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Sorting of LPXTG Peptides by Archetypal Sortase A: Role of Invariant Substrate Residues in Modulating the Enzyme Dynamics and Conformational Signature of a Productive Substrate

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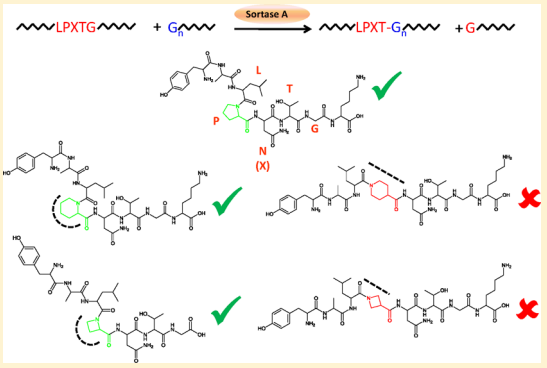
Supporting Information

ABSTRACT: Transpeptidase sortase catalyzes the covalent anchoring of surface proteins to the cell wall in Gram-positive bacteria. Sortase A (SrtA) of *Staphylococcus aureus* is a prototype enzyme and considered a bona fide drug target because several substrate proteins are virulence-related and implicated in pathogenesis. Besides, SrtA also works as a versatile tool in protein engineering. Surface proteins destined for cell wall anchoring contain a LPXTG sequence located in their C-terminus which serves as a substrate recognition motif for SrtA. Recent studies have implicated substrate-induced conformational dynamics in SrtA. In the present work, we have explored the roles of invariant Leu and Pro residues of the substrate in modulating the enzyme dynamics with a view to understand the selection process of a catalytically competent substrate. Overall results of molecular dynamics simulations and experiments carried out with noncanonical substrates and site-directed mutagenesis reveal that the kinked conformation due to Pro in LPXTG is obligatory for productive binding but does not per se control the enzyme dynamics. The Leu residue of the substrate appears to play the crucial role of an anchor to the beta6−beta7 loop directing the conformational transition of the enzyme from an "open" to a "closed" state subsequent to which the Pro residue facilitates the consummation of binding through predominant engagement of the loop and catalytic motif residues in hydrophobic interactions. Collectively, our study provides insights about specificity, tolerance, and conformational sorting of substrate by SrtA. These results have important implications in designing newer substrates and inhibitors for this multifaceted enzyme.



Sortase enzymes belong to the family of cysteine transpeptidases that catalyze covalent anchoring of surface proteins to the peptidogycan in Gram-positive bacteria.[1−3] Almost all Gram-positive bacteria express a housekeeping sortase usually referred to as sortase A meant for anchoring a majority of the surface proteins. Sortase A of *Staphylococcus aureus* (designated SrtA) is considered a prototype housekeeping enzyme.[1,4−6] Several of SrtA substrates are virulence-related and implicated in host−pathogen interactions.[7−10] Therefore, SrtA is considered an important target for therapeutic inhibitor development.[11−14]

The SrtA-mediated anchoring process involves a transpeptidation reaction by which surface proteins containing a LPXTG sorting motif are covalently attached to the peptidoglycan.[15] The transpeptidation is proposed to occur through the mediation of an acyl-enzyme thioester intermediate that is formed by attack of the active site Cys184 thiolate on the T−G peptide bond of the LPXTG protein substrate.[16−19] The acyl-enzyme intermediate is subsequently resolved by the amino group of the terminal Gly residue of the pentaglycine branch in the peptidoglycan. Interestingly, transpeptidation reaction proceeds quite well even in vitro[20] and has enabled many applications of SrtA in protein engineering, labeling, and bioconjugation.[21−34] However, engineering of SrtA with capabilities to recognize a diverse range of peptide substrates is necessary to enlarge the synthetic ambit of this enzyme.

Delineating the underlying principles and mechanisms governing SrtA−substrate interaction, specificity, tolerance, and catalysis is imperative to design therapeutic inhibitors as well as to expand the utility of this multifaceted enzyme. The structure of the catalytically active domain representing residues 60−206 of SrtA elucidated by X-ray crystallography and NMR show an eight-strand β-barrel fold in which the β-strands are connected by multiple loops and two helices.[35−37] The loop regions, especially those connecting the β6 and β7 strands located in the vicinity of the active site, are observed to be highly disordered and mobile as compared to the remainder of the protein. Atomic resolution information on the SrtA−substrate interactions derived from the NMR structure of SrtA linked covalently to a modified LPAT peptide has revealed severe restriction of β6−β7 loop movement, indicating

Received: December 1, 2013
Revised: March 6, 2014
Published: April 2, 2014

dx.doi.org/10.1021/bi4016023 | *Biochemistry* 2014, 53, 2515−2524

conformational transition of the β6−β7 loop from a disordered "open" state to an ordered "closed" state.[37] In contrast, the crystal structure of inactive SrtA (C184A) mutant bound to a native LPETG pentapeptide shows the β6-β7 loop in an "open" conformation.[36]

Interestingly, the substrate peptide displayed different conformations in the two structures. In the NMR structure, LPAT peptide covalently bound to the enzyme assumed a L-shaped "kinked" conformation and was held in the cleft through extensive contacts between Leu of the substrate and residues spanning Val166-Leu169 of the β6−β7 loop.[37] In contrast, crystal structure of LPETG in complex with SrtA (C184A) showed the peptide in an extended conformation with side chains projecting away into the solvent.[36] The extended binding pose was in contradiction with the biochemical experiments and was considered to have arisen due to nonspecific binding of the peptide to the enzyme.[37] On the other hand, "kinked" LPAT substrate conformation captured in the NMR structure of SrtA-substrate covalent adduct appears consistent with site-directed mutations,[38] domain swapping,[39] and random mutagenesis experiments.[40] Curiously, molecular dynamics (MD) simulations have indicated the existence of multiple conformational states for LPXTG peptide in the binding cleft, thereby suggesting that both experimentally determined poses of the substrate (kinked in NMR and extended in crystal) are possible.[41] This possibility, however, should be viewed with the caveat that both the "open" state crystal structure and the "closed" state NMR structure have employed a somewhat artificial peptide substrate bound to a catalytically inactive enzyme. Notwithstanding this, the atomic resolution experimental and simulation data provide compelling evidence for the presence of intrinsic and substrate-induced dynamics in SrtA.

