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Anchoring of Surface Proteins to the Cell Wall of *Staphylococcus aureus*

CYSTEINE 184 AND HISTIDINE 120 OF SORTASE FORM A THIOLATE-IMIDAZOLIUM ION PAIR FOR CATALYSIS*

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Surface proteins of *Staphylococcus aureus* are anchored to the cell wall peptidoglycan by a mechanism requiring a C-terminal sorting signal with a LPXTG motif. Sortase cleaves polypeptides between the threonine and the glycine of the LPXTG motif. The carboxyl group of threonine is subsequently amide-linked to the amino group of peptidoglycan cross-bridges. The three-dimensional structure of sortase revealed the close proximity of the catalytic site residue cysteine 184 with histidine 120; however, no structural evidence for a thiolate-imidazolium ion pair could be detected. We report that alanine substitution of either cysteine 184 or histidine 120 abolishes *in vivo* and *in vitro* sorting reactions. Further, alanine substitution of tryptophan 194, a residue that is in close proximity of histidine 120, reduces the transpeptidase activity of sortase. These results suggest a model whereby sortase forms a thiolate-imidazolium ion pair for the catalysis of its transpeptidation reaction and that the position of tryptophan 194 assists in the formation of this ion pair.

Surface proteins of Gram-positive microbes play important roles during human infection, promoting bacterial attachment to the host tissues or preventing phagocytosis of the invading pathogen (1). Many surface proteins of Gram-positive bacteria are anchored to the cell wall by a mechanism requiring a C-terminal sorting signal with a conserved LPXTG motif, a hydrophobic domain, and a positively charged tail (2). During cell wall anchoring, surface proteins are cleaved between the threonine and the glycine of the LPXTG motif (3) and subsequently amide-linked to the pentaglycine cross-bridges of the cell wall peptidoglycan (4–7). Lipid II, the biosynthetic precursor of cell wall synthesis, is presumed to act as the peptidoglycan substrate of sortase (7).[1] The lipid-linked surface protein intermediate is then incorporated into the peptidoglycan via the transpeptidation and transglycosylation reactions of bacterial cell wall synthesis (7). *Staphylococcus aureus* sortase (SrtA), a 206-amino acid transpeptidase with an N-terminal transmembrane anchor/signal peptide (8), catalyzes the anchoring of surface proteins to peptidoglycan (9, 10). *S. aureus srtA* mutants fail to display about twenty different surface proteins, and are defective in the pathogenesis of animal infections (11).

Purified recombinant sortase lacking its N-terminal signal peptide/membrane anchor catalyzes the transpeptidation reaction of surface protein anchoring *in vitro*, using LPXTG peptides and NH_2-Gly_3 as a peptidoglycan substrate (9). Both the *in vivo* and *in vitro* reactions of sortase can be inhibited with the thiolate reagent methylmethane thiosulfonate (for example MTSET)[2] or parahydroxy-mercurybenzoic acid (12), but not with sulfhydryl reagents such as iodoacetate or iodoacetamide (7, 9). Surface proteins bearing LPXTG motifs as well as sortase genes have been found in many Gram-positive bacteria, and the presence of a single conserved cysteine within a LXTC signature sequence is a distinguishing feature of these enzymes (13, 14). Incubation of staphylococci with hydroxylamine, a strong nucleophile that releases thioester-linked acyl enzyme intermediates, causes the release of surface proteins into the extra-cellular medium (9). Released surface proteins harbor C-terminal threonine hydroxamate, consistent with the notion that hydroxylamine performs a nucleophilic attack when sortase is charged with thioester-linked surface protein (9). Together these results suggest that the single cysteine residue of sortase performs the nucleophilic attack at the peptide bond between the threonine and the glycine of the LPXTG motif.

Truncation of N-terminal sortase residues, up to position 59, does not interfere with the *in vitro* enzymatic cleavage at the LPXTG motif or with the *in vitro* transpeptidation reaction (15). The truncated sortase enzyme was used for structural studies and the three-dimensional fold determined using NMR spectroscopy. Sortase assumes a β-barrel structure (15) (Fig. 1). Strands β7 and β8 of sortase form the floor of a hydrophobic depression that encompasses the active site, with walls constructed by amino acids located in loops connecting strands β3-β4, β2-β3, β6-β7, and β7-β8 (Fig. 1). Leucine (Leu) 97,

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[1] A. Perry, H. Ton-That, S. K. Mazmanian, and O. Schneewind, submitted for publication.

[2] The abbreviations used are: MTSET, [2-(trimethylammonium) ethyl] methane-thiosulfonate; RP-HPLC, reversed phase-high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; Abz, 2-aminobenzoyl; Dnp, diaminopropionic acid (2,4-dinitrophenyl); Spa, staphylococcal protein A; Seb, staphylococcal enterotoxin B.

