPK sequence to undergo transpeptidation.

Table 1

| Peptide designation | Sequence | Observed mass Da | Monoisotopic Average Da | Calculated mass Da |
|---------------------|----------|------------------|-------------------------|-------------------|
| P1                  | YPKNTAEKP | 1174.61          | 1174.63                 | 1175.35           |
| P2                  | VDAHYYPKNTAEKPAGGLNLK | 2478.98 | 2477.3 | 2478.79 |
| P3                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2572.73 | 2571.41 | 2573.05 |
| P4                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2571.85 | 2571.41 | 2573.05 |
| P5                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2571.85 | 2571.41 | 2573.05 |
| P6                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2571.85 | 2571.41 | 2573.05 |
| P7                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2571.85 | 2571.41 | 2573.05 |
| P8                  | VDAHYYPKNTAEKPAGGLNLK | 2305.14 | 2305.39 | 2315.03 |
| P9                  | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2515.42 | 2515.39 | 2517.03 |
| P10                 | ILPFWKWPWPRRGLPNTGK-NH$_2$ | 2728.65 | 2728.5 | 2730.26 |

To accomplish this, a single peptide containing both the pilin motif and sortase-recognition sequence was designed to explore the possibility of oligomerization through isopeptide ligation at Lys$^{183}$. The importance of the tripartite motif (a pilin motif, an E-box motif, and an LPXTG like sortase-recognition motif) and the identity of specific pilin sortases (distinct from a housekeeping sortase) have been established. It is abundantly clear that pilin sortases cleave the scissile T-G peptide bond in the recognition motif and ligate the threonyl carboxylate to an e-amino of a lysyl residue in the pilin motif of another subunit. Some evidence has been provided for the involvement of a Glu residue located in the E-box motif of the pilin motif in pilus assembly (9–12, 33, 35–39).
were analyzed by ES-MS. The mass of peak 3A was determined to be 2292.3 Da, which fit to the calculated mass of the hydrolyzed peptide, VDAHVYPKNTAEAKPGGLPNT (calculated 2292.18 Da). The experimental mass of peak 3B peptide was found to be 2275.23 Da. Interestingly this mass was about 18 units less than that of the hydrolyzed peptide, thus indicating the cyclic nature of this product. Further characterization of peak 3B, employing tryptic mapping, mass spectrometry, and Edman sequencing, was carried out to establish its identity.

20 cycles of Edman degradation produced a single residue in each cycle (corresponding to residues 176–190 of RrgB) in the desired yield except for the 8th cycle where the expected Lys residue (Lys183) was present in very small amounts (supplemental Table S2) suggesting that this residue was derivatized. The RP-HPLC profile of the tryptic digest (supplemental Fig. S4) showed a single peak whose mass (2292.34 Da) was 18 units higher than that of the parent peptide (peak 3B) suggesting that tryptic cleavage occurred at a single peptide bond. Formation of such a product is consistent with a single point hydrolysis at the K-G peptide bond (supplemental Scheme 1) in the cyclized product because the Lys188–Pro189 bond will be recalcitrant to tryptic hydrolysis. Taken together, the results suggest that SrtA catalyzed the intramolecular cyclization through isopeptide ligation of the Thr carboxylate of the sortase-recognition sequence and conserved Lys of the YPK pilin sequence when the two motifs were nested together. Notably in this reaction, desired branching/oligomerization of the peptide was not observed.

**SrtA-mediated Transpeptidation Reaction of Indolicidin-derived Peptides**

The stereochemical proximity of the amino and carboxyl groups and their pK_a appear to be two important thermodynamic factors that facilitate the formation of a peptide bond. Although the proximity effect diminishes the entropic cost of bringing the reacting ends together, lowering the pK_a reduces the enthalpic barrier to peptide bond synthesis (40, 41). Thus, the propensity of an ε-amino of the pilin Lys residue to form isopeptide bond leading to branching or cyclization must be related to its pK_a and proximity effects. The significant lowering of pK_a could occur if the lysine is present in a hydrophobic environment. In fact, isopeptide bonds involving lysine residues in major pilin subunits in *C. diphtheriae, Bacillus cereus*, and *S. pneumoniae*, and minor pilin subunits Cpa in *Streptococcus pyogenes*, and RrgA and RrgC in *S. pneumoniae*, are found to be buried in hydrophobic cores (17, 18, 42–45). The likely mechanism proposed for the formation of such bonds is that a hydrophobic environment alters the pK_a of ionizable groups, in turn favoring a neutral form and facilitating a nucleophilic attack by the unprotonated lysine ε-amino group (46). Based on the above features (and some intuition!) a short, 13-mer antimicrobial peptide indolicidin was considered for further exploration of the sortase-mediated isopeptide ligation reaction. The indolicidin sequence contains five tryptophan residues and a single Lys residue flanked by hydrophobic indole rings (47). The indolicidin sequence length is similar to the pilin motif peptide tested above. Importantly, indolicidin displays antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi. Indolicidin oligomeric derivatives may be useful as antibiotics.