In the present study, we have experimentally defined the chemical/conformational signatures of a minimal productive SrtA recognition motif and used an authentic substrate to delineate the role of fully conserved Leu and Pro residues of the substrate in modulating the dynamics of the β6−β7 loop. Furthermore, we have carried out transpeptidation assays employing LPXTG peptide substrates integrated with non-canonical Pro analogues to establish the relevance of kink or extended conformation to productive binding. Collectively, our results attribute distinct roles to Leu and Pro residues in modulating the dynamics of the β6−β7 loop and unequivocally establish the absolute requirement of "kinked" conformation to productive binding and catalysis.

EXPERIMENTAL PROCEDURES

Materials. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Custom-synthesized oligonucleotide primers were obtained from Sigma. Standard Fmoc-amino acids, preloaded Wang resins and Rink amide resins, were procured from Novabiochem. Fmoc-piperidine-2-carboxylic acid and Fmoc-azetidine-3-carboxylic acid were bought from Peptech Corporation. Fmoc-piperidine-4-carboxylic acid and Fmoc-azetidine-2-carboxylic acid were purchased from Fluka. Fmoc-*cis*-4-fluoroproline and Fmoc-3,4-dehydroproline were purchased from Bachem. Fmoc-4-hydroxyproline was obtained from Advanced Chemtech. Fmoc-5,5,5-DL-trifluoroleucine was obtained from Anaspec. Fmoc-*N*-methyl-Leucine, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) and diisopropylethylamine (DIEA) were purchased from Sigma. All other solvents and reagents used in peptide synthesis were purchased from Applied Biosystems Inc., USA.

Expression and Purification of SrtA. Sortase A sequence encoding residues 60−206 (SrtA) was expressed in *Escherichia coli* BL-21 cells as described previously.[22] The cells containing the *srtA* plasmid were grown until mid log phase (OD_{600} = 0.5) at 37 °C, induced with 0.2 mM IPTG for protein expression and subsequently grown at 30 °C for 3 h. The cells were harvested, suspended in buffer A [10 mM Tris·HCl (pH 7.5), 40 mM NaCl, 2 mM 2-mercaptoethanol], and lysed by sonication. The resulting lysate was centrifuged at 10 000 rpm for 30 min, and the supernatant was collected. SrtA present in the supernatant was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose column as described earlier.[22] The purity of protein was analyzed by SDS-PAGE and mass spectrometry.

Preparation of SrtA Mutants. Sortase A sequence encoding residues 60−206 (SrtA), previously cloned into the pET23b vector (SrtAΔ59-pET23b) was used as the template for the introduction of desired mutations.[22] The mutation was introduced by PCR using QuikChange (Stratagene) protocol using appropriate primers listed in Table S1, Supporting Information. The identity of each mutation was confirmed by DNA sequencing. SrtA mutants were expressed, purified, and characterized as described above for the wild type protein.

Synthesis of Peptide Substrates. Peptides were assembled by standard solid-phase method employing Fmoc chemistry on a peptide synthesizer (Applied Biosystems, ABI 433A or Advanced Chemtech, Model 90) as per standard procedures and using preloaded Wang resin or Rink amide AM resin as starting material. After completion of the synthesis, the respective peptides were cleaved from the resin, and side chains were deprotected with 95% trifluoroacetic acid (TFA). The resin was removed by filtration, and the crude peptide was precipitated in chilled ether. The peptides were purified by reverse phase HPLC using a C-18 preparative column (100 Å, 10 μ, 30 × 250 mm, Phenomenex, gradient: 8−72% acetonitrile in 0.1% TFA in 60 min, flow rate: 30 mL/min). The identity of each purified peptide was confirmed by electrospray-ionization mass spectrometry (Table S2, Supporting Information).

Acetylation of Peptide Substrates. The Fmoc group was removed to generate the free amine on the resin-bound peptide. The resin was washed with DMF/DCM, dried, and treated for 1 h with 10% acetic anhydride in the presence of 10% DIEA prepared in DMF. The acetylated peptide was cleaved from the resin and purified as described above.

Synthesis of Acetyl-Leu-Pro-Asn-Thr-*N*-Methyl Amide. The purified acetyl-LPNT-OH peptide was treated with 5-fold molar excess of methylamine in the presence of EDC for 6 h. The *N*-methyl amide derivatized peptide was isolated using reverse phase HPLC and characterized by ESI-MS (Table S2, Supporting Information).

HPLC Assay for SrtA-Catalyzed Transpeptidation Reaction. Sortase-mediated transpeptidation reaction between LPXTG peptide substrate or analogues (0.5 mM) and aminoglycine (G_3KY) nucleophile (1 mM) was carried out at pH 7.5 (50 mM Tris·HCl, 150 mM NaCl, 5 mM $CaCl_2$, 2 mM 2-mercaptoethanol) in the presence of 50 μM enzyme. Reactions were set up in a volume of 50 μL at 37 °C for indicated time and analyzed by HPLC as described previously.[22] Relevant peak fractions were collected and characterized by mass spectrometry (Table S3, Supporting Information). The yield of the transpeptidation product was

calculated from the peak areas obtained using LCsolution software provided by Shimadzu Corporation, Japan.

Circular Dichroism (CD) Measurements. CD spectra of wild type SrtA and mutants (L169N, L169A, and L169D) were recorded in 10 mM potassium phosphate buffer (pH 7.5) on a J-710 (Jasco, Japan) Spectropolarimeter using a 0.2 cm path-length cell at 25 °C. The concentration of each protein sample was approximately 5 μM. The spectra are represented as mean residue ellipticity (MRE), expressed in deg cm^2 dmol^{-1}.