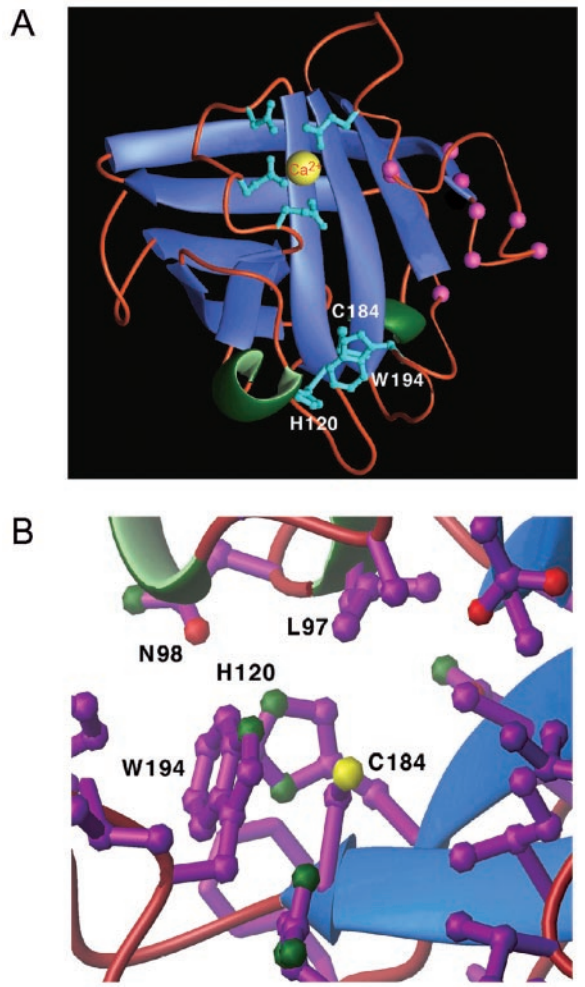

FIG. 1. **The three-dimensional structure and active site of sortase.** *A*, ribbon drawing of the structure of $SrtA_{\Delta N59}$, obtained without peptide substrate (LP*X*TG motif). β strands and α helices are colored *blue* and *green*, respectively. The active site sulfhydryl, Cys^{184}, is positioned at the end of β7, which also includes LITC184, the signature sequence of sortase enzymes. The position of histidine (*H*) 120 and tryptophan (*W*) 194 is indicated. *B*, expanded view of the active site of sortase. His^{120} and Cis^{184} in sortase are positioned in close proximity but do not form a thiolate-imidazolium ion pair in the absence of LP*X*TG substrate. The drawing was adapted from data generated by Ilangovan *et al.* (15, 20).

histidine (His) 120, and cysteine (Cys) 184 are conserved among sortase enzymes and assume close proximity within the active site (13). Catalysis is stimulated by calcium binding near the active site, altering the conformational dynamics of a loop that may also be involved in recognizing the polypeptide substrate (15).

Although structurally unrelated, sortase-catalyzed cleavage at LP*X*TG peptides appears to be mechanistically related to the proteolytic reactions of the papain/cathepsin protein family. The active sites of these proteases contain three conserved residues: Cys^{25}-His^{159}-Asn^{175} (16–18). Prior to substrate binding, Cys^{25} is held in an active configuration through a thiolate-imidazolium ion interaction with His^{159} (19). Analogously, Cys^{184} of sortase may be activated by the imidazole ring of His^{120} to facilitate thiolate formation and subsequent nucleophilic attack on the carbonyl carbon at the scissile peptide bond. In the structure of sortase solved in the absence of substrate, the side chains of Cys^{184} and His^{120} do not interact, with Cys^{184} projecting away from His^{120} into the hydrophobic pocket (Fig. 1) (15, 20). However, substrate binding could initiate a subtle conformational rearrangement in these side chains, enabling sulfhydryl proton extraction and subsequent nucleophilic attack.

