The serine and cysteine proteases SspA and SspB of Staphylococcus aureus are secreted as inactivezymogens, zSspA and zSspB. Mature SspA is a trypsin-like glutamyl endopeptidase and is required to activate zSspB. Although a metalloprotease Aureolysin (Aur) is in turn thought to contribute to activation of zSspA, a specific role has not been demonstrated. We found that pre-zSspA is processed by signal peptidase at ANA29↓, releasing a Leu30 isoform that is first processed exclusively through autocatalytic intramolecular cleavage within a glutamine-rich propeptide segment, 40QQTQSSKQQTPKIQ53. The preferred site is Gln43 with secondary processing at Gln47 and Gln53. This initial processing is necessary for optimal and subsequent Aur-dependent processing at Leu58 and then Val69 to release mature SspA. Although processing by Aur is rate-limiting in zSspA activation, the first active molecules of Val69SspA promote rapid intermolecular processing of remaining zSspA at Glu65, producing an N-terminal 66HANVILP isoform that is inactive until removal of the HAN tripeptide by Aur. Modeling indicated that His66 of this penultimate isoform blocks the active site by hydrogen bonding to Ser237 and occlusion of substrate. Binding of glutamate within the active site of zSspA is energetically unfavorable, but glutamine fits into the primary specificity pocket and is predicted to hydrogen bond to Thr232 proximal to Ser237, permitting autocatalytic cleavage of the glutamine-rich propeptide segment. These and other observations suggest that zSspA is activated through a trypsinogen-like mechanism where supplementary features of the propeptide must be sequentially processed in the correct order to allow efficient activation.

Staphylococcus aureus can colonize and infect virtually every tissue and organ system of the body (1, 2). A key factor in these defining traits is its ability to sustain bacteremia and adhere to tissues. Consequently it has adapted to growth in blood by subversion of plasma proteins (3) and the tissue extracellular matrix (4). Members of the MSCRAMM (microbial extracellular matrix-binding protein) family of adhesion proteins bind extracellular matrix ligands such as collagen, fibronectin, and fibrinogen (4) of which the latter two are also abundant in plasma. Manipulation of other plasma proteins such as IgG and von Willebrand factor is facilitated by staphylococcal Protein A (5), whereas coagulase promotes fibrin clot formation by activation of prothrombin (6), and staphylokinase activates plasminogen (7) to facilitate fibrin clot dissolution. Our studies on secreted proteases are also supportive of S. aureus as a paradigm for the manipulation of plasma and coagulation proteins. The SspA4 glutamyl endopeptidase, also known as V8 protease, moderates adhesion of S. aureus to fibronectin by degrading cell surface fibronectin-binding proteins in which there is a high glutamic acid content, including a conserved motif that is essential for ligand binding (8, 9). SspA is expressed in an operon with a cysteine protease, SspB, and activates the zSspBzymogen by removing its N-terminal propeptide (10). Mature 20-kDa SspB mimics plasma serine proteases as noted by its ability to (i) convert high molecular weight kininogen into heavy and light chains, (ii) hydrolyze the Bz-Pro-Phe-Arg substrate of kallikrein, a serine protease that processes single chain kininogen by excision of the vasoactive peptide bradykinin (RPPGFSPER), (iii) process the N-terminus of the fibrinogen β-chain at the same site as plasmin, and (iv) remove the N-terminal domain of fibrinectin with a specificity equivalent to plasminogen activator (10). We have also found that antibodies to SspB are produced when mice are challenged with hypervirulent strains of community-acquired methicillin-resistant S. aureus and that SspA and SspB are rapidly expressed in neutrophil vacuoles following phagocytosis (11), alluding to a role in modifying vacuolar proteins.

A third gene in the ssp operon encodes a small protein, SspC, now commonly referred to as Staphostatin B, which we identified as an inhibitor of SspB (10) and for which others defined the structural basis of inhibition (12). The need for an inhibitor is
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TABLE 1
Bacterial strains and recombinant plasmids used in this study

| Strain/plasmid | Description | Source/Ref. |
|---------------|-------------|-------------|
| S. aureus | Derivative of NCTC 8325 with increased expression of secreted proteins | 20 |
| RN6390 | Entry level host strain for plasmids | 18 |
| RN4220 | sspA::erm; nonpolar inactivation of sspA in RN6390 | 21 |
| SP6391 | aurol::lacZ transcriptional fusion in strain 8325; deficient in Aur expression | 52 |
| DU5969 | aureol::lacZ allele of DU5969 transduced into RN6390 with d85 | This study |
| 6390aur::lacZ | sarA::km mutation in S. aureus 8325; produces elevated level of secreted proteases | 53 |
| PC1839 | sarA::km transduced from PC1839 into RN6390 | This study |
| E. coli | Host strain for construction of shuttle vectors | Invitrogen |
| Plasmids | pRN5548a lacking bldZ promoter fragment | 10 |
| pYBH | SspA with C-terminal His6 tag, cloned into HindIII and Sal sites of pRN5548a | This study |