Molecular Dynamic Simulation. MD simulations were carried out for SrtA alone and in complex with various peptide substrates using AMBER11 suite of programs,[42] and the ff99SB force field. The initial structure of SrtA was taken from the NMR structure of sortase−LPAT complex (PDB ID: 2KID), from which the covalently attached substrate was removed and the Cys184 was restored for ApoSrt simulations. The protein was solvated using TIP3P water in a truncated octahedral box with a distance of 10 Å between surface of the protein and the walls of the box. SrtA activity is enhanced in the presence of calcium atom which is liganded to residues E105, E108, and D108 of the β3−β4 loop.[37,43] Therefore, all simulations were carried out with a calcium atom placed in this site. AMBER compatible parameters for the calcium atom were obtained from the parameter database hosted by Richard A. Bryce.[44] The charge of the system was neutralized by addition of three chloride ions. For the SrtA−substrate complex simulations, the initial model of the substrate was created by first detaching the covalently bound ligand from the NMR structure. As was shown from experimental studies (vide infra), it was found necessary to block the two ends of the substrate peptide for optimum activity. Accordingly, acetyl (Ac) and *N*-methyl (Nme) end groups were interactively modeled into the ends of the substrate peptide keeping the rest of the molecule in the same conformation as the NMR structure. Initial models of the substrate analogues were also constructed similarly except that side chain atoms following the Cα atom of either the Leu or Pro residues of the substrate were removed, thus converting them to an Ala. Equilibration was carried out essentially using the nine-step protocol as described by Chachra et al.[45] and employed the SANDER module of the AMBER11 suite of programs. Briefly stated, the procedure consists of alternating cycles of energy minimization and restrained molecular dynamics. An initial restraint weight of 5.0 kcal/mol on all non-hydrogen atoms was applied. In subsequent steps, the restraint weights were gradually reduced ending with a 50 ps MD step with a restraint weight of 0.1 kcal/mol only on the backbone atoms of the protein. This was followed by an unrestrained MD simulation for 100 ps also using the SANDER module. The final atomic positions and velocities were used for 100 ns of production MD simulations using the GPU accelerated PMEMD-CUDA program from the AMBER suite. Production simulations were carried out in the NPT ensemble, using a 2 fs integration time step. Temperature and pressures were held at 298.15 K and 1 atm respectively and regulated using Berendsen schemes with the heat-bath coupling and pressure relation time constants both set to 1.0 ps. The SHAKE algorithm was used to constrain all bonds involving hydrogen atoms, short-range electrostatic cut off was kept at 10 Å, beyond which particle mesh Ewald (PME) method was used to calculate long-range electrostatics. Structures were saved every 2 ps, thus providing a total of 50 000 snapshots out of the 100 ns trajectory for analysis. All trajectory analyses were carried using the PTRAJ and CPPTRAJ modules built into the AMBER package. Dynamic cross-correlation maps (DCCM) as a tool to follow protein−substrate communication were calculated essentially as described by Harte et al.[46] and Swaminathan et al.[47]

RESULTS

Definition of a Minimalist SrtA Peptide Recognition Motif. The sortase recognition LPXTG sequence is located near the C-terminus of bacterial protein substrates and is always followed by a stretch of residues. Therefore, it was pertinent to see if an isolated LPXTG pentapeptide would serve as an effective SrtA substrate. We first examined the propensity of three LPXTG pentapeptide sequences (Figure 1A) containing

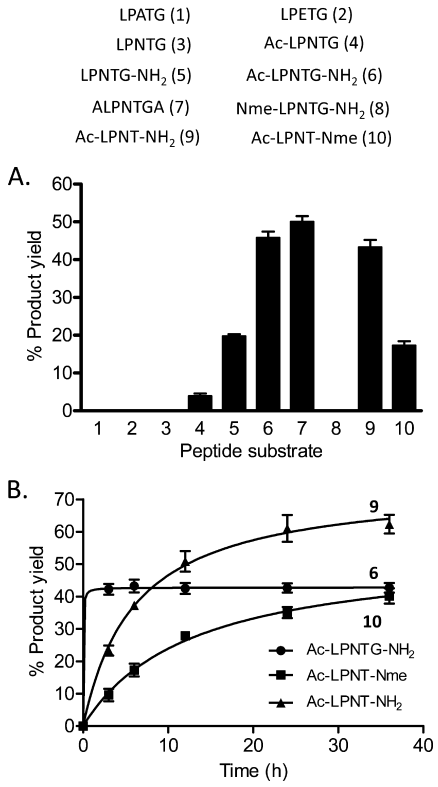


Figure 1. Chemical signature of a minimalist sortase-recognition peptide motif. SrtA-catalyzed transpeptidation reaction of respective substrates with GGGKY as an acceptor peptide. (A) Product yield associated with various peptide substrates obtained after 6 h of transpeptidation reaction. (B) Time course of transpeptidation reaction with tetrapeptide substrates Ac-LPNT-NH$_2$ (peptide 9) and Ac-LPNT-Nme (peptide 10) respectively with GGGKY. The Ac-LPNTG-NH$_2$ (peptide 6) is also included for comparison. Each data point represents mean ± SD of three independent experiments.

Glu, Ala, or Asn respectively at the X position to undergo SrtA-mediated transpeptidation by a RP-HPLC assay using GGGKY as a nucleophile (Figure S1, Supporting Information). Interestingly, these peptides (labeled 1, 2, and 3) with free termini did not yield any detectable transpeptidation product when assayed after 6 h of reaction. To further delineate the chemical signatures of a minimal SrtA recognition motif, we chose LPNTG as a model sequence and carried out systematic modifications by incorporating acetyl and/or amide groups at the N- and C-terminus (Figure 1A). The acetylated (peptide 4) or amidated (peptide 5) versions of LPNTG afforded about 5 or 20% conversion to product, respectively. The product yield

dx.doi.org/10.1021/bi4016023 | *Biochemistry* 2014, 53, 2515−2524

improved to about 45% when both termini of the peptide were blocked (peptide 6), suggesting that charge neutralization at the termini was important. Consistent with this, the presence of an Ala residue (ALPNTGA) at the termini (peptide 7) elicited the same effect and resulted in a similar or slightly better yield (45−50%) of the product.

Together the above results show that the charge neutrality achieved at the termini of the LPXTG motif either by acetylation/amidation or due to the presence of an additional residue exerts a similar effect on the transpeptidation reaction. This could also be interpreted to mean that the amide or peptide linkage (−CONH−) flanking the LPXTG sequence may have some role to play in the recognition of a catalytically competent substrate. To see that this was indeed true, we replaced the acetyl group by a methyl and subjected the N-methylated and C-amidated LPNTG peptide (peptide 8) to a transpeptidation reaction. Interestingly, this peptide behaved much like peptides 1, 2, or 3 (peptide with free termini) and did not form any product, thereby suggesting a crucial role of the peptide bond preceding the Leu residue in the process of productive substrate recognition by SrtA.

