Staphylococcus aureus Sortase A Exists as a Dimeric Protein In Vitro†

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ABSTRACT: We report the first direct observation of the self-association behavior of the *Staphylococcus aureus* sortase A (SrtA) transpeptidase. Formation of a SrtA dimer was observed under native conditions by polyacrylamide gel electrophoresis and fast protein liquid chromatography (FPLC). Subsequent peptide mass fingerprinting and protein sequencing experiments confirmed the dimeric form of the SrtA protein. Furthermore, SrtA can be selectively cross-linked both in vitro and in *Escherichia coli*. Multiple samples of enzyme were subjected to analytical sedimentation equilibrium ultracentrifugation to obtain an apparent K_d for dimer formation of about 55 μM. Finally, enzyme kinetic studies suggested that the dimeric form of SrtA is more active than the monomeric enzyme. Discovery of SrtA dimerization may have significant implications for understanding microbial physiology and developing new antibiotics.

Gram-positive bacteria are the cause of many serious human diseases, including anthrax, pneumonia, and meningitis (*1*). A Gram-positive species of special interest today is *Staphylococcus aureus*. *S. aureus* is the major cause of hospital-acquired infections, such as bacterial endocarditis, metastatic infections, sepsis, and toxic shock syndrome (*2*). Unfortunately, over the past decade there has been a dramatic increase in the prevalence of methicillin-resistant *S. aureus* (MRSA) strains. Today, more than 50% of hospital isolates in the United States are methicillin-resistant. The standard treatment for MRSA has been vancomycin, but alarmingly, in early 2002, the first vancomycin-resistant *S. aureus* strain was isolated (*3*). Therefore, it has become critical to devise new and more potent drugs to treat these bacterial infections.

In order to infect their host, Gram-positive bacteria require the display of certain proteins on their surface (*4*−*7*). Many of these surface proteins are anchored to the cell wall by a 206 amino acid, membrane-bound cysteine transpeptidase first identified in *S. aureus*, known as sortase A (SrtA)[1] (*8*). The sequence of the prototypical staphylococcal SrtA has been widely used to identify orthologous genes in other bacterial genomes, revealing an abundance of sortase enzymes in almost all Gram-positive bacteria (*9*). SrtA mutants display severe defects in virulence, suggesting that inhibitors of this enzyme could serve as a new type of antibiotic.

All of the substrates (surface protein precursors) of SrtA possess an N-terminal signal sequence for secretion and a C-terminal sorting signal. The sorting signal consists of an LPXTG motif (where X can be any amino acid), followed by a hydrophobic domain and a charged tail. SrtA recognizes the LPXTG motif and cleaves between the threonine (T) and glycine (G) residues. It then catalyzes the formation of an amide bond between the carboxyl group of threonine and the amino group of a pentaglycine cross-bridge in a peptidoglycan precursor, thus anchoring the protein to the peptidoglycan cell wall layer (*8*, *10*−*13*).

Given the interest in designing highly selective inhibitors for SrtA, several classes of small molecules that target the active site of the enzyme have been described in vitro (*8*, *14*−*22*). However, none of these compounds has yet been therapeutically useful. A thorough knowledge of the mechanism of the SrtA enzyme will be critical to the identification of an agent that inhibits SrtA in vivo. We report here that SrtA may function as a homodimer. This knowledge may lead to a new understanding of the mechanism of the SrtA enzyme, based upon which, new inhibitors of SrtA may be designed or identified by high through-put screening that targets the SrtA dimerization. It may also open a new path toward regulation of SrtA activity, as dimerization is known to regulate the activities of many other cell surface proteins (*23*−*25*).

MATERIALS AND METHODS

Construction of $SrtA_{\Delta N59}$ and $SrtA_{WT}$ Expression Vectors. Primers designated PsrtA59 (5′-CGATCCATGGGCCAAGCTAAACCTCAAATTCC-3′) and PsrtA59R (5′-CCGCTCGAGTTTGACTTCTGTAGCTACAA-3′) were used to amplify a $SrtA_{\Delta N59}$ sequence (which would express only residues 60−206) from genomic DNA from *S. aureus* subsp. *aureus* (ATCC 700699D) by the polymerase chain reaction. Full-length SrtA ($SrtA_{WT}$) was generated in a similar manner using the primers designated PsrtA (5′-CGATCCATGGGCAAAAA-

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[1] Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; sulfo-HSAB, *N*-hydroxysulfosuccinimidyl-4-azidobenzoate; PCR, polymerase chain reaction; FPLC, fast protein liquid chromatography; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; SrtA, *Staphylococcus aureus* sortase A transpeptidase.

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ATGGACAAATCGA-3′) and PsrtA59R. The two DNA fragments were digested with *Nco*I and *Xho*I (New England Biolabs, Beverly, MA) and cloned into the pET28b expression vector (Novagen, La Jolla, CA) to generate the constructs pET28-SrtA59 and pET28-FSrtA.

Purification of $SrtA_{\Delta N59}$ and $SrtA_{WT}$ Proteins. The pET28-SrtA59 and pET28-FSrtA constructs were transformed into *Escherichia coli* strain BL21 (Novagen). The pET28-SrtA59 transformed cells were grown in 1 L of Luria broth media at 37 °C until the OD_{600} reached 0.6. The culture was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA) and grown for another 8 h at 37 °C. The cells were harvested and lysed under native conditions using the QIAexpression kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Cells containing the pET28-FSrtA construct were grown in 6 L of Luria broth media at 37 °C until the OD_{600} reached 0.6. The culture was then induced with 1 mM IPTG and grown for another 9 h at 37 °C. Since the full-length protein had poor solubility under native conditions, these cells were harvested and lysed under denaturing conditions (8 M urea, 500 mM sodium chloride, 10 mM imidazole, 20 mM 2-mercaptoethanol, 10% glycerol, and 1% Triton X-100, pH 9.5). The lysate was centrifuged, and the supernatant was applied to 0.8 mL Ni-NTA agarose beads (preequilibrated with 10 mM imidazole). The protein was eluted with 8 M urea (with 100 mM NaH_2PO_4 and 10 mM Tris·HCl, pH 4.5). The collected fraction was dialyzed at 4 °C against a linear gradient (6−1 M urea containing 500 mM NaCl, 20% glycerol, and 20 mM Tris·HCl, pH 7.4) for 24 h to refold the full-length protein. Both the $SrtA_{\Delta N59}$ and $SrtA_{WT}$ proteins were then dialyzed against saline buffer (150 mM sodium chloride, 50 mM Tris·HCl, and 5 mM calcium chloride, pH 7.5) for future use. The concentration of protein was determined using Bradford reagent (Pierce Biotechnology, Rockford, IL).