supported by the finding that expression of SspB in Escherichia coli is lethal unless accompanied by Staphostatin B or a Cys → Ser active site mutant in SspB. Moreover, inactivation of sspC in S. aureus promotes a gross disturbance in growth and protein export, supporting a role for Staphostatin B in protecting against activation of zSspB during protein export (13–15). One means by which this could occur would be if zSspB were also activated prematurely. However, although it has long been thought that the metalloprotease Aureolysin is in turn essential for activation of zSspA (16), one study found that zSspA was correctly activated albeit with reduced efficiency in an aurol-deficient strain (17). In the present study, we elucidated a novel stepwise process for activation of zSspA that minimizes the possibility of its untimely activation. We found that Aureolysin is essential for activation of zSspA as the first step in processing of the N-terminal propeptide requires autocatalytic intramolecular cleavage at glutamine. Aureolysin then processes at Leu26 and then Val69 to produce the first active molecules of mature SspA, which then feedback to promote efficient autocatalytic intermolecular processing of remaining zSspA at Glu65. This exposes an N-terminal 66HAN tripeptide that blocks the active site until final Aur-dependent processing exposes the hydrophobic N terminus (69VIL) that is common in the trypsin family proteases. These events must occur in the proper sequence for efficient activation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains and recombinant plasmids used in this study are listed in Table 1. S. aureus and E. coli were cultured at 37 °C in tryptic soy broth (Becton Dickinson and Company) and LB medium (Invitrogen), respectively. Medium was supplemented with agar (15 g liter⁻¹; Difco) or antibiotic (ampicillin, 100 μg ml⁻¹; erythromycin, 10 μg ml⁻¹; chloramphenicol, 10 μg ml⁻¹; or kanamycin, 25 μg ml⁻¹) as required. For production of secreted proteins, cultures were grown for 18 h in tryptic soy broth with vigorous aeration. E. coli DH5α (Invitrogen) was used as a host for construction of shuttle vectors that were transferred to S. aureus RN4220 by electroporation (18) before transfer into other strains as indicated in Table 1. The aurol::lacZ and sarA::km alleles were transferred from the original host strains into S. aureus RN6390 via transduction with d85 (19) as detailed in Table 1.

PCR and Recombinant DNA Procedures—S. aureus genomic DNA was isolated using the DNeasy Tissue kit (Qiagen) following the manufacturer’s protocol for Gram-positive bacteria. Plasmid DNA was isolated using the GenElute Plasmid Miniprep kit (Sigma) following recommended protocols. Polymerase chain reaction was performed with Biotools DNA polymerase (Interscience) in the buffer supplied or with the Expand Long Template PCR System (Roche Applied Science) for products larger than 2 kb. PCR products used in cloning were purified using the QIAquick PCR Purification kit (Qiagen) or Concert Rapid Gel Extraction System (Invitrogen). All plasmids harboring cloned DNA fragments were submitted to the Center for Applied Genomics at the Toronto Hospital for Sick Children for verification of the expected nucleotide sequences.

Purification of Proteases from S. aureus—Mature SspA and SspB were purified from culture supernatant of S. aureus RN6390 sarA::km, which expresses high levels of secreted protease due to a defect in SarA, which is a repressor of protease gene expression (53). The 40-kDa SspB zymogen zSspB was purified from culture supernatant of S. aureus RN6390 sspA::erm, which cannot activate zSspB due to a defect in production of the SspA serine protease. The purification schemes were as described previously (10). Aureolysin was purified from a sarA::km derivative of RN6390. Protein in stationary phase culture supernatant was precipitated with 80% saturation of ammonium sulfate followed by dialysis into 20 mM Tris-Cl, pH 7.8, containing 5 mM CaCl2. Aureolysin was then purified by successive anion exchange steps on HiLoad 16/10 Q Sepharose Fast Flow (Amersham Biosciences) and Tricorn Mono Q medium (Amersham Biosciences). Protein was eluted in linear ascending NaCl gradients. The concentration of purified proteins was determined using the absorbance at 280 nm (A280) combined with extension coefficients calculated using the ExpASy ProtParam tool (ca.expasy.org/tools/protparam.html).

Construction of His₆-SspA and Mutants in S. aureus—The sspa gene with its native promoter was amplified by PCR of genomic DNA from S. aureus RN6390 using primer aagct CCAATTGCCTCAATTCCTTTC and reverse primer tgtcgc TAGTGTAGTGTGATGATGACGTGCTTGGATTGTCTC. The primers incorporate HindIII and PstI sites (in italic lowercase), respectively, and the underlined sequence in the reverse primer incorporates a His6 tag prior to the stop codon at the 3’-end of sspa. The PCR products were cloned
into the HindIII and PstI sites of pRN5548a (10), a variant of pRN5548 (10, 20) that lacks the blaZ promoter. The resulting plasmid, pV8H, was then transferred into S. aureus SP6391sspA::erm (21) and 6390aur::lacZ. To substitute the active site serine of SspA, we first excised sspA from pV8H and ligated it into the complementary HindIII and PstI sites of pUC18. PCR-directed mutagenesis was performed on the ligated mixture using the QuickChange II site-directed mutagenesis strategy (Stratagene) together with the complementary mixture using the QuikChange II site-directed mutagenesis kit (Stratagene). The mutagenized plasmid was transformed into E. coli, and after sequencing to confirm the desired substitution, the 3’-end of sspA containing the Ser237→Ala substitution was excised by digestion with PspGI and PstI and then used to substitute for the same fragment of the wild type pV8H plasmid from S. aureus SP6391. The resulting pV8H-S237A was then transformed into SP6391 and 6390aur::lacZ genetic background. The expected nucleotide changes and integrity of the sspA gene was confirmed by nucleotide sequence analyses.