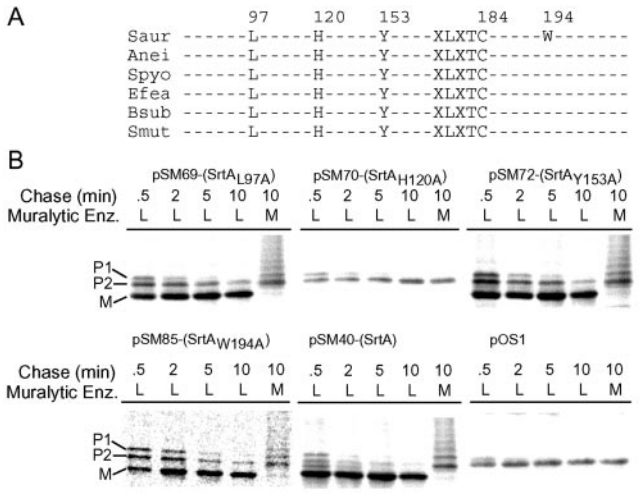

FIG. 2. **His^{120} is required for *in vivo* sortase activity.** *A*, alignment of the conserved residues of *S. aureus* SrtA (*Saur*) with sortase from *A. naeslundii* (*Anei*), *S. pyogenes* (*Spyo*), *E. faecalis* (*Efea*), *B. subtilis* (*Bsub*), and *S. mutants* (*Smut*). *B*, cell wall sorting of Seb-$Spa_{490-524}$ was followed by pulse-labeling staphylococcal cultures. At the indicated time intervals, culture aliquots were precipitated with trichloroacetic acid, and the cell wall was digested with lysostaphin or mutanolysin. Seb-$Spa_{490-524}$ was immune-precipitated with α-Seb, separated on 15% SDS-PAGE, and subjected to phosphorimaging analysis. Surface protein processing was analyzed in *S. aureus* SKM1 ($\Delta srtA$) containing plasmids that encode variant sortase enzymes: pSM40, wild-type SrtA; pSM69, $SrtA_{L97A}$; pSM70, $SrtA_{H120A}$; pSM72, $SrtA_{Y153A}$; and pSM85, $SrtA_{W194A}$. pOS1 is the empty vector lacking a sortase gene.

It is reported here that alanine substitution of either cysteine 184 or histidine 120 abolishes the *in vivo* and *in vitro* reactions of surface protein anchoring. Further, alanine substitution of tryptophan 194, a residue that is in close proximity of histidine 120, reduces the transpeptidase activity of sortase, whereas alanine substitution at Leu^{97} causes little or no effect. These results suggest a model whereby sortase forms a thiolate-imidazolium ion pair for the catalysis of its transpeptidation reaction and that the position of tryptophan 194 assists in the formation of this ion pair.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The primers dN-HQAK-B (AAAGGATCCCAAGCTAAACCTCAAATTCC) and orf6C-B (8) were used to PCR-amplify the *srtA* sequence from the chromosome of *S. aureus* OS2 (21). The amplified DNA fragment was digested with *Bam*HI and inserted into pQE30 (Qiagen)-cut *Bam*HI to generate pHTT27. Plasmid was transformed into *Escherichia coli* XL-1 Blue and selected on Luria agar with ampicillin (100 μg/ml). *S. aureus* strains RN4220 (22) and SKM1($\Delta srtA$) have been described previously (11). Plasmid pGL4 was created by an *Eco*RI/*Bam*HI digest of plasmid pSeb-$Spa_{490-524}$ (23), and cloning of the *seb-spa*$_{490-524}$ reporter gene into *Eco*RI/*Bam*HI digested pT181 (8). *S. aureus* SKM1 (pGL4) was transformed with pSrtA (11) or with the sortase mutant derivatives described in this report.

Site-directed Mutagenesis—Plasmid mutations were generated by PCR amplification using pSrtA or pHTT27 as a template. Primers SRTA-C2A and GSA1–12 introduced a substitution of cysteine 184 with alanine (9). For the substitution of leucine 97, histidine 120, tyrosine 153, and tryptophan 194 the following sets of primers were used: SRTA-L2A-5 (CCAGCAACACCTGAACAAGCAAATAGAGGTGTAAGCTTT) and SRTA-L2A-3 (AAAGCTTACACCTCTATTTGCTTGTTCAGGTGTTGCTGG), SRTA-H2A-5 (AATATTTCAATTGCAGGAGCCACTTTCATTGACCGTCCG) and SRTA-H2A-3 (CGGACGGTCAATGAAAGTGGCTCCTGCAATTGAAATATT), SRTA-Y2A-5 (GGTAATGAAACACGTAAGGCTAAAATGACAAGTATAAGA) and SRTA-Y2A-3 (TCTTATACTTTGTCATTTTAGCCTTACGTGTTTCATTACC), and SRTA-W2A-5 (AATGAAAAGACAGGCGTTGCGGAAAAACGTAAAATCTTT) and SRTA-W2A-3 (AAAGATTTTACGTTTTTCCGCAACGCCTGTCTTTTCATT), respectively. Following PCR amplification, *Dpn*I digestion was

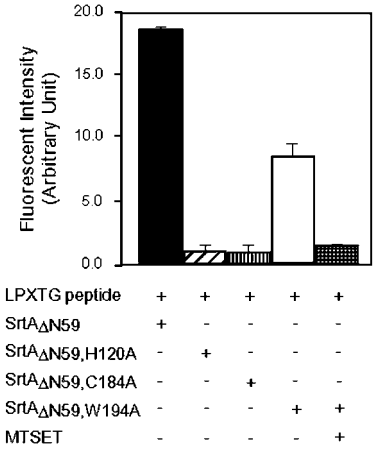

FIG. 3. **His^{120} is required for *in vitro* sortase cleavage activity.** Purified wild-type and mutant sortase enzymes (10 μM) were incubated with Abz-LPETG-Dnp peptide (5 μM) in buffer R at 37 °C. Peptide cleavage was measured as an increase in fluorescent intensity, excited at 320 nm with an emission wavelength at 420 nm.