To further elucidate the chemical features of a minimalist bona fide productive SrtA substrate, we explored the propensity of threonine amide as SrtA substrates. We reasoned that intrinsic amidase activity of SrtA may facilitate acylation of an amide substrate to form an acyl-enzyme intermediate. Accordingly, a tetrapeptide Ac-LPNT-NH_2 (peptide 9) was synthesized and subjected to transpeptidation reaction (Figure S2, Supporting Information). Under standard conditions, SrtA catalyzed the transpeptidation reaction albeit at a slower rate reaching equilibrium in about 36 h (Figure 1B). However, peptide 9 produced a product yield of about 65% that was higher than the equilibrium yield of 40−45% obtained when peptide 6 (Ac-LPNTG-NH_2) was used as a substrate. The reason for the higher conversion in the case of Ac-LPNT-NH_2 is perhaps linked to the reversibility of the transpeptidation reaction, which is somewhat compromised because the ammonia byproduct may not be as good a substrate for reverse reaction as aminoglycine.

SrtA-mediated transpeptidation reaction with an another version of the above peptide (Ac-LPNT-NH-CH_3, peptide 10) carrying a methyl substitution in the amide (threonine methylamide) occurred with a relatively slower rate but produced equilibrium yield (about 40% in 36 h) that compared well with Ac-LPNTG-NH_2 (Figure 1B).

Dynamics of SrtA Interaction with an Authentic Peptide Recognition Motif. We used the above experimentally verified Ac-LPXT-NH-CH_3 (X = Ala) peptide to define the role of invariant Leu and Pro residues of the substrate in modulating the dynamics of SrtA as well as their ability to generate a catalytically competent conformation of the peptide. Toward this, we first undertook the MD simulation studies of SrtA in the absence (designated ApoSrt) and presence of Ac-LPNT-NH-CH_3 (designated Srt-LPAT).

The time-dependent Cα atom RMSD values for both ApoSrt and Srt-LPAT complex simulations were found to vary within an acceptable region (around 2 Å for ApoSrt and 1.6 Å for Srt-LPAT complex), indicating that the enzyme structure was stable through the span of simulation (Figure 2A). Analyses of the time-averaged Cα atom RMSF profiles of ApoSrt and Srt-LPAT complex showed significantly higher values in the region of residues 160−180 that harbors the $\beta6$−$\beta7$ loop in ApoSrt compared to Srt-LPAT complex (Figure 2B). This indicates

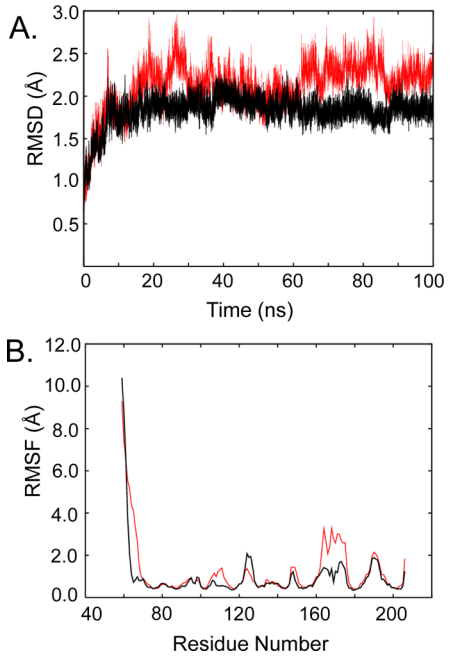


Figure 2. Dynamics of ApoSrt and SrtA bound to Ac-LPAT-Nme (Srt-LPAT). (A) Time-dependent RMSD using initial NMR structure (PDB ID: 2KID) as reference. RMSD was calculated only for Cα atoms in the protein. ApoSrt (red) and Srt-LPAT (black). (B) Root mean squared fluctuations (RMSF) of Cα atoms of ApoSrt (red) and Srt-LPAT (black).

that the $\beta6$−$\beta7$ loop is highly mobile in the Apo-enzyme but becomes conformationally locked in the enzyme−substrate complex.

To further assess the motion of the $\beta6$−$\beta7$ loop, we analyzed the time-dependent RMSD specifically for residues spanning this loop. This was done by first superimposing snapshots from the trajectories onto their initial structures using all protein Cα atoms except for 10 residues at the N-terminus and the residues of the $\beta6$−$\beta7$ loop (residues 158−178). RMSD was then calculated only for Cα atoms lying within the $\beta6$−$\beta7$ loop. The time-dependent RMSD profile for the $\beta6$−$\beta7$ loop depicted in Figure S3A, Supporting Information clearly shows that the loop in the Srt-LPAT complex remains stable with an RMSD of about 3.5 Å, compared to the corresponding region in the initial structure. In the case of ApoSrt, however, the RMSD values increased considerably with time reaching almost up to 6 Å. We then compared the RMSD of snapshots from the ApoSrt trajectory with the SrtA crystal structure (1T2O) as reference. As expected, the RMSD values obtained are generally high and mostly vary within a 3−4 Å band. However, on a number of occasions, the RMSD value decreases to as low as 2.5 Å, a value even lower than the average RMSDs obtained when the NMR structure (from which the simulation has started) was used as reference. This indicates that the continuum of conformations spanned by the MD trajectory of ApoSrt covers both the crystal and the NMR structure of the protein (Figure S3B, Supporting Information). Comparison of the ApoSrt and Srt-LPAT complex simulations further indicates that the $\beta6$−$\beta7$ loop is kept stable in the "closed" conformation (similar to the NMR structure of the complex) due to interactions with the Ac-LPAT-NH-CH_3 substrate, in the absence of which it remains disordered as found in the crystal structure.

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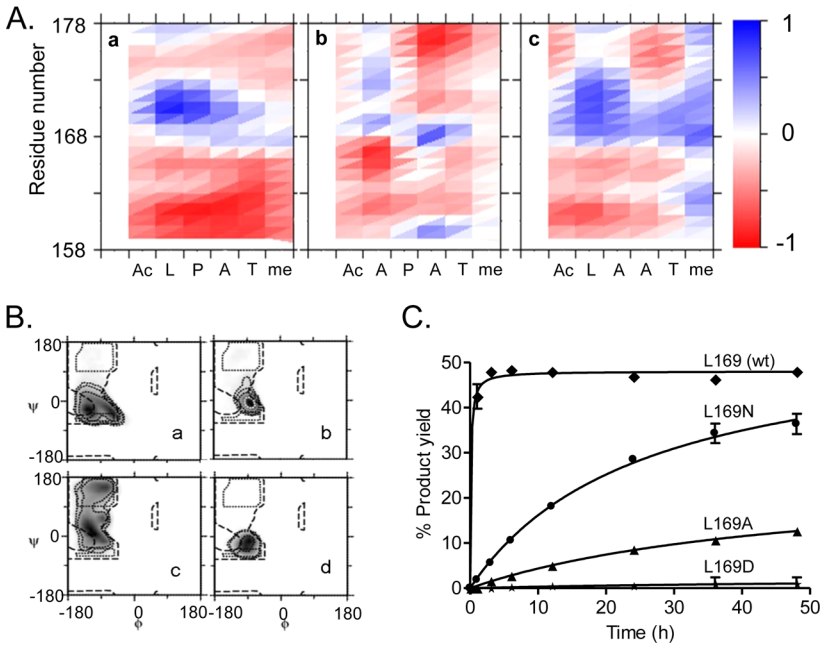