**Purification of His-Tagged SspA Variants**—His-tagged SspA variants were isolated from culture supernatant using magnetic Ni-NTA (Fe–Ni2+) beads (Qiagen) for SDS-PAGE and N-terminal sequence analysis or Ni-NTA-agarose slurry (Qiagen) for purification of larger quantities of protein. Briefly the supernatant from a late exponential phase culture (5 h) was mixed with an equal volume of ice-cold 40 mM sodium phosphate buffer, pH 7.4, containing 1 mM NaCl and 40 mM imidazole, and rocked with Ni-NTA beads for 30 min at 4°C. After washing the magnetic beads three times in phosphate buffer containing 20 mM imidazole and 500 mM NaCl, bound protein was eluted by boiling in 1× SDS-PAGE reducing buffer. Alternatively Ni-NTA-agarose slurry was mixed with culture supernatant and packed into a disposable 5-ml polycarbonate column (Pierce) followed by application of 50 ml of wash buffer and elution with 500 mM imidazole elution buffer. Samples were desalted into 20 mM Tris-Cl, pH 7.4, using either PD-10 columns (Amersham Biosciences) or Slide-A-Lyzer dialysis cassettes (Pierce).

**Purification of zSspA**—The zSspA zymogen was purified from 4-h culture supernatant of RN6390aur::lacZ harboring pV8H. Protein was precipitated from culture supernatant with 5 mM CaCl2 for Aur or without calcium for SspA. The zSspA glutamyl endopeptidase activity was measured by monitoring the release of p-nitroaniline (pNA) from Z-Phe-Leu-Glu-pNA (Bachem) at 37°C. Assays were conducted in triplicate wells of a microtiter plate in a 100-µl volume containing 40 mM Tris-HCl, pH 7.4, and 0.4 mM substrate. Absorbances were read at defined time points on a Bio-Rad model 3550 microplate reader equipped with a 405-nm filter. Stock solutions of 4-nitroaniline (Sigma) were used to create a standard of absorbance versus nmol of pNA to allow calculation of specific activity.

**Production of Antibodies**—Purified SspB zymogen, native SspA, or Aureolysin were emulsified in Freund’s complete adjuvant (Sigma) and administered by subcutaneous injection of 100 µg of protein in New Zealand White rabbits. Booster injections administered at 2-week intervals consisted of 100 µg of protein emulsified in Freund’s incomplete adjuvant (Sigma). Antibodies were affinity-purified from the respective antisera obtained after the second boost using the reagents and protocols provided with the AminoLink Plus Immobilization kit (Pierce).

**SDS-PAGE, Western Blotting, and N-terminal Sequencing**—Proteins in cell-free culture supernatant were precipitated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid. After washing with 70% ethanol, the pellets were air-dried and then solubilized in 1× SDS-PAGE sample buffer. After SDS-PAGE, proteins were either visualized by staining with Coomassie Brilliant Blue R-250 or transferred to polyvinylidene difluoride membrane (Millipore) using standard buffer and transfer conditions for Western immunoblots or CAPS transfer buffer using modified conditions (22) when required for N-terminal sequence analysis. Western blot procedures followed standard blocking and washing protocols. Primary antibodies were antigen-specific, and secondary antibody was alkaline phosphatase-conjugated AffiniPure goat anti-rabbit (heavy and light chains) IgG (Jackson ImmunoResearch Laboratories). For N-terminal sequence analysis, blotted proteins were visualized by brief staining with 0.1% Coomassie Blue in 40% methanol. The appropriate bands were excised and submitted to the Toronto Angiogenesis Research Center Proteomics Core Facility in the Sunnybrook Research Institute for mass determination.

**Enzyme Assays and Substrates**—SspA glutamyl endopeptidase activity was measured by monitoring the release of p-nitroaniline (pNA) from Z-Phe-Leu-Glu-pNA (Bachem) at 37°C. Assays were conducted in triplicate wells of a microtiter plate in a 100-µl volume containing 40 mM Tris-HCl, pH 7.4, and 0.4 mM substrate. Absorbances were read at defined time points on a Bio-Rad model 3550 microplate reader equipped with a 405-nm filter. Stock solutions of 4-nitroaniline (Sigma) were used to create a standard of absorbance versus nmol of pNA to allow calculation of specific activity.

**Production of Antibodies**—Purified SspB zymogen, native SspA, or Aureolysin were emulsified in Freund’s complete adjuvant (Sigma) and administered by subcutaneous injection of 100 µg of protein in New Zealand White rabbits. Booster injections administered at 2-week intervals consisted of 100 µg of protein emulsified in Freund’s incomplete adjuvant (Sigma). Antibodies were affinity-purified from the respective antisera obtained after the second boost using the reagents and protocols provided with the AminoLink Plus Immobilization kit (Pierce).