**FIGURE 2. SrtA-catalyzed isopeptide ligation of LPXTG-embedded indolicidin sequence (P4).** The reaction of P4 with SrtA was carried out as described under “Materials and Methods.” The reaction mixture was analyzed by RP-HPLC (C18 column, Phenomenex, 100 Å, 5 μ, 4.6 × 250 mm, gradient: 4–72% acetonitrile in 0.1% TFA over 130 min, flow rate: 1 ml/min). The product peaks are numbered 1–6 in order of elution from the column.

*SrtA Catalyzed the Isopeptide Ligation of a LPXTG Peptide to Indolicidin*

The ability of native indolicidin, ILPWKWPWWPPR-NH_{2} (designated P3), to act as a nucleophile donor in SrtA-mediated transpeptidation was investigated. Incubation of this peptide with a LPXTG substrate, YALPNTGK, yielded a new product in addition to the hydrolyzed peptide, YALPNT (supplemental Fig. S5A). The mass of the new peptide (5A) was found to be 2565.43 Da, which fit to the calculated mass of an adduct (2564.37 Da) of YALPNT and P3 suggesting that this was a transpeptidation product (supplemental Fig. S5B). Two N-terminal peptide sequences namely, ILPWKWP and YALPNT, could be discerned from Edman degradation confirming that the transpeptidation generated a branched peptide through isopeptide ligation involving the Thr carboxylate and ε-amino group of indolicidin Lys residue. Thus sortase-catalyzed intermolecular transfer of the LPXTG peptide to indolicidin occurred in much the same way as the pilin motif peptide.

*SrtA Produced a Cyclized Peptide and a Gamut of Isopeptide-linked Branched Oligomers from an Indolicidin Sequence Nested with an LPXTG Motif*

Next the intramolecular isopeptide ligation of indolicidin peptide containing the LPXTG motif separated by a triglycine linker was investigated just as was done in case of the pilin motif peptide. Accordingly, a 22-mer peptide sequence ILPWKWPPWWPPRGGGLPNTGK-NH_{2} (designated P4) was synthesized with an aim to examine if this peptide undergoes SrtA-catalyzed reaction in a manner similar to that observed in pilin motif peptide P2 or forms branched oligomer(s). Interestingly, incubation of P4 with sortase led to an array of reaction products. The RP-HPLC profile revealed six new peaks in addition to the parent peptide and SrtA. These peaks were designated as peaks 1–6 based on their order of elution from the reverse-phase column (Fig. 2). The individual peaks were collected and characterized by mass spectrometry (supplemental Table S3) and related methods as described below.

**Peak 1**—The experimentally determined mass of peak 1 was found to be 20338.2 Da (Fig. 3A), which fits to the calculated...
Mass spectrometric characterization of peak 1 obtained from reaction of SrtA and P4 (see Fig. 2). A, ES-MS analysis of peak 1 provided an experimental mass of 20338.2 Da, which corresponds to a covalent adduct of SrtA (17854.08 Da) and hydrolyzed peptide (ILPWKWPWPWGGGLPNT, 2502.32 Da). B, MALDI-TOF analysis of the tryptic digest of peak 1 obtained from the RP-HPLC fractionation of the reaction of SrtA with P4 (see supplemental Table S4 for the mass data).
mass of a covalent adduct of SrtA and the hydrolyzed peptide (ILPWKWPWPPRRGGGLPNT, supplemental Table S3). Ten cycles of amino acid sequencing of peak 1 yielded two sequences corresponding to the first 10 residues of SrtA and indolicidin, respectively, indicating that the adduct was branched and may be a thioester acyl-enzyme intermediate. The co-presence of indolicidin and sortase peptide fragments as well as a unique fragment with m/z 2553.26 seen in the MALDI-TOF spectrum (Fig. 3B, supplemental Table S4) of the peak 1 tryptic digest corresponding to a branched sequence derived from active site residues 176–190 (DKQLTTLITCD-DYNK) and RGGGLPNT sequence of P4 (supplemental Table S4, entry 9) confirmed the identity of peak 1 as an acyl-enzyme intermediate.