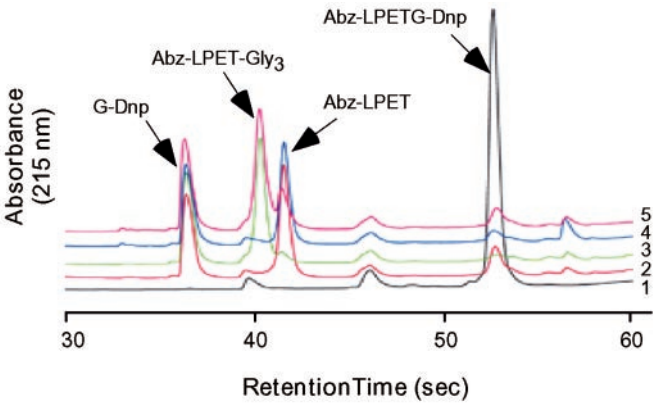

FIG. 4. **Trp^{194} is not absolutely required for *in vitro* sortase transpeptidation activity.** Abz-LPETG-Dnp peptide was incubated with wild-type sortase or W194A mutant in the presence or absence of NH_2-Gly_3 at 37 °C. The reactions were stopped by filtration, reaction products were separated by RP-HPLC on C-18 column, and products were analyzed by monitoring the absorbance at 215 nm and mass spectrometry of eluted peaks. Reaction 1: Abz-LPETG-Dnp, no enzyme; reaction 2, Abz-LPETG-Dnp, SrtA$_{\Delta N59}$; reaction 3, Abz-LPETG-Dnp, NH_2-Gly_3, SrtA$_{\Delta N59}$; reaction 4, Abz-LPETG-Dnp, SrtA$_{\Delta N59,W194A}$; reaction 5, Abz-LPETG-Dnp, NH_2-Gly_3, SrtA$_{\Delta N59,W194A}$.

used to select against parental DNA. Plasmids (pSrtA, pHTT27, or mutant derivatives) were transformed into *E. coli* XL-1 Blue, and transformants selected on LB agar containing ampicillin. Mutant plasmids were purified, and the presence or absence of nucleotide changes was determined by DNA sequencing (Table I).

Purification of Recombinant Sortase: E. coli—XL-1 Blue cells (10^{12} colony-forming units) harboring plasmids that encode either wild-type or mutant sortase were suspended in 30 ml of C buffer (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 7.2) and were lysed in a French pressure cell at 14,000 p.s.i. The extract was centrifuged at 29,000 × *g* for 30 min, and the supernatant applied to 1 ml of nickel-nitrilotriacetic acid resin, pre-equilibrated with C buffer. The column was washed with 40 ml of C buffer, and SrtA$_{\Delta N}$ protein was eluted in 4 ml of C buffer with 0.5 M imidazole.

Pulse-Chase Experiments—Staphylococcal cultures were grown overnight in tryptic soy broth media supplemented with chloramphenicol (10 μg/ml) and tetracycline (2.5 μg/ml) at 37 °C. Overnight cultures were diluted in the same media and grown to mid-logarithmic phase. Cells were harvested by centrifugation, washed, and resuspended in minimal medium lacking methionine and cysteine. Cells were labeled with 10 μCi of ^{35}S-labeled Promix (Amersham Biosciences, Inc.) for 2 min. After 2 min of labeling, 50 μl of chase solution were added (100 mg/ml casamino acids, 10 mg/ml each methionine and cysteine), and at timed intervals (0, 1, 5, and 20 min after the chase) 250 μl of cells were removed and transferred into an Eppendorf tube, and all further processing of surface proteins was quenched by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. Total cells and precipitated molecules were collected by centrifugation at 14,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 14,000 × *g* for 10 min, and dried. Samples were suspended in one ml of 0.5 M Tris-HCl, pH 6.3, and peptidoglycan digested by adding either 150 μg of mutanolysin or 100 μg of lysostaphin and incubation for four h at 37 °C with intermittent mixing of samples. Digests were precipitated by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. The precipitate was collected by centrifugation at 14,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 14,000 × *g* for 10 min and dried. Samples were solubilized by boiling in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0. Forty-μl samples were transferred to 1 ml of RIPA buffer containing 1 μl of rabbit α-Seb antibodies for immuno-precipitation of Seb-Spa$_{490-524}$. Antigen-antibody complexes were captured on 50 μl of pre-swollen protein A CL-4B-Sepharose, washed five times with RIPA buffer, and solubilized by boiling in sample buffer. Immuno-precipitates were separated on 14% SDS-PAGE, dried, and analyzed on a phosphorimaging device.

Kinetic Analysis of Recombinant Sortase Enzymes—Abz-LPETG-Dnp was dissolved in dimethyl sulfoxide and added to the kinetic reaction at a final concentration between 1–100 μM (Me_2SO concentrations were kept constant for this experiment). Peptide cleavage was monitored at emission wavelength of 420 nm with excitation wavelength of 320 nm. Kinetic constants K_m, V_{max}, and k_{cat} were calculated from the curve fit for the Michaelis-Menten equation using the Lineweaver-Burk plot as described previously (10).