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**Mass Spectrometry**—Mass spectrometry was conducted at the Toronto Angiogenesis Research Center Proteomics Core Facility in the Sunnybrook Research Institute. For mass determination, purified protein samples were exchanged into 5% formic acid by ultrafiltration, then diluted 20-fold in 50% acetonitrile and 2% formic acid, and analyzed by electrospray ionization-hybrid quadrupole time of flight mass spectrometry (QStar-XL, Applied Biosystems/AB Sciex) through infusion at 6 µl·min⁻¹ flow rate. The machine was cleaned by infusing 100 µl of 50% trifluoroethanol to eliminate carryover between samples and was calibrated immediately before each sample. Spectra obtained over 3 min of steady spray were accumulated and used for molecular weight reconstruction with “Bayesian Protein Reconstruct” in the BioAnalyst 1.1.5 software package (Applied Biosystems/AB Sciex) using default settings unless otherwise indicated. For protein identification, proteins were digested with trypsin (Promega) using the in-gel digestion pro-
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FIGURE 1. Domain organization of \(z\)SspA. The signal peptide is labeled \(SP\), and the N-terminal propeptide is shaded in a gray gradient with an internal segment of predicted intrinsic disorder shaded light gray. The amino acid sequence of the N-terminal propeptide beginning at Leu\(^{10}\) is provided above the diagram with the glutamine-rich segment of predicted intrinsic disorder also shaded in light gray. The \(^{15}\)VLP segment indicates the experimentally determined N terminus of mature SspA, which contains a trypsin-like serine protease protein family domain (Tryp, Spt; Pfam PF00089). A second region of predicted intrinsic disorder and its corresponding amino acid sequence is shown at the C terminus of the protein. Additional details are provided under “Results” and “Discussion.”

RESULTS

Temporal Analysis of Zymogen Activation—The 33-kDa \(z\)SspA has a predicted 29-amino acid signal peptide followed by a small N-terminal propeptide harboring a glutamine-rich segment that is predicted to be a region of intrinsic disorder (Fig. 1). The mature protease begins at Val\(^{69}\) and has a trypsin-like serine protease domain where His\(^{119}\), Asp\(^{161}\), and Ser\(^{237}\) form the catalytic triad. This is followed at the C terminus by another predicted disordered segment of unknown function consisting mainly of Pro, Asp, and Asn. Because the 53-kDa \(z\)Aur and 40-kDa \(z\)SspB proteins have larger 20-kDa propeptides, we could determine the activation status of each protease in culture supernatant during growth of \(S. aureus\) RN6390 (Fig. 2). There was no evidence of \(z\)Aur at any time point. Mature \(z\)Aur was reactive polypeptide was evident (Fig. 3). Subsequent N-terminal sequencing established that the largest polypeptide corresponded to unprocessed \(z\)SspA, having been cleaved only at the predicted signal peptidase site ANA\(^{12}H13\). The two intermediate polypeptides had N-terminal sequences consistent with processing at QQTQ\(^{6}Q6\)SSKQQ in the predicted disordered segment and at KGGN\(^{56}L58\)KPLE, which complies with the specificity of Aur. The smallest isof orm was processed at QRE\(^{6}Q6\)HANV just proximal to Val\(^{69}\) of mature SspA. This is unexpected, we performed mass spectrometry analysis on native SspA purified from \(S. aureus\) culture supernatant (Table 2). Three mass isof orms were evident in the purified
SspA protein that by integration of peak areas in the mass spectrum were present in a 100:48:21 ratio. The most abundant corresponded to the expected mass of native SspA with an N-terminal ValAla. The next most abundant appeared to be a variant of SspA that lacked two C-terminal alanine residues. The least abundant species corresponded to the expected mass of native SspA with an N-terminal Ser237Ala active site substitution mutant polypeptide recovered from supernatant of an aur:sspA or aur:sspA strain as indicated. The arrow on the right margin points to the same SerAla and LeuAla isoforms, respectively, as defined for the left panel. Neither isoform is present when Ser237Ala zSspA is expressed in the aur strain.

FIGURE 4. Left panel, processing of wild type SspA and Ser237Ala zSspA isolated from supernatant of an aur: lacZ strain

| Source of SspA (probable isoform) | Mass | Area |
|----------------------------------|------|------|
| Native SspA (6390±a:K:mm; 18 h)  | 29,023.31 | 29,023.86 | 312,887.2 | 100 |
| 66vILP/DNPDA 256            | 28,881.15 | 28,882.55 | 150,437.3 | 48  |
| 66HANVILP/DNPDA 256        | 29,345.63 | 29,347.28 | 66,733.7 | 21  |
| His6-SspA (aur: lacZ) *       |      |      |      |      |
| Purified on Hi-Trap Ni2+ 4 *  |
| 44SSKQ  | 32,645.11 | 32,645.30 | 738,384.3 | 97  |
| 48QTPK//DNPDAAH3             | 32,214.68 | 32,214.84 | 763,269.6 | 100 |
| 54KGG (Table 2)               | 31,518.87 | 31,519.02 | 240,347.9 | 31  |
| Eluted from Fe-Ni2+ 4        |
| 44SSKQ/DNPDAH3                | 32,645.87 | 35,645.30 | 120,238.6 | 100 |
| 44SSKQ//DNPDAAH3              | 34,212.52 | 34,212.01 | 106,980.2 | 89  |
| 54KGG//DNPDAAH3,4              | 31,519.75 | 31,519.02 | 17,223.0 | 14  |

* The C terminus of SspA is modified by inclusion of a His6 (H6) tag, which is reflected in the heavier mass values.
* Culture supernatant protein was precipitated with ammonium sulfate followed by dialysis, binding on Hi-Trap Ni2+ matrix, elution, and desalting.
* Protein was captured directly from 5-h culture supernatant using Fe-Ni2+ beads followed by elution and immediate processing for mass spectrometry.
* It was necessary to reduce the signal to noise threshold value to detect this isoform.
* Absolute area is the total intensity (counts/s) integrated over the m/z range of the ions contributing to the mass information for each isoform collected over 3 min.