**Peak 2**—The mass of peak 2 (supplemental Table S3) was found to be 2502.07 Da, which matched well with the calculated mass (2502.32 Da) of the hydrolyzed peptide.

**Peak 3**—The observed mass for peak 3 was 5170.18 Da, which fit to a composition of one molecule of the parent peptide (P4) and one molecule of the hydrolyzed peptide (calculated mass, 5170.77 Da) suggesting a dimeric nature of this species (supplemental Table S3). Tryptic digestion and ES-MS analyses were carried out to delineate if the dimer was linear or formed through isopeptide ligation involving the Thr carboxyl of one molecule to the Lys (ε-amine) of the other. Toward this, the tryptic digest of peak 3 was resolved by RP-HPLC (supplemental Fig. S6) and individual tryptic fragments labeled A to G were subjected to mass spectrometric analyses. The mass data (supplemental Table S5) of the tryptic fragments were commensurate with the branched nature of the dimer. Finally, 10 cycles of Edman degradation on tryptic fragment peak D (2345.19 Da) produced two sequences (supplemental Table S6) confirming peptide branching and intermolecular isopeptide ligation between Lys and Thr.

**Peak 4**—ES-MS of peak 4 revealed the presence of two molecular species with mass of 2484.26 and 7654.57 Da, respectively (supplemental Table S3). Indeed further fractionation of peak 4 on RP-HPLC using an altered gradient gave rise to two distinct peaks labeled 7A and 7B (supplemental Fig. S7). The mass of 7A (2484.2 Da) was about 18 Da less than that of the hydrolyzed peptide suggesting that 7A may be a cyclized product. To establish that this was indeed the case, peptide 7A was subjected to trypsin digestion. RP-HPLC fractionation of the digest (supplemental Fig. S8) predominantly revealed a single peptide whose mass was found to be 2346.68 Da. The mass of this peptide was 138 Da (2484 – 2346 Da) less than the parent peptide (7A) and was equivalent to 18 units less than the mass of an Arg residue (156 Da). This could be interpreted to mean that cleavage occurred at both Arg sites leading to the excision of an Arg residue and that the Lys site was resistant to proteolysis due to its engagement in the cyclization reaction (supplemental Scheme 2). The mass of 7B (7658.03 Da) could fit to a covalent adduct composed of one molecule of the parent peptide (P4) and two molecules of the hydrolyzed peptide suggesting the trimeric nature of the molecule (supplemental Table S3).

**Peaks 5 and 6**—The mass of peak 5 was determined to be 10,139 Da (supplemental Table S3), which was in agreement with the calculated mass of an adduct comprising one molecule of the parent peptide (P4) and three molecules of the hydrolyzed peptide. Likewise, peak 6 gave a mass of 12,623 Da that fit to the composition of one molecule of the parent peptide (P4) and four molecules of the hydrolyzed peptide. These peaks were not characterized any further and were inferred, by analogy and pattern, as tetramer and pentamer, respectively.

Thus SrtA action on P4 yielded a stable thioester acyl-enzyme intermediate, a cyclic monomer, and a gamut of oligomers (dimer, trimer, tetramer, and pentamer) in which one molecule of the parent peptide (P4) was linked to one or more molecules of the hydrolyzed peptide through an isopeptide


**Sortase-catalyzed Ligation of Isopeptide Bond**

**TABLE 2**

Kinetic parameters of SrtA-catalyzed reaction

| Substrate varied | Product monitored | $K_m$ | $V_{max}$ |
|------------------|------------------|-------|----------|
| Gly-Gly-Gly       | YALPETGGG        | 0.043 | 0.038    |
| Agmatine          | (NH$_2$)$_3$C = N-(CH$_3$)$_3$(NH-CO)-TEPLAY | 12.18 | 0.030 |
| YALPETGK (Hydrolysis) | YALPET | 1.59 | 0.001 |

linkage comprising the e-amine of Lys and $\alpha$-carboxyl group of Thr. This is in contrast to the results seen in the case of the LPXTG-appended pilin motif peptide (P2) where only a cyclized product was formed. The difference in the reaction product profile may be related to the presence or absence of a stable acyl-enzyme intermediate.