HPLC Purification of Cleaved Products—The procedure was following as previously described (10). Briefly, a reaction mixture consisting of 10 μM of fluorescent peptides, 15 μM of recombinant enzymes in 520 μl of Buffer R (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) was incubated either in the presence or absence of 5 μM of NH_2-Gly_3 at 37 °C for 16 h. The reaction was stopped by centrifugation on Centricon-10 (Millipore) at 5,000 × *g* to remove the enzyme. The eluate was subjected to RP-HPLC purification on a C-18 column (2 × 250-mm, C18 Hypersil, Keystone Scientific). The elution of cleaved products was monitored at 215 nm, and 1-min fractions were collected. Vacuum dried fractions were stored at 4 °C for ESI-MS analysis.

RESULTS

Conserved Residues in the Active Site of Sortase—Homology searches in data base revealed sortase homologs in many different Gram-positive bacteria. Fig. 2*A* reports an alignment of all those sortase residues that are absolutely conserved within the active site of *S. aureus* SrtA. *Actinomyces naeslundii*, *Bacillus* subtilis, *Enterococcus faecalis, Streptococcus pyogenes,* and *Streptococcus mutants* sortase display absolute conservation of leucine 97, histidine 120, tyrosine 153, leucine 181, threonine 183, and cysteine 184 (8). Fig. 1 shows that tryptophan 194 of SrtA is in close proximity with His^{120} and Cys^{184}, suggesting that it may be involved in electron interaction within the active site as has been reported for the cysteine protease SpeB (30). It should be noted that tryptophan 194 is not a conserved residue of sortase.

Effect of Alanine Substitutions of Sortase on in Vivo Surface Protein Anchoring in S. aureus—To examine the role of the conserved residues of sortase in surface protein anchoring to the cell wall of *S. aureus*, site-directed mutagenesis was performed to substitute each codon, *i.e.* leucine 97, histidine 120, tyrosine 153, cysteine 184, and tryptophan 194, with an alanine codon. Plasmids encoding wild-type or mutant sortase genes were purified, DNA sequenced for confirmation, and then and transformed into *S. aureus* SKM1 (Δ*srtA*). Surface protein anchoring to the cell wall was assessed by subjecting *S. aureus* cultures expressing various sortase enzymes to pulse-labeling experiments with [^{35}S]methionine and following the fate of Seb-Spa$_{490-524}$, an engineered surface protein. Wild-type staphylococci synthesize surface protein precursor bearing an N-terminal signal peptide and a C-terminal sorting signal (P1 precursor in Fig. 2*B*). Following export across the cytoplasmic membrane and signal peptide cleavage, sortase cleaves the P2 precursor between the threonine and the glycine of the LPXTG

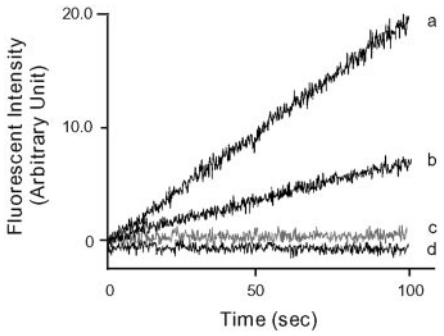

FIG. 5. **Kinetic Analysis of sortase-catalyzed cleavage of LPXTG peptides.** Wild-type sortase $SrtA_{\Delta N59}$ (*a*) cleaved Abz-LPETG-Dnp peptide at a faster rate than $SrtA_{\Delta N59,W194A}$ (*b*). Sortase mutants $SrtA_{\Delta N59,H120A}$ (*c*) or $SrtA_{\Delta N59,C184A}$ (*d*) did not display enzymatic activity. Reaction mixture contained 5 μM of Abz-LPETG-Dnp and 10 μM of enzymes in 500 μl of buffer R at 37 °C.

motif to generate mature, cell wall-anchored surface protein (*M*) that can be released from the peptidoglycan with lysostaphin digestion (*L*) (7). Deletion of the *srtA* gene in *S. aureus* strain SKM1 (*pOS1*, vector control) prevents surface protein cleavage at the LP*X*TG motif and causes accumulation of the P2 precursor species. The anchoring defect of strain SKM1 can be complemented *in trans* by transforming mutant staphylococci with pSM40, a plasmid that encodes the wild-type *srtA* gene.

Transformation of *S. aureus* SKM1 with plasmids pSM34 ($SrtA_{C184A}$) or pSM70 ($SrtA_{H120A}$) failed to complement the anchoring defect of mutant staphylococci and led to the accumulation of surface protein P2 precursor species that were not cleaved at the LP*X*TG motif (10) (Fig. 2*B*). Thus, histidine 120 and cysteine 184 are absolutely required for the *in vivo* transpeptidase activity of sortase that anchors surface proteins to the cell wall of *S. aureus*. In contrast, transformation of *S. aureus* SKM1 with plasmids pSM69 ($SrtA_{L97A}$), pSM72 ($SrtA_{Y153A}$), or pSM85 ($SrtA_{W194A}$) restored the anchoring defect of mutant staphylococci as Seb-$Spa_{490-524}$ was cleaved at the LP*X*TG motif. It should be noted, however, that the processing of Seb-$Spa_{490-524}$ by $SrtA_{L97A}$ and $SrtA_{Y153A}$ was slowed as compared with wild-type sortase (pSM40). $SrtA_{W194A}$ cleaved surface proteins even more slowly than $SrtA_{L97A}$ and $SrtA_{Y153A}$ (Fig. 2*B*).