SspA isoforms, respectively, as defined for the left panel. Neither isoform is present when Ser237Ala zSspA is expressed in the aur strain.

Confirmation of Autocatalytic and Aur-dependent Cleavage—To determine whether the Aur-independent processing was autocatalytic, we constructed a Ser237Ala active site substitution mutant, zSSpA(S237A). When processing of zSSpA(S237A) was compared with wild type zSSpA in the sspA background, the Q→44SSKQ isoform was not evident (Fig. 4). There was also reduced processing of Leu30Ala zSSpA, accumulation of the Leu58Ala isoform, and a limited amount of mature Val69SSpA. In the aur strain (Fig. 4, right panel), zSSpA(S237A) remained unprocessed. When this unprocessed N-terminal Leu30(S237A) isoform was incubated with a mixture of the Ser44, Gln48, Lys54, and His66 self-processed isoforms purified from supernatant of the aur strain, there was no obvious conversion to smaller

| TABLE 2 | Mass values for isoforms of native SspA and self-processed His6-SspA isolated from supernatant of an aur: lacZ strain |
|---------|-------------------------------------------------------------|
| Source of SspA (probable isoform) | Mass | Area |
| Native SspA (6390±a:K:mm; 18 h) | 29,023.31 | 29,023.86 | 312,887.2 |
| 66vILP/DNPDA 256 | 28,881.15 | 28,882.55 | 150,437.3 |
| 66HANVILP/DNPDA 256 | 29,345.63 | 29,347.28 | 66,733.7 |
| His6-SspA (aur: lacZ) * | | | |
| Purified on Hi-Trap Ni2+ 4 * | | | |
| 44SSKQ | 32,645.11 | 32,645.30 | 738,384.3 |
| 48QTPK//DNPDAAH3 | 32,214.68 | 32,214.84 | 763,269.6 |
| 54KGG (Table 2) | 31,518.87 | 31,519.02 | 240,347.9 |
| Eluted from Fe-Ni2+ 4 | | | |
| 44SSKQ/DNPDAH3 | 32,645.87 | 35,645.30 | 120,238.6 |
| 44SSKQ//DNPDAAH3 | 34,212.52 | 34,212.01 | 106,980.2 |
| 54KGG//DNPDAAH3,4 | 31,519.75 | 31,519.02 | 17,223.0 |

* The C terminus of SspA is modified by inclusion of a His6 (H6) tag, which is reflected in the heavier mass values.
* Culture supernatant protein was precipitated with ammonium sulfate followed by dialysis, binding on Hi-Trap Ni2+ matrix, elution, and desalting.
* Protein was captured directly from 5-h culture supernatant using Fe-Ni2+ beads followed by elution and immediate processing for mass spectrometry.
* It was necessary to reduce the signal to noise threshold value to detect this isoform.
* Absolute area is the total intensity (counts/s) integrated over the m/z range of the ions contributing to the mass information for each isoform collected over 3 min.
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**FIGURE 5.** SDS-PAGE of Leu\(^{30}\)ZspA(S237A) (first lane) after incubation for 30 min with native SspA (A) or Aur (B) at the indicated ratios. The lane labeled Std is the mixture of zSspA intermediates recovered from culture supernatant of an sspA strain after 5 h of growth. For native SspA treatment (A) the mixtures were supplemented with 5 mM diisopropyl fluorophosphate serine protease inhibitor after the 30-min incubation and directly processed for SDS-PAGE. For the native Aur treatment (B), the Aur metalloprotease shown in the rightmost lane co-migrated with unprocessed Leu\(^{30}\)ZspA(S237A). Therefore, the samples were mixed with Fe-Ni\(^{2+}\) beads followed by extensive washing to remove native Aur, traces of which are evident in the sample that was treated at a 1:1 ratio. For C, the mixture of self-processed (QE)-zSspA polypeptides purified from supernatant of an aur culture was incubated with buffer alone (Nil), with Aur at a 1:1 ratio or with SspA at a 100:1 ratio, and the resulting monoisoform Val\(^{69}\)SspA or His\(^{66}\)SspA was recovered on magnetic beads. The lane labeled Std is the same as in A and B, and the rightmost lane contains purified native SspA (nSspA).

polypeptides over a 2-h incubation even at a direct 1:1 ratio (data not shown). Consequently the self-processed isoforms cannot promote reciprocal intermolecular cleavage of remaining zSspA at these same sites. The initial processing of zSspA at Glu\(^{65}\) is therefore exclusively intramolecular and autocatalytic.