**A Long-lived and Stable Acyl-enzyme Intermediate Is Important for Branched Oligomer Assembly**

The time course studies of SrtA reaction with P4 revealed that the acyl-enzyme intermediate was formed rather rapidly and remained stable throughout the course of the reaction (Fig. 4). The stability of the acyl-enzyme intermediate was further probed by SDS-PAGE. Analysis of the reaction mixture (24 h) revealed the presence of a band migrating slightly above the band corresponding to SrtA, indicating the presence of a species with molecular weight higher than that of SrtA (Fig. 5A, lane 1). Detection of both bands by Western blotting with anti-polyhistidine antibody indicated the presence of SrtA as a component in the higher molecular weight species (Fig. 5B, lane 1). That the high molecular band was an acyl-enzyme intermediate was confirmed using the RP-HPLC purified (peak 1) standard; peak 1 migrated at the same position as the higher molecular weight species (Fig. 5A, lane 4) and was detected by Western blotting with anti-polyhistidine antibody (Fig. 5B, lane 4).

How critical is the requirement that the acyl-enzyme intermediate be “stable and long-lived” for a facile oligomerization reaction through isopeptide ligation? The formation of a thio-ester acyl enzyme intermediate involving threonyl carboxyl of the LPXTG motif and thiol group of active site Cys$^{184}$ is the first step in sortase-mediated peptide ligation. This intermediate can be subsequently attacked by an amine nucleophile ($\alpha$ or e) resulting in transpeptidation (peptide ligation) or simply be attacked by a water molecule leading to hydrolysis. Thus the hydrolytic reaction competes with transpeptidation. In fact apparent $K_m$ values for hydrolysis of the YALPETGK substrate (1.6 mM) were found to be about 8-fold lower as compared with agmatine (4-aminobutyl guanidine, $K_m$ ~ 12 mM), which can be considered as a surrogate for e-amine of Lys residue (Table 2). Taking the proteolysis factor into account and considering that e-amine is not a preferred nucleophile, transpeptidation through an isopeptide linkage is likely to be facilitated in conditions that enhance the stability of the thioester acyl-enzyme intermediate. We surmised that one or more of the hydrophobic tryptophan residues of indolicidin might be determinants of strong nucleophilic capacity of the e-amine group of the lysine residue and/or acyl-enzyme intermediate formation. Hence, synthetic analogs of P4 (P5, P6, and P7), wherein individual tryptophan residues flanking Lys$^5$ were substituted by an Ala residue, were created and their site-specific impact on sortase-mediated isopeptide ligation reaction was studied.

Under identical conditions, P5 (W4A analog) yielded low levels of acyl-enzyme intermediate, cyclic peptide, hydrolyzed peptide, and a small amount of dimeric species as compared with the wild type peptide (Fig. 6A). P6 (W6A analog) produced an acyl-enzyme intermediate that was even lower in amounts as compared with P5 and showed very little or no hydrolytic or transpeptidation products (Fig. 6B). The analog P7 (W4A,W6A), in which both Trp residues were replaced by Ala, produced a significant amount of hydrolyzed and cyclic products, a small amount of the acyl-enzyme intermediate, and no branched oligomers (Fig. 6C). Thus the product profile of the P7 (W4A,W6A) analog was very similar to that of the pilin motif peptide (P2). The overall results emanating from studies of sortase action on Ala analogs of indolicidin, which show diminished levels of the acyl-enzyme intermediate and an absence of the oligomeric species, highlight the importance of a
long-lived acyl-enzyme intermediate in facilitating the formation of branched oligomers.