Figure 3. Dynamic cross-correlation map (DCCM) of SrtA in complex with its cognate and decoy substrates. (A) DCCM was generated for each MD simulation trajectory following the protocol described in the methods. Only correlations between the substrate and residues 158−178 corresponding to the $\beta6-\beta7$ region of SrtA are shown. Positive correlations are shown in shades of blue, and negative correlations are depicted in shades of red. Correlations of larger magnitude are shown in darker colors. Panel a, SrtA complexed to Ac-LPAT-Nme (Srt-LPAT). Panel b, SrtA complexed to Ac-APAT-Nme (Srt-APAT). Panel c, SrtA complexed to Ac-LAAT-Nme (Srt-LAAT). (B) Backbone conformational dynamics of Leu169. The backbone torsion angles for Leu169 of SrtA is presented in the form of Ramachandran plots. Panel a, ApoSrt. Panel b, Srt-LPAT. Panel c, Srt-APAT. Panel d, Srt-LAAT. (C) Influence of L169 mutations on SrtA activity. The transpeptidation reaction was carried out using YALPNTGK and GGGKY as substrates as described in Figure 1. Each data point represents the mean ± SD of three independent experiments.

Leu and Pro Residues of the Substrate Exert Distinct Effects on SrtA Dynamics. To determine the specific protein−ligand interactions that help to maintain the productive substrate conformation, we carried out MD simulations of Srt-substrate complexes in which Leu or Pro substrate residues were replaced by an Ala. We used dynamic correlation map of Srt-substrate complexes, namely, Srt-LPAT (native), Srt-APAT (Leu to Ala), and Srt-LAAT (Pro to Ala) to identify correlated motions of substrate and protein residues (Figure 3A). The SrtA-substrate dynamic cross-correlation map (DCCM) in the case of native substrate (LPAT) shows strong positive correlations between Leu and Pro residues of the substrate and the $\beta6-\beta7$ loop residues in the vicinity of Leu169 (Figure 3A, panel a). This correlation is somewhat weakened or diffused when the substrate Pro residue is replaced by Ala (LAAT) but completely abrogated in the case of Leu to Ala (APAT) replacement (Figure 3A, panels b and c).

To further clarify the role of substrate residues in dictating SrtA dynamics, we investigated the backbone conformation of Leu169 located in the $\beta6-\beta7$ loop together with the substrate residues. Leu169 is an evolutionarily conserved residue implicated in substrate interactions.[15,38] Toward this, we compared the Ramachandran plots of Leu169 as well as substrate residues from the simulation of each SrtA−substrate complex. The data presented in Figure 3B clearly show that the dynamics of Leu169 is strongly dependent on the presence of the type of substrate (native or mutant) in the active site cleft. Leu169 becomes highly dynamic when the Leu residue of the substrate is substituted by Ala (Figure 3B, panel c). Interestingly, the backbone conformation of the substrate does not change significantly in either in case of the wild type substrate or in the case of Leu to Ala substitution (Figure S4A,B, Supporting Information). On the other hand, Pro to Ala substitution in the substrate does not perturb the dynamics of Leu169 (Figure 3B, panel d) but causes considerable alterations in substrate backbone dynamics (Figure S4C, Supporting Information). The results indicate that interactions emanating from Leu and Pro residues of the substrate have different manifestations. For Leu to Ala substitution, destabilization of the substrate seems to be related to the increased dynamics of the $\beta6-\beta7$ loop and consequent inability of the decoy to induce proper conformation for substrate recognition. In the case of Pro to Ala substitution, it appears to influence the conformational fit of the substrate in the active site.

Leu169 May Be a Crucial Site for Substrate Anchoring on the $\beta6-\beta7$ Loop. The severe restriction of conformational dynamics of Leu169 in SrtA−substrate complex confers a critical role for this residue in substrate immobilization.[38] This is also borne out by the fact that earlier Ala scan studies of residues representing sequence 167−172 of the $\beta6-\beta7$ loop found that Leu169 mutation exerted the highest deleterious impact on enzyme activity.[38] The Leu169 to Ala mutation led to about a 90-fold decrease in enzyme activity as compared with a meager 2−5 fold for other residues of the loop.[38] We surmised that the dramatic diminution of enzyme activity in the Ala169 mutant that arises due to loss of interactions associated with Leu residue may be somewhat restored if an isosteric residue was substituted for Leu169 instead of an Ala. For this we considered Asn because it is nearly isosteric to Leu and therefore unlikely to cause any significant stereochemical perturbations. An Asp residue at this site was also considered to clearly evaluate the impact of Asn residue.

As a prelude to experimental validation of Leu169 mutations, we carried out MD simulations of SrtL169N−LPAT and