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Proteins were analyzed on either a 12% SDS−polyacrylamide gel electrophoresis (PAGE) under denaturing conditions or a 12% native polyacrylamide gel under native conditions and visualized by Coomassie Blue staining (*26*). For Western blot analyses, proteins were transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Bioscience, Piscataway, NJ) in a Tris−glycine-buffered electrophoresis tank (*26*). The membranes were then probed with an anti-His C-terminal primary antibody (Invitrogen) and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA). The Phospha GLO AP substrate (KPL Incorporation, Baltimore, MD) was applied to visualize the signals, which were detected by exposing the membrane to BioMax light film (Eastman Kodak Co., Rochester, NJ).

In Vitro Chemical Cross-Linking. Both the purified $SrtA_{WT}$ and $SrtA_{\Delta N59}$ proteins were dialyzed against 100 mM NH_4HCO_3 buffer (pH 7.6) and lyophilized. Egg albumin and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO) as lyophilized powders. For cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Sigma-Aldrich), the proteins were dissolved in 0.1 M 2-(4-morpholino)ethanesulfonic acid (Fisher Scientific, Fair Lawn, NJ), pH 4.5, to give a 2.5 mg/mL solution. This was added to a freshly prepared EDC aqueous solution (0.8 mg/mL). The final concentration of the proteins was 1.25 mg/mL. After 1 h at room temperature, the reaction was quenched with 2% (v/v) 2-mercaptoethanol. For cross-linking with *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB; Pierce Biotechnology), the lyophilized proteins were dissolved in 1× PBS (20 mM NaH_2PO_4 and 150 mM NaCl, pH 7.4) to a concentration of 4 mg/mL. Freshly prepared sulfo-HSAB solution (2 mg/mL in DMSO/H_2O, 1:2 v/v) was then added to the protein solution prepared above to give a final concentration of 3.7 mg/mL. After incubation at room temperature (protected from light by foil) for 1 h, the mixture was irradiated by exposure to UV light for 5 min. The cross-linked products were analyzed by SDS−PAGE and visualized by Coomassie Blue staining and Western blotting as described above.

Chemical Cross-Linking in E. coli. BL21 cells transformed with the pET28-FSrtA construct and cells transformed with the pET28-SrtA59 construct were grown and induced as described above. EDC was then added to the media to a final concentration of 0.7 mg/mL. The cells were harvested 40 min later. $SrtA_{WT}$ was purified under denaturing conditions and analyzed by Western blotting as described above. $SrtA_{\Delta N59}$ was purified under native conditions and analyzed by Coomassie Blue staining as described above.

Protein In-Gel Digestion and Peptide Fingerprint Mapping by MS. The proposed dimer and monomer bands of $SrtA_{\Delta N59}$ were sliced from an SDS−polyacrylamide gel. The gel pieces were subjected to in-gel trypsin digestion. Protein was then extracted from the gel and analyzed by MALDI-TOF mass spectrometry and peptide fingerprint mapping analyses in the Analytical Instrumentation Core Facility at the University of Texas at Austin.

Protein Sequencing. The $SrtA_{\Delta N59}$ protein was analyzed by SDS−PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore) using a Bio-Rad semi-dry electrophoretic transfer cell. The proposed protein dimer on the membrane was sequenced using the Applied Biosystems 477A pulse liquid-phase sequencer (Protein Microanalysis Facility, The University of Texas at Austin) (*27*).

Size-Exclusion Chromatography. Purified $SrtA_{\Delta N59}$ protein was applied to a Superdex 200 column (300 × 10 mm) preequilibrated with 50 mM NH_4HCO_3 buffer. The sample was eluted with the same buffer at 0.5 mL/min at 4 °C using an AKTA FPLC (Amersham Pharmacia Biotech). Eluted protein was detected by monitoring at an absorbance of 280 nm. Proteins used as molecular weight standards were cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa), purchased from Sigma.

Analytical Sedimentation Equilibrium Ultracentrifugation. $SrtA_{\Delta N59}$ protein purified under native conditions was used for these analyses. All experiments were carried out on a Beckman Optima XL-1 analytical ultracentrifuge with the rotor speed set to 20000, 30000, and 40000 rpm at 25.0 °C. All experiments were performed in triplicate using six-channel centerpieces. Ultracentrifuge cells were assembled containing 26 or 52 μM $SrtA_{\Delta N59}$ in saline buffer (150 mM sodium chloride, 50 mM Tris·HCl, and 5 mM calcium chloride, pH 7.5). Scans were performed by a monochromator inside the ultracentrifuge with a wavelength of 242 or 246 nm. Ultrascan II version 8.0 software (*28*) was used to estimate the approximate time it would take for the

samples to achieve equilibrium at different concentrations and rotor speeds. Thirteen scans were then performed for each cell at the time points 0:30, 18:00, 21:00, 24:00, 27:00, 42:00, 45:00, 48:00, 51:00, 66:00, 69:00, 72:00, and 75:00 (hour:minute). Scanning data were extracted and fitted using Ultrascan II 8.0. A partial specific volume ($\bar{\nu}$) of 0.7349 mL/g (predicted by Sednterp 1.07 software, www.bbri.org/RASMB/rasmb.html) and a buffer density (ρ) of 1.0 g/mL were used in the analysis. The data were globally and simultaneously fitted to a one-component ideal species model (eq 1) and a monomer−dimer equilibrium model (eq 2), where X = radius, X_r = reference radius, A = amplitude of monomer*, M = molecular weight of monomer*, E = extinction coefficient, R = gas constant, T = temperature, B = baseline*, ω = angular velocity, L = path length, and $K_{1,2}$ = monomer−dimer equilibrium constant* (the asterisk indicates that this parameter can be floated) (*28*).