**Processing by Aur Is Essential and Rate-limiting in Activation**—The Leu\(^{58}\) isoform could only be detected by trichloroacetic acid precipitation of culture supernatant protein or by expressing zSspA(S237A). To confirm that the Leu\(^{58}\) isoform was generated by Aur and to determine whether its transient nature was due to subsequent (i) rapid Aur-dependent processing at Val\(^{69}\), (ii) intramolecular processing at Glu\(^{65}\), or (iii) feedback from the first molecules of Val\(^{69}\)SspA to promote intermolecular processing at Glu\(^{65}\), we compared the ability of either native SspA or Aur to process the inactive monoisoform Leu\(^{30}\)ZspA(S237A). When assayed at several dilutions in the amount of native SspA starting at a direct 1:1 ratio, the Leu\(^{30}\)ZspA(S237A) was efficiently processed at Glu\(^{65}\) (Fig. 5A), whereas the specificity of Aur processing was dictated by the ratio of zSspA:Aur (Fig. 5B). At a 1:1 ratio, zSspA was processed to the Val\(^{69}\) isoform, whereas a 10:1 ratio produced mainly Leu\(^{58}\), and at 100:1 the Leu\(^{30}\)ZspA was largely unprocessed. Therefore, intermolecular processing of zSspA by mature SspA at Glu\(^{65}\) is ~10- and 100-fold more efficient than Aur-dependent processing at Leu\(^{58}\) and Val\(^{69}\), respectively. This explains the accumulation of the Leu\(^{58}\) isoform and its slow conversion to mature Val\(^{69}\) as observed in Fig. 4 when temporal processing of zSspA(S237A) was monitored.

**Comparison of His\(^{66}\) and Val\(^{69}\) Isoforms**—For a stringent comparison of the His\(^{66}\) and Val\(^{69}\) isoforms of SspA, the heterogeneous mixture of self-processed polypeptides purified from culture supernatant of an aur culture was treated in vitro with native SspA, which results in their conversion to monoisoform His\(^{66}\)SspA. Alternately the polypeptides were treated with native Aur for conversion to monoisoform Val\(^{69}\)SspA. The in vitro processed proteins where then captured on Fe-Ni\(^{2+}\) beads followed by washing to remove the native proteases and then elution and desalting. The resulting His\(^{66}\) and Val\(^{69}\) isoforms, which were indistinguishable from one another by SDS-PAGE (Fig. 5C), were then assayed relative to native SspA for their ability to convert the Leu\(^{30}\)zSspA(S237A) into smaller isoforms (Fig. 6A). Native SspA and the in vitro activated Val\(^{69}\)SspA both efficiently processed Leu\(^{30}\)zSspA(S237A), but the in vitro processed His\(^{66}\)isoform did not (Fig. 6A). The 40-kDa zSspB is also a very sensitive indicator of the status of SspA activity as we had demonstrated previously that SspA could convert zSspB to mature SspB at ratios that surpassed 500:1 zSspB:SspA (10). However, when zSspB was incubated with His\(^{66}\)SspA at a 100:1 ratio, there was no obvious conversion to mature SspB, whereas the in vitro prepared Val\(^{69}\)SspA facilitated its complete conversion to mature protease (Fig. 6B). The in vitro prepared His\(^{66}\)SspA was also inactive when tested with the small synthetic substrate Z-Phe-Leu-Glu-pNA (Fig. 6C), whereas the in vitro activated Val\(^{69}\)SspA was only 3-fold less active than native SspA.

**A Model for Active Site Blocking by His\(^{66}\)**—The similarity of SspA to the trypsin family of proteases extends to the N-terminal Val\(^{69}\) insofar as trypsin, chymotrypsin, and plasmin have hydrophobic residues at their N termini (28). In the structure of trypsin, the N-terminal Ile\(^{1}\) is buried and stabilizes the active site by hydrogen bonding and salt bridges to the side chains of Asn\(^{132}\) and Asp\(^{194}\), respectively (28–30). Although the N-terminal Val\(^{69}\) of SspA is located on one side of the primary specificity pocket, it was observed to form hydrogen bonds to the side chains of Thr\(^{32}\) and Asn\(^{261}\) (31). More importantly, the positively charged terminal NH\(_2\) group of Val\(^{69}\) was proposed...
to have an important role in coordinating negatively charged glutamate-containing substrate peptides within the active site cleft. We therefore performed modeling experiments to predict how this would be influenced by the HAN tripeptide (Fig. 7). Our model predicts that the HAN motif extends toward the active site, placing His66 adjacent to the catalytic His119. The OH group of the catalytic Ser237 lies between these two His residues and forms hydrogen bonds with His119 and His66. The model predicts that His66 occludes the active site for all substrates.

**FIGURE 7.** Model for active site blocking by His66 of the penultimate zSspA activation intermediate. The N-terminal His66 is predicted to extend into the active site, placing it opposite to the catalytic His119, with the OH group of Ser237 lying in the middle. Hydrogen bonding of catalytic Ser237 and Asp161 to the catalytic His119 that was observed in the crystal structure of mature V8 protease is shown by dotted red lines as is the predicted hydrogen bonding of Ser237 to His66 in this isoform. The NH groups on the imidazole side chains of His66 and His119 are approximately equidistant to the OH of Ser237. Consequently it is predicted that Ser237 can form hydrogen bonds with both His residues. This figure and Fig. 9 were drawn with the use of the computer graphics software package PyMol (54).