The incorporation of surface proteins into the cell wall of *S. aureus* can be measured by digesting the peptidoglycan with two different muralytic enzymes. Lysostaphin, a glycyl-glycine endopeptidase (24), cleaves the pentaglycine cross-bridge and solubilizes surface proteins as a single uniform species (25). Muramidase cuts the β1–4 glycosidic bond between MurNac-GlcNac and disrupts the glycan strands of mature peptidoglycan without affecting its peptide backbone (26). Muramidase solubilizes surface protein as a large spectrum of fragments with linked peptidoglycan, each of which migrates more slowly on SDS-PAGE than the lysostaphin-released counterpart (23, 27). This assay was used to assess whether the sortase mutants linked surface protein to the cell wall of *S. aureus* (Fig. 2*B*). After 10 min of incubation, all pulse-labeled precursor was cleaved by $SrtA_{L97A}$, $SrtA_{Y153A}$, or $SrtA_{W194A}$. Muramidase digestion of the labeled cells released a spectrum of surface protein fragments, each of which migrated more slowly than the lysostaphin-released counterpart (Fig. 2*B*). In contrast, no cell wall anchoring was observed when the *srtA* mutant was transformed with pOS1 (vector control) or pSM70 ($SrtA_{H120A}$) (Fig. 2*B*).

Effect of Alanine Substitutions of Sortase on in Vitro Hydrolysis and Transpeptidation Reactions—Purified recombinant sortase $SrtA_{\Delta N59}$ was used to study the *in vitro* hydrolysis and transpeptidation reactions of surface protein anchoring. Fluorescence of the Abz fluorophore (*a*) within the peptide *a*-LPETG-*d* is quenched by the close proximity of the Dnp quencher (*d*). When the peptide is cleaved by sortase and the fluorophore is separated from Dnp, an increase in fluorescence is observed. The *a*-LPETG-*d* peptide is about a 10-fold better substrate for SrtA than the previously reported *d*-QALPETGEE-*e* and was therefore used for *in vitro* sorting reactions. Incubation of $SrtA_{\Delta N59}$ resulted in cleavage of *a*-LPETG-*d* even in the absence of the peptidoglycan substrate (Fig. 3). A similar result has been previously reported, and these data are consistent with a slow hydrolysis of peptide substrate by sortase. As expected from the *in vivo* studies, purified $SrtA_{\Delta N59,H120A}$ and $SrtA_{\Delta N59,C184A}$ failed to cleave *a*-LPETG-*d* in the absence of the peptidoglycan substrate (Fig. 3). It should be noted that the mutant sortases were folded and presumably assumed their native three-dimensional fold as suggested by protease protection and NMR spectroscopy experiments (data not shown). Purified $SrtA_{\Delta N59,W194A}$ was capable of cleaving *a*-LPETG-*d* in the absence of the peptidoglycan substrate as revealed by an increase in fluorescence when the substrate was incubated with the mutant sortase (Fig. 3). Nevertheless, $SrtA_{\Delta N59,W194A}$ displayed about a 2-fold reduction in the overall activity of sortase. Cleavage of the *a*-LPETG-*d* peptide still occurred in a manner requiring the active site cysteine and formation of a thiolate ion, because all hydrolysis activity of $SrtA_{\Delta N59,W194A}$ could be inhibited by addition of MTSET, a compound reactive with cysteine thiolate (28) (Fig. 3). Although we did attempt the purification of $SrtA_{\Delta N59,L97A}$ and $SrtA_{\Delta N59,Y153A}$, this could not be achieved as the mutant proteins aggregated in the *E. coli* cytoplasm. We presume that the mutant sortase enzymes are not folded into the correct three-dimensional structure.

To determine whether sortase mutants catalyze the transpeptidation reaction of surface protein anchoring *in vitro*, purified enzymes were incubated with *a*-LPETG-*d* and NH_2-Gly_3. Each reaction was quenched by centrifuging samples through a Centricon-10 membrane (Millipore), thereby separating enzymes from reaction substrates and products. Substrate and product samples were subjected to RP-HPLC and ESI-MS. Wild-type $SrtA_{\Delta N59}$ cut *a*-LPETG-*d* peptide and transferred NH_2-Gly_3 to the carboxyl group of threonine as evidenced by the appearance of the *a*-LPET-Gly_3 peak (Fig. 4, *traces 2* and *3*). Consistent with the results described above, $SrtA_{\Delta N59,C184A}$ and $SrtA_{\Delta N59,H120A}$ failed to catalyze the transpeptidation as no *a*-LPET-Gly_3 product peak could be detected (data not shown). $SrtA_{\Delta N59,W194A}$ catalyzed the transpeptidation reaction of surface protein anchoring (Fig. 4). Unlike wild-type $SrtA_{\Delta N}$, $SrtA_{\Delta N59,W194A}$ did accumulate some