$$C(X) = e^{[\ln(A)+M\omega^2(1-\bar{\nu}\rho)(X^2-X_r^2)]/2RT} + B \quad (1)$$

$$C(X) = e^{[2\ln(A)+\ln(2/EL)+\ln(K_{1,2})+2M\omega^2(1-\bar{\nu}\rho)(X^2-X_r^2)]/2RT} + e^{[\ln(A)+M\omega^2(1-\bar{\nu}\rho)(X^2-X_r^2)]/2RT} + B \quad (2)$$

Determination of Kinetic Constants. EDC-mediated cross-linked $SrtA_{\Delta N59}$ was purified by size-exclusion chromatography with saline buffer (50 mM Tris·HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM 2-mercaptoethanol, pH 7.5). The monomeric and dimeric fractions of $SrtA_{\Delta N59}$ were separated, collected, and used for the following enzymatic assays. The peptide substrate *o*-aminobenzoyl-LPETG-(2,4-dinitropheyl)-diaminopropionic acid [Abz-LPETG-Dap(Dnp)] was synthesized on the basis of the well-established Fmoc/piperidine chemistry on a PAL resin (Protein Microanalysis Facility, The University of Texas at Austin). The peptide GlyGly-GlyGlyGly was purchased from BACHEM (Torrance, CA). Assays were performed in a 100 μL assay buffer (50 mM Tris·HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM 2-mercaptoethanol, pH 7.5) containing $SrtA_{\Delta N59}$ (100 nM for monomer, 50 nM for dimer), pentaglycine (2 mM), and varying concentrations of Abz-LPETG-Dap(Dnp) (0−300 μM). Reactions were initiated by the addition of enzyme and were monitored by measuring the increase in fluorescence for 10 min (λ_{ex} = 317 nm, λ_{em} = 420 nm) at 37 °C on a Spex FluoroMax-3 spectrofluorometer (Jobin Yuon Co., Edison, NJ). Initial velocities (V_0) were calculated as units of fluorescence per unit time using eq 3, where m is the slope during the linear phase of the cleavage, [S] is substrate concentration, and I_0 and I_{100} are the fluorescence intensities of substrate solution before and after complete cleavage, respectively. The slope (m) was measured in three independent experiments. The velocities (υ) were determined from the progress curves (the steady-state rates in the case of biphasic curves) at various substrate concentrations. The velocities obtained were then fit to eq 4 using KaleidaGraph software (version 3.6) to afford the apparent $K_{m,app}$ and $k_{cat,app}$ values (*29*).

$$V_0 = m[S]/(I_{100} - I_0) \quad (3)$$

$$\nu = k_{cat}ES/(S + K_m) \quad (4)$$

RESULTS AND DISSCUSSION

Expression of Cloned Sortase A. In order to obtain *S. aureus* SrtA protein for study, the SrtA gene was cloned, overexpressed, and purified from *E. coli*. A C-terminal His6 tag was used to facilitate the purification of the protein. A His6 tag has been shown to have no effect on SrtA activity (*30*). Both a full-length and a truncated version of SrtA were generated for further study.

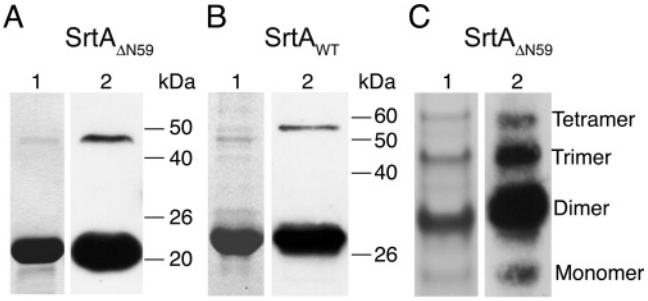

FIGURE 1: $SrtA_{\Delta N59}$ and $SrtA_{WT}$ protein expression. Purified $SrtA_{\Delta N59}$ (A, C) and $SrtA_{WT}$ (B) proteins were visualized by Coomassie Blue staining (lanes 1) and Western blotting (lanes 2) using an anti-His6 antibody. Denaturing conditions were applied in panels A and B, while native conditions were applied in panel C.

Like most other membrane proteins, full-length SrtA ($SrtA_{WT}$) is difficult to purify in sufficient amounts. However, the truncated SrtA protein ($SrtA_{\Delta N59}$), with the N-terminal 59 amino acids deleted, was readily expressed and purified. This hydrophobic N-terminal region of SrtA normally functions as a signal peptide for secretion and a stop-transfer signal for membrane anchoring. However, the signal peptide, membrane anchor, and a short linker domain of sortase enzymes (*S. aureus* SrtA residues 26−59) display no amino acid conservation between species. In contrast, the core (residues 60−206) is present in all SrtA orthologues examined, suggesting that this part of protein comprises the catalytically active domain (*31*, *32*). It is known that $SrtA_{\Delta N59}$ retains the same transpeptidation activity as the full-length SrtA enzyme (*31*, *32*).

Sortase A Forms a Dimer in Vitro. After purification under denaturing conditions, $SrtA_{\Delta N59}$ was analyzed by SDS−PAGE. When Coomassie stained, there appeared to be an intense band around 20 kDa along with a faint band around 45 kDa (Figure 1A, lane 1). The calculated molecular mass of a $SrtA_{\Delta N59}$ monomer is 17.9 kDa, while that of a dimer is 35.8 kDa. Therefore, these data suggested that these two bands represented monomeric and dimeric forms of $SrtA_{\Delta N59}$, respectively. In order to verify the composition of the bands, Western blotting analysis was performed. As demonstrated in Figure 1A, lane 2, both bands contained proteins with a C-terminal His6 tag, indicating that $SrtA_{\Delta N59}$ is present in both bands. The higher molecular mass band could therefore correspond to either a $SrtA_{\Delta N59}$ dimer or $SrtA_{\Delta N59}$ interacting with another, as yet unidentified, protein.