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**Evolution of SspA Activation Mechanism—S. aureus harbors the coa gene encoding coagulase, whereas all other staphylococci belong to the coagulase-negative group typified by *Staphylococcus epidermidis*, a member of the commensal microflora. An sspA paralogue is also present in two *S. epidermidis* genomes that have been sequenced (32, 33) that encodes a glutamyl endopeptidase that is 59% identical and 78% similar to SspA of *S. aureus*. However, in these situations, the gene is not associated with an operon. Therefore, there would be no requirement for the SspA paralogue to activate a downstream cysteine protease as in the case of the ssp operon of *S. aureus*. In this regard, although the mature SspA from *S. epidermidis* is well conserved with SspA of *S. aureus* (Fig. 8), this does not extend to the N-terminal propeptide. Strikingly the only conserved feature of the propeptide is the presumed metallo-protease-dependent processing site NIKP, similar to the N↓ LKP site we identified in *S. aureus*. In addition to this distinct difference in the propeptide, there is also no predicted disordered segment at the C terminus. Similar observations are noted in the genomes of *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus*, which also possess an sspA paralogue that is not associated with an operon (data not shown). One exception to date among the coagulase negative staphylococci is an equivalent of the ssp operon in *Staphylococcus warneri* (34) that duplicates the sspABC arrangement seen in *S. aureus*. In this situation, the SspA paralogue has an N-terminal propeptide and C-terminal disordered segment that closely mimics SspA of *S. aureus* (Fig. 8). An interesting difference is that the HAN motif is altered to RAN. When the 11 completed *S. aureus* genomes are compared with one another, 10 have HAN, but strain MRSA 252 has RAN. These observations suggest that the activation mechanism of SspA co-evolved with acquisition of sspB and sspC to comprise an operon. Specific features include a glutamine-rich disordered segment as the first site of autocatalytic processing and pressure to maintain a basic amino acid residue at position −3 of the propeptide, which would permit hydrogen bonding of an N terminal of the side chain to the side chain of the active site Ser237. We also note that after the first Aur-dependent processing step at Leu58, the remaining segment of the propeptide shows some resemblance to the trypsinogen activation peptide, which must be removed to activate trypsinogen precursor (29, 35–37).

**DISCUSSION**

Our study established that zSspA is activated by a unique mechanism involving sequential processing of its small N-terminal propeptide. The intermediate and final steps are strictly dependent on Aur, but maturation is initiated by intramolecu-
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lar autocatalytic cleavage at Gln\textsuperscript{43}. Although the reason for this remains to be established, it is possible that this initial processing promotes better presentation of the remaining propeptide as a substrate for Aur such that subsequent cleavage at Leu\textsuperscript{58} and Val\textsuperscript{69} occurs in a processive manner through a protein complex to liberate the first active molecules of Val\textsuperscript{69}SspA, which can then promote efficient intermolecular processing of remaining SspA at Glu\textsuperscript{65}. This produces a penultimate His\textsuperscript{66} isoform that remains inactive until the HAN tripeptide is removed by Aur-dependent cleaving at Val\textsuperscript{69}. We propose that this sequential series of intramolecular and intermolecular processing events involving both autocatalysis and Aur-dependent cleavage has evolved to minimize the likelihood of untimely activation of zSspA during protein secretion.

Mechanistically a number of factors may contribute to inhibition of the penultimate intermediate by the HAN motif, for which we must consider the relationship of SspA to the trypsin family of serine proteases, and the activation mechanism of trypsinogen. Although the structure of zSspA has not been solved, the trypsinogen and trypsin structures are 85% identical with the differences being restricted to the S1 substrate binding site and the oxyanion hole, which are unformed in trypsinogen, rendering the zymogen inactive (28, 29, 36). On removal of the short activation peptide APFDDDDK by enterokinase (35), the newly exposed N-terminal Ile\textsuperscript{16} stabilizes four previously disordered peptide segments known as the activation domain, defined by amino acids 16–19, 142–152, 184–194, and 216–223 (28). Each segment contributes one amino acid to a salt bridge and hydrogen-bonding network that stabilizes the active site. The most significant of these is a salt bridge between the terminal NH\textsubscript{3}\textsuperscript{+} group of Ile\textsuperscript{16} and the side chain of Asp\textsuperscript{194}, which is adjacent to the active site serine. This is strengthened by hydrogen bonding of Asn\textsuperscript{142} to the terminal NH\textsubscript{3}\textsuperscript{+} group of Ile\textsuperscript{16}. Similar interactions occur in SspA where the terminal NH\textsubscript{3}\textsuperscript{+} group of Val\textsuperscript{69} hydrogen bonds to side chains of Thr\textsuperscript{232} and Asn\textsuperscript{261} (31); hydrophobic interactions also occur between the Val\textsuperscript{69} side chain and the hydrocarbon moiety of Pro\textsuperscript{257}. These interactions have two important implications with respect to the maintenance of an inactive zymogen. First, it is apparent that addition of even a single amino acid to the N-terminal Val\textsuperscript{69} could perturb these forces that stabilize the active site and constitute the primary specificity pocket. Second, when the HAN motif of the penultimate intermediate is exposed, it is likely that its conformational mobility is quickly restricted due to the proposed hydrogen bonding of His\textsuperscript{66} to the active site Ser\textsuperscript{237} and the observed hydrophobic interaction of the Val\textsuperscript{69} side chain with Pro\textsuperscript{257}. Potentially this limits the surface exposure of the HAN motif, ensuring that Aur-dependent processing must take place slowly over a period of several hours as suggested in Fig. 4 where autocatalytic processing is negated by the S237A substitution.