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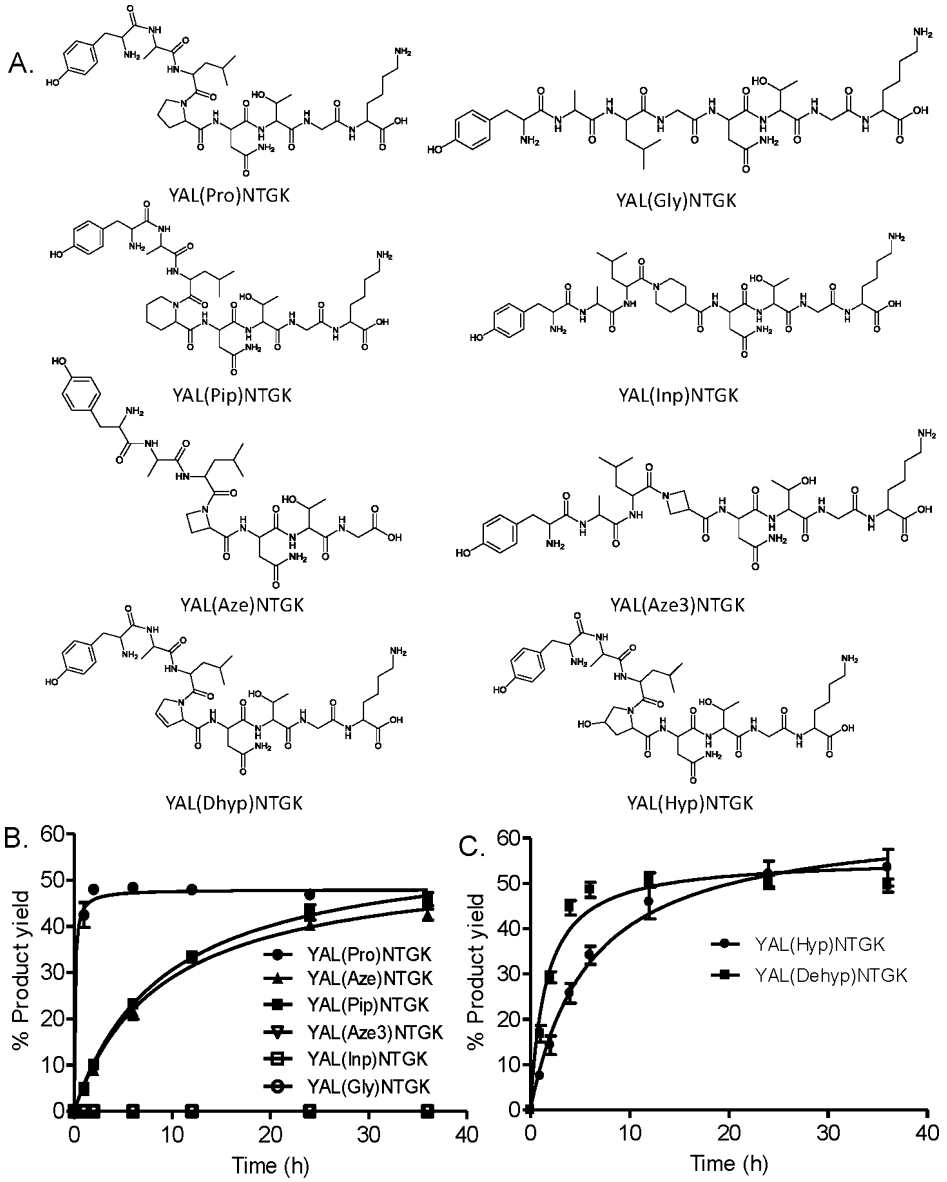

Figure 4. SrtA-catalyzed transpeptidation reaction of LPXTG peptide substrates containing Pro mimics or related isomers. (A) Structure of YALXNTGK synthetic peptide substrates where X represents Pro (proline), Gly (glycine), Pip (piperidine-2-carboxylic acid), Inp (piperidine-4-carboxylic acid), Aze (azetidine-2-carboxylic acid) and Aze3 (azetidine-3-carboxylic acid), Hyp (4-hydroxyproline) and Dhyp (3,4-dehydroproline), respectively. (B) Time-course of SrtA-catalyzed transpeptidation reaction with the above Pro-, Gly-, Pip-, Inp-, Aze-, and Aze3-containing substrates. (C) Time-course of SrtA-catalyzed transpeptidation reaction with Hyp- and Dehyp-containing substrates. The reaction of the peptide substrates was carried out with GGGKY under identical conditions as described in Figure 1. Each data point represents the mean ± SD of three independent experiments. Note that Gly, Inp, and Aze3 peptides were nonproductive substrates.

SrtL169D−LPAT complexes to evaluate the impact of mutations on correlated motions between Leu169 and substrate Leu residue. Interestingly, the DCCM pattern in the case of Asn mutant showed a strong positive correlation with the substrate, and the conformational dynamics of mutated residue Asn169 was comparable with that of Leu169 of the wild type SrtA in complex with the substrate (Figure S5A, panel a). In contrast, the DCCM map of Asp169 mutant showed very little or no correlations, and the Ramachandran plots revealed enhanced dynamics of Asp169 (Figure S5A, panel b, and S5B). The nativelike dynamic behavior of Asn mutant raised the possibility of considerable enzymatic activity in Asn169 mutant.

We created Asn and Asp mutants for Leu169 as described in methods. The previously reported Ala mutant[38] was also created to serve as a control in our studies. SrtA mutants displayed a single protein band on SDS-PAGE (Figure S6A, Supporting Information). The mass of the mutants was consistent with the Asn, Asp, or Ala substitution. CD spectra of the mutants (Figure S6B) were very similar to the native SrtA, indicating that gross secondary structure of the wild type protein was maintained in the mutants.

Next we assessed the enzymatic activity of the mutants in a transpeptidation assay using YALPNTGK and GGGKY as substrates (Figure 3C). SrtA containing Leu169 to Asp mutation was relatively very sluggish and yielded only about 2% product after 48 h of reaction, indicating a deleterious effect of charge and thereby emphasizing that the presence of the hydrophobic side chain is important at this site. Under identical conditions, the Ala mutant produced about 10% transpeptidation product. In contrast, the Asn mutant displayed a

dx.doi.org/10.1021/bi4016023 | *Biochemistry* 2014, 53, 2515−2524

considerable gain of function and yielded more than 30% product as compared to 45−48% obtained with the wild type SrtA.

"Kinked" Conformation of the Substrate Is Obligatory for Catalytic Turnover. We examined the role of peptide conformation in sorting of a productive substrate by incorporating nonproteinogenic mimics of the Pro residue in the LPXTG motif. For this we considered isomeric pairs of Pro homologues endowed with contrasting conformational preference as a tool to experimentally verify the role of kinked conformation in productive substrate recognition. We surmised that homologues of Pro such as azetidine-2-carboxylic acid (Aze) or piperidine-2-carboxylic acid (pipecolic acid, Pip) should be tolerated by SrtA because these moieties can induce a kink conformation in much the same way as a Pro residue. In contrast, related isomers such as azetidine-3-carboxylic acid (Aze3) and piperidine-4-carboxylic acid (Inp) or a glycine (Gly) residue that supports an extended conformation should not turn over. Accordingly, the Pro residue in a YALPNTGK substrate was replaced with Gly, Aze, Aze3, Pip, and Inp, and the respective substrates (Figure 4A) were tested by a standard transpeptidation assay using GGGKY as an amine nucleophile (Figure S7). Interestingly, SrtA-catalyzed transpeptidation reaction with peptides containing Aze and Pip produced similar equilibrium yield as that of the Pro-containing native substrate (about 45%) but proceeded with a relatively slower rate (Figure 4B). On the other hand, no product could be detected with Aze3-, Inp-, or Gly-containing peptides even after 36 h of reaction, indicating that these were not recognized by SrtA (Figure 4B). These results suggested that kinked conformation is highly preferred over the extended conformation.