These same experiments were then repeated with $SrtA_{WT}$ with similar results. On a Western blot detected with an anti-His6 antibody, two bands were observed. We detected a more intense band around 26 kDa and a less intense band around 55 kDa (Figure 1B, lane 2). A monomer of $SrtA_{WT}$ is predicted to have a mass of 24.7 kDa, while the dimer should have a mass of 49.5 kDa.

$SrtA_{\Delta N59}$ was also purified under native conditions (Materials and Methods) and analyzed by native polyacrylamide gel electrophoresis (Figure 1C, lanes 1 and 2). In contrast to the results under denaturing conditions, when Coomassie

stained, four protein bands were observed (Figure 1C, lane 1). Western blotting with an anti-His6 antibody showed that these four protein bands contain $SrtA_{\Delta N59}$ (Figure 1C, lane 2). We suspected that these four bands correspond to the monomeric, dimeric, trimeric, and tetrameric forms of $SrtA_{\Delta N59}$, respectively (Figure 1C, lanes 1 and 2). Because the putative dimeric $SrtA_{\Delta N59}$ band was the most intense, we believe that the dimeric form of $SrtA_{\Delta N59}$ is the preferred state.

To eliminate the possibility that the oligomerization of $SrtA_{\Delta N59}$ was due to a nonspecific protein aggregation, we performed a serial dilution experiment. The dimeric band could be readily detected on a native gel with a protein concentration of 22 μM, to as little as 1.3 μM by Western blot (data not shown). Hexahistidine (His6) is one of the most commonly used tags for protein affinity chromatography. In most cases His6-tagged proteins are little affected in their biophysical and biochemical properties, as shown in the case of $SrtA_{\Delta N59}$ (*33*). However, at least in one case, the His6 tag has been reported to partially induce the dimerization of a monomeric protein pi(30.5) mutant (*34*). To eliminate the possibility that oligomerization of $SrtA_{\Delta N59}$ was induced by its C-terminal His6 tag, site-directed mutagenesis has been used to generate a His6-tagged $SrtA_{\Delta N59}$ mutant in which a single amino acid mutation at the protein−protein interface completely disrupted $SrtA_{\Delta N59}$ dimerization (to be published).

Confirmation of Dimerization in Vitro. We next confirmed that $SrtA_{\Delta N59}$ was the sole protein component of the higher molecular mass bands observed by SDS−PAGE. The protein from the putative dimeric band on the $SrtA_{\Delta N59}$ gel was extracted and directly sequenced. Only one protein sequence was found. The first seven amino acids of this sequence, GQAKPQI, were identical to that of $SrtA_{\Delta N59}$.

The protein from this putative dimeric band was then subjected to protein in-gel digestion and peptide fingerprint mapping by mass spectroscopy. Only one protein was detected from this assay, and its fingerprint corresponded to the known sequence of $SrtA_{\Delta N59}$ with a confidence value of 83% (NCBI Protein Database: gi l4277822, chain a, structure of sortase).

Next, size-exclusion chromatography was used to observe the oligomerization state of $SrtA_{\Delta N59}$ under native conditions. Three peaks were observed after the purified $SrtA_{\Delta N59}$ was applied to a Superdex 200 gel filtration column (Figure 2, peaks a, b, and c). The calculated molecular masses of two major peaks were 41 kDa (peak b) and 26 kDa (peak c), which correspond to a dimer and monomer of $SrtA_{\Delta N59}$, respectively. The fractions containing peaks b and c were then eluted, digested with trypsin, and subjected to peptide mass fingerprinting analysis. They corresponded to the $SrtA_{\Delta N59}$ sequence with confidence limits of 71% and 99%, respectively (data not shown). These data confirm that the major peak b (41 kDa) consists of the dimeric form of $SrtA_{\Delta N59}$, while peak c (26 kDa) consists of the monomeric form of $SrtA_{\Delta N59}$. The third, smaller protein peak a has a calculated molecular mass of 56 kDa, which suggests that this peak consists of a trimeric form of $SrtA_{\Delta N59}$. The tetrameric form of $SrtA_{\Delta N59}$ was not detected in this assay.

The attainment of the monomer−dimer equilibrium is a slow process. Figure 1C demonstrates that when $SrtA_{\Delta N59}$ is run on a polyacrylamide gel under native conditions, the

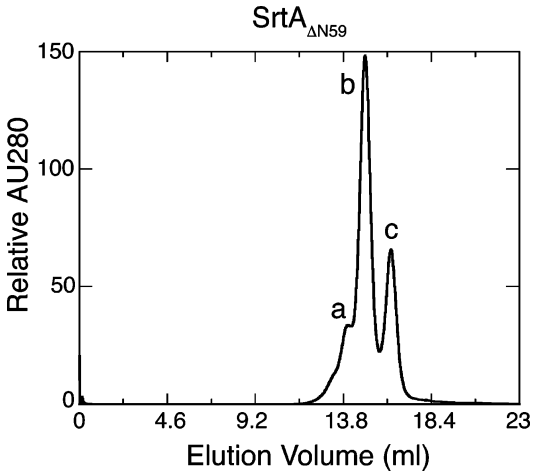

FIGURE 2: Size-exclusion chromatography of $SrtA_{\Delta N59}$ protein. $SrtA_{\Delta N59}$ protein was purified under native condition and incubated at 4 °C overnight before being analyzed by size-exclusion chromatography.

dimeric form predominates. Similar results were obtained when $SrtA_{\Delta N59}$ was subjected to size-exclusion chromatography. About 62% of the total protein was found in a dimeric state (Figure 2, peak b), about 27% was found as a monomer (Figure 2, peak c), and about 11% was found as a trimer. The protein sample used in these experiments had been incubated overnight at 4 °C. If, instead, a freshly prepared sample is used, different results were found. When a freshly prepared sample of $SrtA_{\Delta N59}$ was subjected to size-exclusion chromatography under native conditions, the majority of the protein existed as a monomer (about 50%), while the dimeric form was about 45%, and the trimeric form was less than 5% of the total protein.