TABLE I
Strains and plasmids used in this study

Strain	Plasmid	Sortase	Reference
S. aureus SKM1	pOS1	—	(11, 21)
S. aureus SKM1	pSM34	$SrtA_{C184A}$	(10)
S. aureus SKM1	pSM40	SrtA	This study
S. aureus SKM1	pSM69	$SrtA_{L97A}$	This study
S. aureus SKM1	pSM70	$SrtA_{H120A}$	This study
S. aureus SKM1	pSM72	$SrtA_{Y153A}$	This study
S. aureus SKM1	pSM85	$SrtA_{W194A}$	This study
E. coli XL1-Blue	pHTT16	$SrtA_{\Delta N,C184A}$	(9)
E. coli XL1-Blue	pHTT27	$SrtA_{\Delta N59}$	(15)
E. coli XL1-Blue	pHTT45	$SrtA_{\Delta N59,C184A}$	This study
E. coli XL1-Blue	pHTT46	$SrtA_{\Delta N59,H120A}$	This study
E. coli XL1-Blue	pHTT50	$SrtA_{\Delta N59,L97A}$	This study
E. coli XL1-Blue	pHTT51	$SrtA_{\Delta N59,Y153A}$	This study
E. coli XL1-Blue	pHTT53	$SrtA_{\Delta N59,W194A}$	This study

TABLE II
Kinetic analysis of SrtA$_{\Delta N59}$ and SrtA$_{\Delta N59,W194A}$

The substrate peptide Abz-LPETG-Dnp was incubated with SrtA$_{\Delta N59}$ or SrtA$_{\Delta N59,W194A}$. Substrate cleavage between the threonine and glycine was measured as an increase in fluorescence. The slope was collected in the linear phase of the kinetic curve within the first 100 seconds as shown in Fig. 5. Kinetic constants K_m, V_{max}, and K_{cat} were calculated from the curve fit for the Michaelis-Menten equation using the Lineweaver-Burk plot.

Enzyme	K_m	V_{max}	K_{cat}	K_{cat}/K_m
	μM	$\mu M\ s^{-1}$	s^{-1}	$\mu M^{-1}\ s^{-1}$
SrtA$_{\Delta N59}$	1.16×10^2	4.77×10^{-1}	5.69×10^{-1}	4.91×10^{-3}
SrtA$_{\Delta N59,W194A}$	2.06×10^2	3.97×10^{-1}	2.19×10^{-1}	1.06×10^{-3}

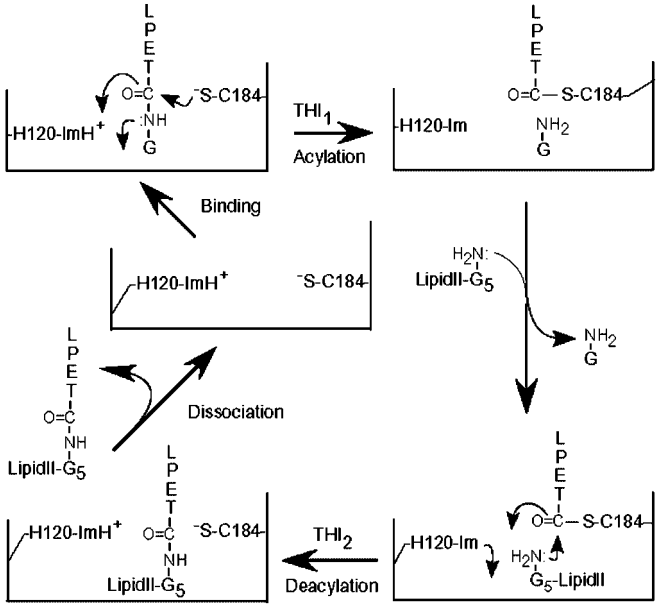

FIG. 6. **Mechanistic model for sortase-catalyzed anchoring of surface proteins.** The drawing depicts the active site of sortase with Cys^{184} and His^{120} positioned at its lateral walls, forming a thiolate-imidazolium ion pair (*ImH*$^+$). Substrate (*LPET-(CO-NH)-G*) binding to the active site (*Binding*) is followed by the nucleophilic attack of the thiolate ion (S^-), tetrahedral intermediate (*THI$_1$*) and acyl enzyme formation (*LPET-(CO-S)-C184*) (*Acylation*). The peptidoglycan substrate (lipid II-G$_5$-NH$_2$) attacks the acyl enzyme intermediate and, after formation of a second tetrahedral intermediate (*THI$_2$*), deacylates the active site (*Deacylation*), releasing cell wall-anchored surface protein product (*LPET-(CO-NH-)-G$_5$-lipid II*) (*Dissociation*).

hydrolysis product in addition to the transpeptidation product (Fig. 4, *traces 4* and *5*), suggesting that the mutant enzyme did not catalyze the transpeptidation reaction with the same fidelity as wild-type sortase (Fig. 4).