To further corroborate the impact of conformation and tolerance of Pro mimics, we integrated 4-hydroxyproline (4Hyp) and 3,4-dehydroproline (Dhyp) in the YALPNTGK peptide substrate (Figure 4A). SrtA effected the turnover of both these substrates (Figure S8, Supporting Information). The time course revealed that the transpeptidation reaction reached equilibrium in about 12 h and produced an yield of 45% or more, which was similar to that obtained with native (Pro) substrate (Figure 4C). The recognition of the above two substrates is consistent with their ability to form a kink structure. Collectively, the results unequivocally establish the profound impact of kink conformation in the productive recognition of LPXTG peptide substrates by SrtA.

Fluoro Derivatives of Leu and Pro Residues in the LPXTG Substrate Are Well Tolerated. A hydrophobic effect appears to dominate the interactions of the LPXTG peptide substrate and Leu169 or vicinal residues of the $\beta6-\beta7$ loop.[37,38,48] The hydrophobic interaction is presumably further fortified by Leu181 and Ile182 of the critical TLITC catalytic sequence motif spanning residues 180−184 of the $\beta7$ strand.[37,48] We surmised that LPXTG substrates containing hydrophobic surrogates of Leu or Pro residues but endowed with similar conformational attributes should be tolerated by SrtA. We considered 5,5,5-trifluoroleucine (tfL) and *cis*-4-fluoroproline (fP) for exploring this aspect of substrate recognition by SrtA. The choice was based on the fact that both tfL and fP have been incorporated in proteins and generally seen not to exert untoward influence on protein structure and activity.[49] Importantly, fP can form a "kink" structure in much the same way as a natural Pro residue.

We incorporated tfL for Leu residue in YALPNTGK substrate using Fmoc-derivatized diastereoisomeric mixture of tfL (Figure 5A). While Leu contains one chiral center at Cα, placement of a trifluoro group at the Cγ position creates an additional chiral center and results in the formation of two diastereoisomers (2S,4S and 2S,4R) in tfL. Accordingly formation of two isomeric forms of the YA(tfL)PNTGK peptide is expected. The synthetic peptide indeed eluted as two distinct peaks of the same molecular mass (916.34 and 916.24 Da), indicating that the origin of the peaks was related to the diastereoisomeric tfL (Figure S9A, Supporting Information).

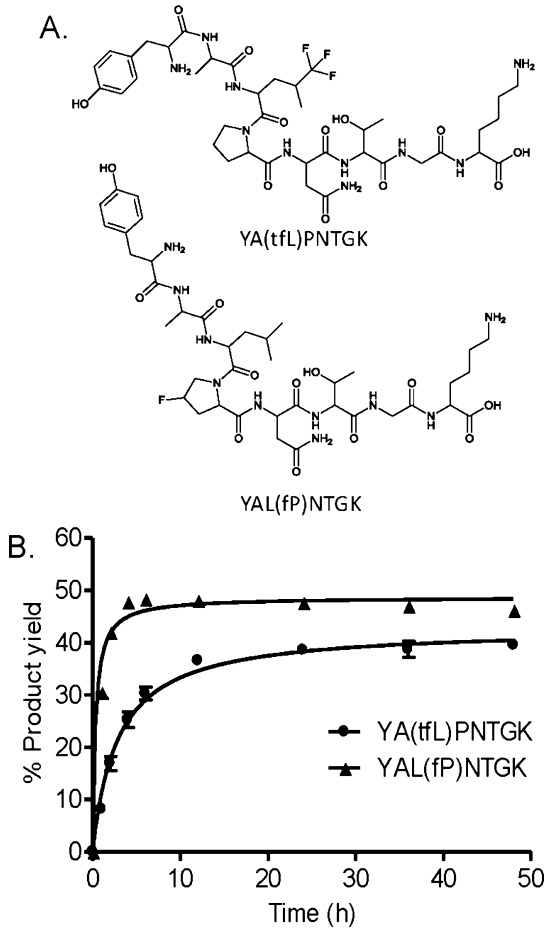

Figure 5. Tolerance of noncanonical fluoro analogues of Leu and Pro residues in the LPXTG motif by SrtA. The transpeptidation reaction of YA(tfL)PNTGK (tfL= 5,5,5-DL-trifluoroleucine) or YAL(fP)NTGK (fP = *cis*-4-fluoroproline) was carried out with GGGKY as described in Figure 1. (A) Structure of the peptide substrates. (B) Time course of the transpeptidation reaction. Each data point represents the mean ± SD of three independent experiments.

Next we tested the propensity of the above tfL-containing peptide to undergo SrtA-catalyzed transpeptidation reaction in the presence of GGGKY. The RP-HPLC analysis of SrtA-mediated transpeptidation reaction yielded two product peaks, indicating that the products were related to the starting tfL isomers (Figure S9A, Supporting Informatiojn). Consistent with this, the two product peaks yielded the same mass (1193.41 and 1193.39 Da) that fit to the mass of a transpeptidation product. These results show that SrtA tolerated both 2S,4S and 2S,4R isomers of 5,5,5-trifluoroleucine in much the same way as Leu in the LPXTG peptide substrate. The overall equilibrium yield (cumulative) of the product was found to be about 35% (Figure 5B).

dx.doi.org/10.1021/bi4016023 | *Biochemistry* 2014, 53, 2515−2524

Likewise, a YALPNTGK peptide in which the Pro residue was replaced by *cis*-4-fluoroproline (fP) was also accepted by the enzyme (Figure S9B, Supporting Information). The SrtA-catalyzed transpeptidation reaction proceeded well with this peptide and produced an equilibrium yield of about 45% or more (Figure 5B). Collectively, the tolerance of noncanonical fluoro derivatives of Leu and Pro residues in the LPXTG peptides highlight the importance of the hydrophobic effect in substrate recognition.