$SrtA_{\Delta N59}$ has a strong tendency to form oligomers. Under native conditions, oligomerization of $SrtA_{\Delta N59}$ into dimeric and trimeric forms was consistently observed after both polyacrylamide gel electrophoresis and size-exclusion chromatography analysis. A possible tetrameric form of $SrtA_{\Delta N59}$ was also detected by polyacrylamide gel electrophoresis but not by size-exclusion chromatography. This is probably due to the detection limit of the latter method. Detailed mass spectroscopic analyses and protein sequencing results excluded the possibility of formation of heterooligomers between $SrtA_{\Delta N59}$ and other proteins. Furthermore, even under denaturing conditions, $SrtA_{\Delta N59}$ dimers were still observed. Treatment with various concentrations of dithiothreitol was also unable to eliminate the dimeric band on a denaturing polyacrylamide gel (data not shown). This indicates that the formation of $SrtA_{\Delta N59}$ dimers is not caused by the random disulfide bond formation between protein surfaces. The surface of $SrtA_{\Delta N59}$ is highly charged. There are 18 positively charged lysine and arginine residues and 18 negatively charged aspartic acid and glutamic acid residues on its surface, according to its X-ray structure (*32*). Experiments were carried out to check the ion strength dependence of dimer formation. $SrtA_{\Delta N59}$ was incubated with the buffers containing increasing salt concentrations from 5 mM to 1 M at 4 °C overnight. The samples were subsequently analyzed by native PAGE. The ratio of dimer to monomer $SrtA_{\Delta N59}$ remained unchanged regardless of the salt concentration (data not shown). These data suggest that dimers of $SrtA_{\Delta N59}$ are not stabilized by electronic interac-

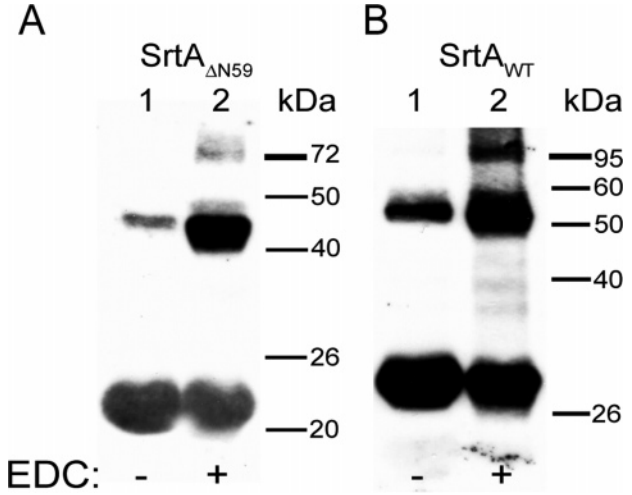

FIGURE 3: In vitro EDC-mediated cross-linking. SrtA$_{\Delta N59}$ (panel A) and SrtA$_{WT}$ (panel B) proteins were subjected to EDC-mediated chemical cross-linking in vitro. Both cross-linked (lanes 2) and un-cross-linked (lanes 1) samples of protein were then analyzed by SDS−PAGE and Western blotting using an anti-His6 antibody. An equal amount of protein was loaded into each lane.

tions, which is supported by our recent site-directed mutagenesis studies (to be published). Ca^{2+} concentration is consistently used at 5 mM in buffers throughout all of the SrtA$_{\Delta N59}$ experiments in this study. We did not observe that the presence of calcium influences the native PAGE gel and the gel filtration behaviors.

Sortase A Can Be Cross-Linked in Vitro. To further confirm that SrtA selectively forms a dimer, a series of in vitro chemical cross-linking experiments were also performed. Purified SrtA$_{\Delta N59}$ and SrtA$_{WT}$ proteins were subjected to cross-linking using EDC, a compound that couples carboxylic acid groups with primary amines (*35*). When the products of these reactions were subjected to Western blot analyses (Figure 3, lanes 2), the putative dimeric bands were much more intense, compared to the dimeric bands from un-cross-linked proteins (Figure 3, lanes 1). Both the SrtA$_{\Delta N59}$ and SrtA$_{WT}$ proteins exhibited extra bands with higher molecular masses in addition to the putative dimeric bands after cross-linking. In the case of SrtA$_{\Delta N59}$, it appeared around 72 kDa where the band was not detected in the un-cross-linked protein (Figure 3A). While in that of SrtA$_{WT}$, EDC-mediated cross-linking resulted in a band above 95 kDa besides the monomeric and dimeric bands (Figure 3B). On the basis of their apparent molecular mass, these bands may correspond to those of SrtA tetramers. These data are consistent with the previous observation of formation of the sortase A tetramer, which may result from the SrtA dimer−dimer interaction. It is interesting that no trimeric SrtA band was observed.

We then repeated these experiments by using another, more efficient and photoreactive cross-linker, sulfo-HSAB (*36*). In these experiments we also cross-linked egg albumin, a protein that does not normally form dimers, and lysozyme, which has a strong tendency to dimerize (*37*, *38*). When these proteins were subjected to sulfo-HSAB-mediated cross-linking, both SrtA$_{\Delta N59}$ and lysozyme formed dimers, and as expected, dimerization was not apparent with egg albumin (Figure 4). These experiments support the notion that SrtA naturally forms a homodimer.

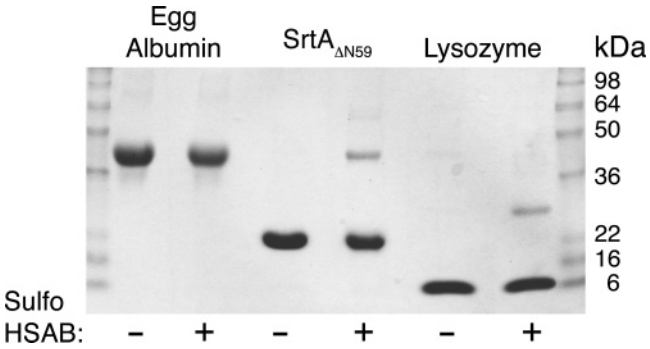

FIGURE 4: In vitro sulfo-HSAB-mediated cross-linking. Egg albumin, SrtA$_{\Delta N59}$, and lysozyme proteins were subjected to sulfo-HSAB-mediated chemical cross-linking in vitro. Both cross-linked (+ lanes) and un-cross-linked (− lanes) samples of protein were then separated by SDS−PAGE and visualized by Coomassie Blue staining.