Kinetic Measurements of Sortase Enzymes—To measure the rate of cleavage by different sortase mutants, *a*-LPETG-*d* was incubated with purified proteins, and the increase in fluorescence was measured over time at the wavelength of 420 nm. The rate of cleavage by SrtA$_{\Delta N59,W194A}$ was reduced by about 2-fold as compared with the rate of cleavage by wild-type SrtA$_{\Delta N59}$ (Fig. 5). SrtA$_{\Delta N59,C184A}$ and SrtA$_{\Delta N59,H120A}$ did not cleave substrate peptide between the threonine and the glycine of the LPXTG motif (Fig. 5). Kinetic constants were calculated for the hydrolysis catalyzed by wild-type and mutant enzymes (Table II). The affinity of SrtA$_{\Delta N59,W194A}$ for *a*-LPETG-*d* substrate was decreased (K_m 2.06×10^2 increases as compared with that of the wild-type SrtA$_{\Delta N59}$, K_m 1.16×10^2). Overall, SrtA$_{\Delta N59}$ catalyzed the sorting reaction four times more efficiently than SrtA$_{\Delta N59,W194A}$ as determined by the K_{cat}/K_m ratios for both enzymes (Table II).

DISCUSSION

The active site of cysteine proteases in the cathepsin/papin family contains three conserved residues: Cys^{25}-His^{159}-Asn^{175} (16–18). Prior to substrate binding, Cys^{25} is held in an active configuration through a thiolate-imidazolium ion interaction with His^{159} (19). A hydrogen bond between the side chain oxygen of Asn^{175} and the Nϵ2 of His^{159} has been proposed to stabilize the active site. However, this interaction does not appear to be essential, as Asn^{175} can be substituted with glutamine or alanine without complete loss of enzymatic activity (29). In a similar manner, Cys^{184} of sortase may be activated by the imidazole ring of His^{120}, which most likely facilitates thiolate formation and subsequent nucleophilic attack on the carbonyl carbon at the scissile peptide bond. In the structure of sortase solved in the absence of substrate, the side chains of Cys^{184} and His^{120} do not interact, with Cys^{184} projecting away from His^{120} into the hydrophobic pocket (Fig. 1). It is conceivable that during catalysis, substrate binding initiates a subtle conformational rearrangement in these side chains (for example rotation of the χ1 and χ2 angles of Cys^{184} and His^{120}, respectively), enabling sulfhydryl proton extraction and subsequent nucleophilic attack (15). Substrate-induced activation of sortase may be advantageous, preventing spurious proteolysis reactions without the need for more elaborate inactivation mechanisms.

As cysteine proteases assemble their active site residue as a catalytic triad that stabilizes the thiolate-imidazolium pair (Cys^{25}-His^{159}-Asn^{175}), what is the mechanism of ion pair stabilization in sortase? In this report we have focused on conserved residues of sortase that are present in the active site. Neither Lue^{97} nor Tyr^{153} appear to play a role in catalysis, while Trp^{194} may contribute, but is definitively not essential for the transpeptidation reaction of surface protein anchoring. Trp^{194} is located close proximity to His^{120} and Cys^{184}. The polarity of the organic ring structure may exert an electron interaction within catalysis triad composed of HCW as has been proposed for some cysteine proteases such as streptococcus virulence factor SpeB (30). Residue Asn^{98} of sortase could also play a stabilizing role for the thiolate-imidazolium ion pair, since the chemical shift positions of the imidazole nitrogen atoms of His^{120} in the NMR structure indicate that this residue is fully protonated and therefore poised to donate a hydrogen bond from its Nϵ2 proton to the side chain carbonyl group of Asn^{98}. However, Asn^{98} is not a conserved residue in other sortase enzymes (13), and this prediction certainly requires experimental verification.

Fig. 6 proposes a molecular mechanism of surface protein anchoring and electron rearrangements within the active site of sortase. (i) The first step in the reaction pathway corresponds to the association of enzyme and substrate to form a Michaelis complex. (ii) A subtle conformational change of His^{120} results in the formation of a thiolate imidazolium ion pair with cysteine 184. (iii) The thiolate attacks the peptide bond, and the cysteine residue is acylated by cleaved surface protein, while the amino group of the cleaved peptide bond is released. (iv) The amino group of the pentaglycine cross-bridge of lipid II precursor attacks the thioester of the acyl-enzyme and forms the second product. This deacylation step results in the regeneration of the free enzyme. Several enzyme substrate

intermediates and/or transition states are presumed to exist along this pathway and our future work must delineate these mechanisms and achieve a detailed understanding of sortase catalysis by combining biochemical, genetic, and structural studies. These studies may permit the rational design of inhibitors that could be useful for anti-infective therapy of humans diseases caused by otherwise drug-resistant microbes.

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