DISCUSSION

The presence of intrinsic dynamics in SrtA and its role in substrate binding have been revealed by NMR[37] and simulations,[41,50] but the mechanism of recognition remains to be fully elucidated. Here we have explored the role of individual LPXTG residues in the binding process and delineated the chemical/conformational attributes of a productive substrate.

In our endeavor to understand the selection process of a catalytically competent substrate, we first examined the chemical signatures of a minimalist SrtA recognition motif. We considered this an important issue in view of the fact that the X-ray crystallographic studies used the native LPETG pentapeptide in the SrtA−substrate complex.[36] NMR studies, in contrast, used a CbZ-protected LPAT peptide in which the threonyl carboxylate was substituted with a $-CH_2SH$ group and linked to the active site Cys184 through a disulfide producing a thioacyl rather than a natural thioester acyl-enzyme intermediate.[37] Interestingly, the native LPXTG pentapeptide sequences (X = A, E, or N) turned out to be recalcitrant substrates but could be converted into a productive substrate by capping their ends with acetyl and amide groups, respectively. These results suggest that the LPETG peptide seen in the crystal structure of the SrtA−substrate complex is not bound to the enzyme in a catalytically competent conformation. Furthermore, facile transpeptidation of an acetylated and amidated LPNT sequence establishes this tetrapeptide as the minimalist recognition motif of SrtA. Importantly, this tetrapeptide motif that produces a higher yield of the product by disfavoring the reverse reaction can be very useful in N-terminal labeling of proteins.

Comparative MD simulations of SrtA in Apo form and bound to the productive substrate (Ac-LPNT-NH-CH_3) show that the conformational space spanned by ApoSrt covers both the X-ray and NMR conformations. The bound substrate however considerably restricts the conformational freedom of the $\beta6-\beta7$ loop, which by-and-large resemble the conformation of the loop as seen by NMR studies. These results are consistent with the recent simulation studies by Kapell et al.[41] but with an important difference in that we do not observe any major conformational variability of the bound substrate. The reason for this difference may possibly be related to the fact that Kapell et al. used a nonproductive LPATG pentapeptide sequence in their studies as against a productive peptide substrate in the present work wherein the N- and C-termini were modified by acetyl- and *N*-methylamide groups, respectively. It is quite conceivable that charge neutralization at the peptide termini of the catalytically competent substrate provided crucial additional interactions facilitating anchoring of the bound peptide more efficiently to the enzyme active site.

MD simulations of SrtA complexed to decoy substrates (Leu or Pro substituted to Ala; APAT and LAAT respectively) provide important cues to the mechanism of conformational dynamics and substrate recognition in SrtA. The loss of dynamic cross-correlation of the APAT substrate with the $\beta6-\beta7$ loop as reflected in the enhanced dynamics of Leu169 confer a pivotal role for Leu of the substrate in controlling the protein dynamics. This result is consistent with the earlier studies of Bentley et al.,[38] who reported about a 93-fold decrease in k_{cat}/K_m for L169A mutation. The drastic loss of native enzyme activity in Ala169 or Asp169 mutants and retention of the bulk of the enzyme activity in isosteric Asn169 mutation reported in here unequivocally establishes the involvement of the Leu169 residue. Taken together, the functional properties of Leu169 mutants and the tolerance of 5,5,5-trifluoroleucine for the Leu residue in the substrate provides experimental evidence for the preponderance of hydrophobic interactions in SrtA substrate recognition.

The preservation of native (LPAT) like correlated motion between the LAAT decoy substrate and $\beta6-\beta7$ loop suggests that the Pro residue does not dictate the protein dynamics. Since substitution of the Pro residue in LPXTG motif is not tolerated by the enzyme,[15] it is legitimate to assume that the Pro residue may have a major role in generating a productive binding conformation. The transpeptidation experiments with noncanonical Pro analogues are instructive in this regard. Among all tested analogues, those with kink forming propensity were only tolerated. Besides, contraction (azetidine), expansion (pipecolic acid), derivatization (fluoro, hydroxy), or rigidification (double bond) of the Pro ring was not detrimental to the transpeptidation reaction, thereby further supporting the intimate association between kink conformation and catalysis.

In summary, we have delineated the chemical and conformational signatures of a catalytically competent substrate of SrtA. Our results in conjunction with previous work[41,50] highlight the complexity of the substrate recognition processes in SrtA catalysis. As noted by Moritsugu et al.,[50] binding of both the substrate peptide as well as allosteric effects from the bound calcium atom to bring about the conformational change of the $\beta6-\beta7$ loop may be required for productive substrate binding and catalysis. These findings are complemented by our experimental results using substrate analogues containing nonproteinogenic amino acids, which clearly demonstrate the requirement of a kinked conformation of the peptide. Dissection of the substrate−enzyme interactions by the use of decoy substrates shows that the Leu and Pro side chains make distinctly different contributions toward the specificity of the enzyme for its substrate, with the Leu side chain directing the conformational change of the enzyme in an induced-fit fashion and the Pro side chain stabilizing the bound conformation of the substrate. These results can be reconciled with an induced fit and/or conformational selection model of substrate recognition in SrtA.[51−53] In this connection, the tolerance of fluoro analogues of Leu/Pro residues in the substrate peptide is an important result that can be exploited in the further interrogation of sortase−substrate interactions by ^{19}F-NMR spectroscopy and facilitate the rational design of inhibitors of this important drug target.

ASSOCIATED CONTENT

Supporting Information

Sequence of mutagenic primers; table for mass spectrometric characterization of substrate peptides and their respective transpeptidation products and HPLC traces for transpeptidation reaction with various substrate peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by core grants to the National Institute of Immunology from Department of Biotechnology (DBT), Government of India, and an extramural grant (BT/PR5037/MED/15/77/2012) to R.P.R. from DBT. T.B. and V.S.P. are recipients of a research fellowship from the Council of Scientific & Industrial Research, India.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

SrtA, sortase A; ApoSrt, apo-sortase A; Aze, azetidine-2-carboxylic acid; Aze3, azetidine-3-carboxylic acid; Pip, piperidine-2-carboxylic acid; Inp, piperidine-4-carboxylic acid; Hyp, 4-hydroxyproline; Dhyp, 3,4-dehydroproline; fP, *cis*-4-fluoroproline; tfL, 5,5,5-trifluoro-DL-leucine; MD, molecular dynamics; DCCM, dynamic cross-correlation map; RMSD, root mean square deviation; CD, circular dichroism

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