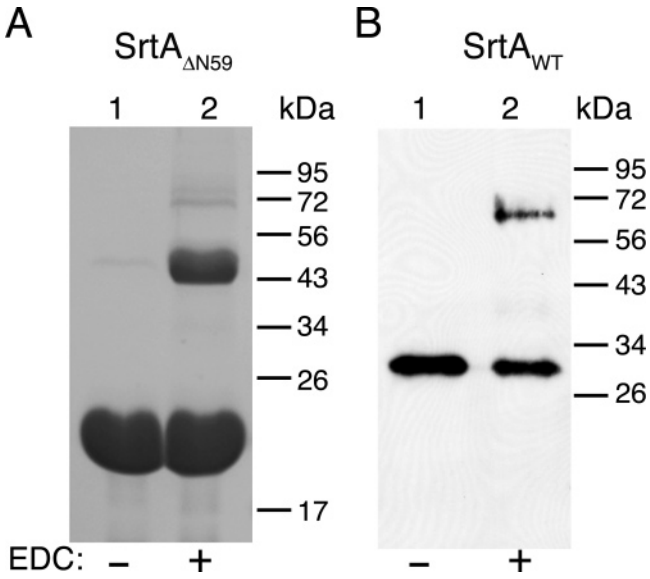

FIGURE 5: EDC-mediated cross-linking in *E. coli*. SrtA$_{\Delta N59}$ (panel A) and SrtA$_{WT}$ (panel B) proteins purified from cells after EDC treatment (lanes 2), as well as that lacking EDC treatment (lanes 1), were separated by SDS−PAGE. For purified SrtA$_{WT}$, Western blotting with an anti-His6 antibody was necessary. Within each panel, equal amounts of protein were loaded into lanes 1 and 2.

Sortase A Selectively Forms a Homodimer in E. coli. We next demonstrated that SrtA can also be cross-linked in *E. coli*. An *E. coli* culture containing cells expressing SrtA$_{\Delta N59}$ was briefly treated with EDC (*39*). SrtA$_{\Delta N59}$ protein was then purified under denaturing conditions and examined by Coomassie staining. Two bands were apparent (Figure 5A, lane 2). As with the in vitro results (Figure 3A, lane 2), the mass of these bands corresponded to a monomer and dimer of SrtA$_{\Delta N59}$. The dimer band was then sliced from the gel and subjected to in-gel digestion with trypsin. Peptide mass fingerprinting indicated that only one protein was present and that its fingerprint corresponded to the known sequence of SrtA$_{\Delta N59}$ with a confidence value of 59% (data not shown).

This experiment was then repeated with SrtA$_{WT}$. When protein purified from EDC-treated *E. coli* cells expressing SrtA$_{WT}$ was examined by Western blotting, two bands were apparent (Figure 5B, lane 2), whose mass corresponded to a monomer and dimer of SrtA$_{WT}$. These experiments demonstrate that SrtA forms a homodimer selectively in a complex

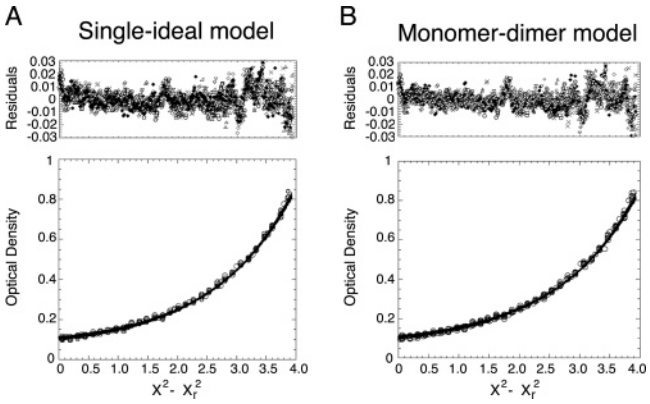

FIGURE 6: Analytical sedimentation equilibrium ultracentrifugation. $SrtA_{\Delta N59}$ protein was purified under native conditions and subjected to analytical sedimentation equilibrium ultracentrifugation under various conditions. The data were subjected to a single-ideal model (panel A) and a monomer−dimer association model (panel B).

mixture of proteins and dimerization of SrtA occurs under more biologically relevant conditions.

Determination of the Dissociation Constant (K_d) of the SrtA Dimer. In order to quantitatively determine the equilibrium between the $SrtA_{\Delta N59}$ monomer and dimer, this protein was next subjected to analytical equilibrium sedimentation ultracentrifugation. Scans were taken at a certain wavelength at different time points (Materials and Methods). After about 18 h, the scanned data points get overlaid, which indicates that the protein solution reaches its equilibrium state from the starting protein concentration at 20000 rpm. The experiment was continued for 75 h in total and repeated at different rotor speeds (20000, 30000, and 40000 rpm) from two starting protein concentrations (26 or 52 μM). Representative equilibrium state data from different scans and time points are shown in Figure 6. Data points were first globally and simultaneously fitted to a single ideal species model (which assumes the protein solution is a pure, single component system that conducts hydrodynamically ideal behavior), indicated by a solid line (Figure 6A). From this fitting, we can estimate the global molecular mass, which is the superposition of the distributions of the individual species in the case of more than one species in solution. The analyses of the data gave an apparent molecular mass of 25370 ± 1310 g/mol. This is between the monomer MW of 17849.2 g/mol and the dimer MW of 35698.4 g/mol, suggesting that the $SrtA_{\Delta N59}$ solution is composed of both monomeric and dimeric components and sustains monomer−dimer equilibrium under experimental conditions.