**Distance of Separation between Lys (ε-amine) and Thr (α-Carboxyl) Is Crucial for Facile Peptide Cyclization**

Both pilin motif (P2) as well as the indolicidin (P4) sequence appeared to be good sortase substrates for intramolecular cyclization through isopeptide linkage. Thus these two peptide substrates should contain some common structural traits that favor cyclization reaction. Far-UV circular dichroic spectra of the peptides, P2 and P4, respectively, were recorded to see if the peptides displayed any secondary structure (Fig. 7). The CD spectrum of pilin peptide (P2) displayed a negative band at 198 nm and was featureless suggesting that this peptide lacked rigid elements of the secondary structure. Indolicidin analog (P4) was characterized by a single negative band around 200 nm suggesting largely randomized conformation.

The proximity brought about by folding or distance between the reacting Lys (ε-amine) and Thr (α-carboxyl) in an extended conformation should be important factors in cyclization. Notably, P2 and P4 are predominantly unstructured but have a similar sequence length and the reactive Lys and Thr residues in each are separated by 13 or 14 intervening residues. To see if this distance between the reacting ends was the cause for facile cyclization, the above peptides were shortened by excising out the triglycine (GGG) residues that were originally meant to separate the pilin/indolicidin sequence from the sortase-recognition motif. Accordingly, synthetic peptides, P8 and P9, consisting of the pilin/indolicidin sequence embedded with the LPXTG sequence in contiguity were made and reacted with SrtA. Interestingly, the cyclization reaction was completely abrogated in both cases. Although pilin peptide (P8) formed the hydrolyzed product (supplemental Fig. S9), indolicidin peptide (P9) yielded the acyl-enzyme intermediate and oligomers but not the cyclized product (supplemental Fig. S10 and supplemental Table S7).

That the distance relationship between reactive amino acids was indeed crucial for cyclization was further corroborated by engineering the original sequence length but substituting Gly-Gly-Gly residues in the indolicidin peptide by Ala-Ala-Ala residues (P10). RP-HPLC profile of the reaction between this peptide P10 and SrtA revealed the presence of the cyclized product along with the acyl-enzyme intermediate and the branched oligomers (data not shown). The retention of cyclization in the Ala-Ala-Ala analog (P10) reinforces the importance of optimum distance of separation between reacting ε-amine and the

**FIGURE 7. CD spectra of peptides.** The spectra of peptides, P2 (solid line) and P4 (broken line), were recorded at a concentration of about 40 μM using a 0.2-cm path-length cell at 25 °C. The spectra are represented as the mean residue ellipticity (MRE), expressed in degree cm² dmol⁻¹.

**FIGURE 8. Reaction of SrtA with P4 in the presence and absence of the dimer.** A, standard reaction of SrtA (0.1 mM) with P4 (0.2 mM). B, reaction of SrtA with P4 as in A but in the presence of 0.4 mM of the dimer. The numbers in parentheses indicate the experimental mass (Da) of the respective reaction products.
The enduring presence of a stable acyl-enzyme intermediate, as revealed by the time course experiment, appears central to the SrtA-catalyzed isopeptide ligation-linked oligomerization of indolicidin. Two possible mechanisms can be envisaged for generation of the branched oligomers from the acyl-enzyme intermediate. The first mechanism is reminiscent of the proposal for the covalent assembly of pilus catalyzed by pilin sortases that involves the growth of the dendrimeric chain on the enzyme through sequential reactions involving two acyl-enzyme intermediates (14). For example, a dimer could be formed when the acyl-enzyme intermediate is resolved by nucleophilic attack of the \( \text{e-amino} \) group of indolicidin Lys\(^5\) from another molecule of the acyl-enzyme intermediate. Likewise, the enzyme-linked dimer can be further attacked by Lys\(^5\) in the acyl-enzyme to produce a trimer. Likewise, the reaction continues to produce tetramer and higher branched oligomers. In general, the number of branched chain \( (n) \) may depend on the nature of the substrate and steric factors.