Therefore, the same data sets were further subjected to a monomer−dimer equilibrium model (Figure 6B) in order to estimate dissociation constants. A starting protein concentration of 26 μM spanning three different speeds gave an average dissociation constant (K_d) of 55.7 ± 6.6 μM. A starting protein concentration of 52 μM at three speeds gave an average K_d of 53.4 ± 6.9 μM. Combining these data gives an average K_d of 54.6 ± 6.9 μM. Compared to other dimeric enzymes whose K_d values range from nanomolar for HIV-1 protease (*40*) to high micromolar for caspase 9 (*24*), the K_d of 54.6 μM suggests a moderate binding affinity for the $SrtA_{\Delta N59}$ dimer in vitro. However, the K_d was measured with the truncated version of SrtA in a dilute solution. For $SrtA_{WT}$ in vivo, the incidence of dimerization may actually be much greater, due to the fact that full-length SrtA is anchored to the cell membrane, thus increasing its effective concentration. An example is the outer membrane phospholipase A (OMPLA) of Gram-negative bacteria (*41*). OMPLA has moderate binding affinity in vitro using analytical ultracentrifugation, similar to that of $SrtA_{\Delta N59}$. Study shows that OMPLA is present in the outer membrane as a monomer and dimerization of OMPLA is induced to activate this enzyme. Thus we also hypothesize that there is equilibrium between the SrtA monomer and dimer in vivo that can be regulated, similar to OMPLA.

Table 1: Kinetic Parameters for the Dimeric and Monomeric Fractions of $SrtA_{\Delta N59}$[a]

	enzyme fraction		
	monomer (100%)	dimer (20%)	published data
$K_{m,app}$ (μM)	100.3 ± 11.3	61.2 ± 17.0	8.2−5500[b]
$k_{cat,app}$ (s^{-1})	0.121 ± 0.01	0.117 ± 0.01	1.2×10^{-4} to 0.57[b]

[a] Duplicate data sets for each experiment were used to calculate the steady-state velocity at different Abz-LPETG-Dap(Dnp) concentrations for both the dimeric and monomeric fractions of $SrtA_{\Delta N59}$ enzyme after EDC cross-linking and FPLC purification. The initial velocity was then normalized and plotted as in Figure 7. $K_{m,app}$ and $k_{cat,app}$ values were determined by a double-reciprocal plot of the substrate dependence of velocity using eq 4. [b] See refs *29* and *43*−*46*.

Kinetic Studies of Monomeric and Dimeric Sortase A. In order to determine whether there was a functional difference between a monomer and dimer of SrtA, we measured the kinetic constants for both the monomeric and dimeric forms of $SrtA_{\Delta N59}$ using a fluorescent quenching assay (*29*, *31*, *42*, *43*).

SrtA catalyzes two sequential reactions following a ping-pong mechanism (*43*). The first step is the hydrolysis of the primary substrate LPXTG to form an acyl-enzyme, and the second step is ligation to the secondary substrate, pentaglycine. It is thought that the first step is the rate-determining step in transpeptidation (*43*). Therefore, we have focused on the $k_{cat,app}/K_{m,app}$ of the hydrolysis of the LPETG substrate. Numerous assays have been used by several groups, including fluorescent and HPLC assays (*29*, *43*−*46*). These reports give a surprisingly wide range of K_m values (8.2 μM to 5.5 mM), k_{cat} values (1.2×10^{-4} to 0.57 s^{-1}) ,and k_{cat}/K_m values (6×10^{-3} to 4.91 mM^{-1} s^{-1}) (Table 1). We chose the fluorescent quenching assay to avoid the precipitation of peptide substrates at high concentration in the HPLC method. A synthetic peptide, Abz-LPETG-Dap(Dnp), was used as the substrate for enzyme kinetics in the presence of 2 mM Gly_5. Abz-LPETG-Dap(Dnp) consists of a peptide with a fluorescent group (Abz) at the N-terminus, the LPXTG motif, and a quencher [Dap(Dnp)] at the C-terminus. Synthetic pentaglycine was used as the nucleophile to substitute for the pentaglycine cross-bridge of the peptidoglycan cell wall. It has been shown that, under these conditions, SrtA catalyzes the cleavage of Abz-LPETG-Dnp, releasing a fluorescent signal, and generates the product Abz-LPET-GGGGG. Fluorescence was monitored over time (10 min) using various amounts of substrate. After 10 min of reaction time, the substrate turnover was less than 10% (data not shown). Thus, the rate obtained represented the initial rate. Fitting of the rate vs Abz-LPETG-Dap(Dnp) concentration to eq 4 generated $K_{m,app}$ and $k_{cat,app}$ for both the monomeric and dimeric forms of $SrtA_{\Delta N59}$. To obtain a dimer-enriched fraction, we

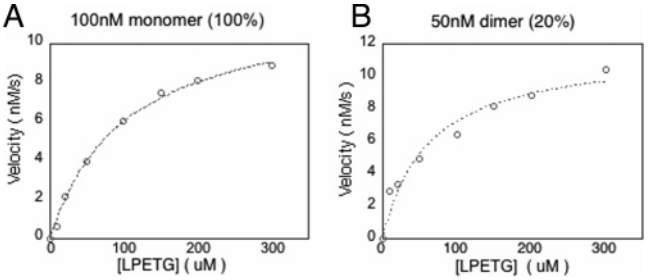

FIGURE 7: Steady-state rate plotted against the concentration of Abz-LPETG-Dap(Dnp). Purified $SrtA_{\Delta N59}$ was subjected to EDC cross-linking and then separated by size-exclusion chromatography. A monomeric fraction containing 100% monomer of $SrtA_{\Delta N59}$ and a dimer-enriched fraction containing 20% $SrtA_{\Delta N59}$ dimer were collected. Each fraction was then used to catalyze the SrtA transpeptidation reaction using Abz-LPETG-Dap(Dnp) as its substrate. Kinetic constants were determined using the Michaelis−Menten equation, where the rate is plotted against Abz-LPETG-Dap(Dnp) concentration, for both the monomeric fraction (panel A) and the dimeric fraction (panel B).

first chemically cross-linked $SrtA_{\Delta N59}$ using EDC. The mixture of proteins was then separated by FPLC under native conditions to collect the monomeric and dimeric forms. However, despite many attempts, the dimeric fraction always contained some un-cross-linked protein. Denaturing SDS−PAGE gel analysis of the putative dimeric fraction indicated that it contained about 20% covalently linked protein dimer. Therefore, we used the two fractions of SrtA directly from FPLC for the assays. Results are shown in Figure 7 and Table 1.

We obtained a $k_{cat,app}$ value of 0.121 ± 0.01 s^{-1} for the monomeric SrtA and that of 0.117 ± 0.01 s^{-1} for the dimer-enhanced fraction (Table 1). These values are in the same order of magnitude as the k_{cat} value of 0.57 s^{-1} reported by Schneedwind et al. using the same fluorescent quenching assay (*29*). Ellestad and co-workers reported a k_{cat} value of 0.096 min^{-1} under the same conditions (*43*). It is still not clear why such discrepancy occurs (*43*). For our study, it is important to note that there is no difference in the $k_{cat,app}$ values of the monomeric and dimeric fractions of $SrtA_{\Delta N59}$ (Table 1). We obtained the $K_{m,app}$ value of 61.2 μM for the dimer-enhanced fraction of $SrtA_{\Delta N59}$ and 100.3 μM for the monomeric fraction. Our $K_{m,app}$ value for monomeric $SrtA_{\Delta N59}$ is in agreement with those reported K_m values, 116 μM from Schneewind and co-workers (*29*) and 117 μM from Ellestad and co-workers (*43*). These authors used $SrtA_{\Delta N59}$ at a concentration of 1.5 μM, which is well below the measured K_d of 55 μM. Under this condition, $SrtA_{\Delta N59}$ mainly exists as a monomer. The $SrtA_{\Delta N59}$ dimer-enhanced fraction has a smaller K_m than the monomeric fraction. Although $K_m \neq K_d$, K_m is a measure of the dissociation constant for all enzyme-bound species (*47*). In this case, it suggests that the $SrtA_{\Delta N59}$ dimer has higher binding affinity toward the substrate than the SrtA monomer. Perhaps dimeric $SrtA_{\Delta N59}$ adopts a conformation that is more favorable for substrate binding than monomeric $SrtA_{\Delta N59}$ (*45*).

The apparent $k_{cat,app}/K_{m,app}$ value for the transpeptidation reaction with the monomeric fraction of $SrtA_{\Delta N59}$ was calculated as 1.21 mM^{-1} s^{-1}, while that of the dimer-enriched fraction was 1.91 mM^{-1} s^{-1}; both are slightly lower than the published $k_{cat,app}/K_{m,app}$ of 4.91 mM^{-1} s^{-1} using the same fluorescent assay, but on the same order of magnitude (*29*). The discrepancy may result from the difference of enzyme preparation and purification. In addition, cross-linking itself may alter the activity of $SrtA_{\Delta N59}$, which may account for the lower $k_{cat,app}/K_{m,app}$ values of the monomeric and dimer-enhanced fractions of $SrtA_{\Delta N59}$. Nevertheless, these data indicate that the dimer-enriched fraction is about 1.5 times more active than the monomeric form under these experimental conditions. However, the concentrations of monomeric $SrtA_{\Delta N59}$ and dimer-enhanced $SrtA_{\Delta N59}$ are 100 and 50 nM, respectively, which is well below the measured K_d (55 μM). Under this condition, the dimeric fraction may contain only about 20% covalently linked dimer. Therefore, we estimate that the dimeric form may in fact be as much as 6−8 times more active than the monomeric form. Regardless of the absolute value, these results clearly demonstrate that dimeric SrtA is more active than monomeric SrtA in vitro. We are currently conducting an in vivo assay to explore the difference in activity between dimeric and monomeric SrtA on the *S. aureus* cell surface.

This difference in activity between the monomeric and dimeric forms of $SrtA_{\Delta N59}$ may mean that dimerization of $SrtA_{\Delta N59}$ is required for proper functioning of the enzyme, as is the case for several other enzymes, such as the human cytomegalovirus protease, the apoptotic protease caspase 9, HIV protease, and many G-protein-coupled receptors (*23*−*25*, *40*). At least one bacterial membrane protein (OMPLA) has also been found to be regulated by protein dimerization (*41*). If this turns out to be correct, it may be possible to develop new reagents that disrupt dimerization.

CONCLUSIONS

The SrtA enzyme has increasingly become the subject of research due to its critical role in the virulence of Gram-positive bacteria. A complete understanding of its mechanism of action may lead to new inhibitors, which can serve as antibiotics to fight the growing numbers of antibiotic-resistant bacteria, including MRSA. Several chemical and kinetic mechanisms have been proposed to explain the actions of this enzyme (*17*, *29*, *31*, *42*, *44*, *48*). On the basis of these mechanisms, several inhibitors have been identified in vitro. However, the lack of working inhibitors in vivo suggests that the mechanism of SrtA needs to be investigated more thoroughly. Indeed, recent studies reveal that interactions with Ca^{2+} can alter the conformation of SrtA and, thereby, its activity (*45*).

Our studies demonstrate that the SrtA protein forms a homodimer and that its dimeric form is more active than its monomeric form. This knowledge should contribute to the formulation of a comprehensive mechanism for SrtA. It is also important to note that dimerization of SrtA is very selective, even in a complex mixture of proteins, in *E. coli*. However, the fact that the dissociation constant was only in the middle micromolar range suggests there is an equilibrium between the monomeric and dimeric forms. On the basis of our data, we hypothesize that the in vivo activity of SrtA may be regulated by unidentified protein(s)/small molecule(s) in response to certain stimuli.

It is possible that agents that disrupt SrtA dimerization can serve as potential antiinfectious drug candidates. Therefore, it is very important to study the forces that govern the protein−protein interactions between SrtA. Our initial chemi-

cal cross-linking experiments and site-directed mutagenesis data (to be published) have shed light on this aspect, suggesting that hydrophobic interactions and hydrogen bonds may play important roles. However, in order to further understand the SrtA mechanism, we are currently working on obtaining an X-ray structure of the dimeric form of SrtA.

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