**Mechanism of SrtA-mediated Isopeptide-linked Oligomerization**

The active site cysteine of SrtA performs a nucleophilic attack on the Thr-Gly bond of the LPXTG motif of a molecule of P4 (red circle) to form an acyl-enzyme intermediate. A, the intermediate undergoes hydrolysis to form the hydrolyzed peptide (green circle). B, intramolecular transpeptidation leads to the formation of a cyclic peptide. C, nucleophilic attack by the \( \text{e-amino} \) of Lys\(^5\) of P4 leads to the formation of a branched dimer. D, the acyl-enzyme intermediate is attacked by the \( \text{e-amino} \) of Lys\(^5\) of the dimer to produce a trimer. Likewise, the reaction continues to produce tetramer and higher branched oligomers. In general, the number of branched chain \( (n) \) may depend on the nature of the substrate and steric factors.
The second mechanism could be visualized as a reaction between an acyl-enzyme intermediate and the free monomeric or oligomeric peptide. Here the acyl-enzyme intermediate is attacked by the Lys\textsuperscript{ε} amine of the free parent peptide to produce the branched dimer. The stability and longevity of the intermediate makes it amenable to a subsequent nucleophilic attack by the Lys\textsuperscript{ε} of the dimer to produce a trimer and likewise. This mechanism is consistent with the fact that a single species of the acyl-enzyme intermediate composed of only one molecule of the peptide could be seen by SDS-PAGE, Western blotting, and RP-HPLC. To further clarify the mechanism, transpeptidation reaction of SrtA and P4 (0.2 mM) was carried out in the presence of excess (0.4 mM) branched dimer (Fig. 8). ES-MS analysis of the acyl-enzyme intermediate isolated from the RP-HPLC yielded the same mass as observed for the acyl-enzyme intermediate present in the reaction that was carried out without the added dimer suggesting the absence of any acyl-enzyme intermediate built from dimer or higher oligomers. Interestingly, the presence of the excess dimeric species blocked formation of the cyclized peptide and resulted in enhanced utilization of the acyl-enzyme intermediate leading to the increased yield of the higher oligomeric products. Taken together the results show that SrtA-catalyzed oligomerization of LPXTG-containing polypeptides follow a mechanism that is distinct from that of the pilin sortases (illustrated in Fig. 9).

**DISCUSSION**

Our results show that SrtA-mediated isopeptide ligation can ensue from intermolecular as well as intramolecular transpeptidation leading to peptide branching and/or cyclization. The occurrence of facile oligomerization in the case of P4, P9, and P10 and its diminution or absence in P2, P5, P6, P7, and P8, clearly indicates that isopeptide-linked peptide oligomerization is intimately associated with the longevity of the acyl-enzyme intermediate. Thus, oligomerization is facilitated in situations that give rise to a long-lived acyl-enzyme intermediate because \( \varepsilon \)-amine is a relatively weak nucleophilic substrate and cannot effectively compete with the hydrolytic reaction. The observation of stable acyl-enzyme complexes comprising pilin sortase and pilin polymers is consistent with this idea (14).

In contrast, the cyclization reaction can proceed even with the intermediary of transient or relatively less stable acyl-enzyme intermediates, as observed in the case of P2 and P7, because entropic effects emanating from stereochromical positioning of the reactive threonyl and lysyl residues can overwhelm the hydrolytic reaction. Thus distance between the reacting ends should be a major force driving the isopeptidyl-linked cyclization reaction. The natural propensity of SrtA to ligate LPXTG and aminoglycine has been earlier exploited for generating circular proteins. SrtA was shown to catalyze the cyclization and oligomerization of proteins equipped with oligoglycine and the LPXTG sequence, at the N and C terminus, respectively (27, 48). The importance of spacing between the termini for facile cyclization was emphasized but not characterized (27). Our results with the pilin motif and indolicidin-derived peptides illustrate that an intervening sequence of about 13 to 14 residues in an extended conformation may be optimum to cover the distance between the reactive Lys and Thr residues.

Stability and protease resistance of a polypeptide chain can be enhanced by engineering isopeptide bonds. Isopeptide bonds are found in several natural proteins but are not made by the genetic machinery. These are either formed enzymatically or by an autocatalytic process. Therefore, innovative strategies are required for site-specific incorporation of isopeptide bonds in peptide/proteins. Enzymes such as transglutaminase can introduce isopeptide cross-links in proteins by condensing side chains of Lys and Gin residues but they lack stringent sequence specificity. Pilin sortases that catalyze the covalent assembly of pilus by joining pilin subunits through isopeptide linkages are specific but they are characterized by low catalytic efficiency. In light of this, catalysis of isopeptide ligation by quintessential SrtA is quite fascinating. The branched oligomerization of indolicidin derivatives represents an inspiring example for future exploration of SrtA as a tool for the semisynthesis of multivalent polypeptide ligands. Besides, the mechanistic cues obtained from the studies will facilitate the strategic design of experiments to favor cyclization over branched oligomerization.

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