Because zSspA undergoes autocatalytic cleavage at glutamine in the absence of Aur, its active site must be structured. Therefore, although a trypsinogen-like activation mechanism predicts that a newly exposed N-terminal hydrophobic amino acid (Val\textsuperscript{69}) would play an essential role in bringing order to the active site, our present data suggest that this is not so for SspA. Rather and as previously predicted (31), the N-terminal Val\textsuperscript{69} seems to function primarily as a scaffold for placing a terminal NH\textsubscript{3}\textsuperscript{+} group in optimal proximity to the active site where it helps to neutralize the negative charge of glutamate-containing substrate peptides. This would not be a requirement for glutamine-containing peptides, and molecular modeling of the QTQSS propeptide segment showed that Glu\textsuperscript{63} would fit nicely into the primary specificity pocket of zSspA and form a hydrogen bond with the side chain of Thr\textsuperscript{232} and Asn\textsuperscript{261} (Fig. 9). Our data can be explained by intramolecular cleavage where the propeptide threads through the active site. The glutamine-rich segment that is susceptible to autocatalytic processing is also a region of predicted intrinsic disorder, which would facilitate its ability to thread through the active site. Glutamate would not bind in the primary specificity pocket of zSspA because this would bury a negative charge and would be energetically unfavorable. This supports another mechanism for maintaining inactivity of zSspA, namely that additional amino acids beyond Val\textsuperscript{69} will occlude all substrates from the active site.

A third explanation is supported by the evolutionary conservation of a basic amino acid at the −3 position of the propeptide sequence, either histidine or arginine. Our modeling data indicate that the side chain of His\textsuperscript{66} will hydrogen bond to the catalytic OH group of Ser\textsuperscript{237} and occlude the active site. A related observation was noted in high resolution structures of the extracellular lipase BsL of Bacillus subtilis that has the same catalytic triad as trypsin (38). Two different structural conformers were observed. In one, the O\textsuperscript{\textdegree} of the catalytic Ser\textsuperscript{77} correctly formed a hydrogen bond with the N\textsuperscript{\textdegree} atom of the catalytic His\textsuperscript{155}, but in another, it formed a hydrogen bond to the sidechain atom of His\textsuperscript{76}, which is adjacent to His\textsuperscript{155} in the structure. It was proposed that this served to modulate the transition of the active site between active and inactive conformations. The His\textsuperscript{66} isoform of zSspA as well as strains with the Arg\textsuperscript{66} variant would have side chains containing an N\textsuperscript{\textdegree} atom that hydrogen bonds to the hydroxyl group of the active Ser\textsuperscript{237} side chain (Fig. 8), thus blocking the primary specificity pocket.

Contrary to this reasoning, it was reported that the mature SspA paralogue of S. warneri was active as the RANVILP iso-
form (34). However, this violates the principals of the trypsin family of serine proteases, which have a hydrophobic amino acid at the N terminus. The apparent activity of the RAN isoform in S. warneri could be due to the presence of the VILP isoform. Using mass spectrometry, we noted that native SspA purified from culture supernatant after 18 h of growth contains a significant amount of the HAVNVL isoform (Table 2), which cannot be resolved from the mature Val$^69$SspA by SDS-PAGE or chromatography. The N-terminal sequencing used to define the S. warneri isoforms may not have been sensitive enough to detect minor amounts of the active VILP isoform.

As it is evident that just three additional amino acids are sufficient to keep SspA inert, this brings into question the function of the remainder of the zSspA propeptide. Although not a recognized classification scheme, we may assign microbial serine proteases into three groups based on their propeptides: (i) those with large propeptides of known function, (ii) those with no N-terminal propeptides, and (iii) proteases with minimal propeptides. It appears that zSspA does not fit into any of these. In the first group, subtilisin of B. subtilis has an 83-amino acid propeptide that acts as an intramolecular chaperone to promote folding of the propeptide domain and to maintain the protease as an inactive precursor during protein export (39, 40). The propeptide alone is intrinsically disordered but when mixed with mature protease adopts a flower-like structure with its stem inserted into the active site (41). This dual inhibitor/chaperone paradigm is maintained by the larger 174-amino acid propeptide of α-lytic protease expressed by Lysobacter enzymogenes even though it has no obvious sequence or structural similarity to the subtilisin propeptide (42, 43). This propeptide defines the Pro_AL_Protease protein family domain (Pfam 02983), which is also found in serine proteases of the Actinomycetales family of soil bacteria, including Thermomonospora, Rarobacter, and several proteases of Streptomyces (44). Although this latter group includes a glutamic acid-specific propeptide, SpgE (45), the small N-terminal propeptide of SspA has no obvious similarity to this or any other propeptide in this family.

Proteases with no propeptides include the exofluvial toxins of S. aureus and several serine protease-like (Spl) proteins also produced by S. aureus for which the structures of each can be superposed on the Cα trace of the SspA/V8 protease (31, 46–48). The exofluvial toxins are glutamyl endopeptidases that primarily cleave a single host protein, desmoglein (49), whereas the Spl proteins are encoded by tandem gene repeats in a genomic island structure and are either very restricted in their substrate specificity or could not be demonstrated to have protease activity (46, 50). Presumably because they are restricted in their substrate specificity, there is no need for an intramolecular inhibitor. For the SplC protein, structural determination revealed that the active site is well ordered, but an essential catalytic histidine residue is pushed away from the active site by conflict with glycine residues in a flexible loop that passes over the active site. It was proposed that contact with substrate containing peptides would trigger a conformational change of the flexible loop, allowing the catalytic histidine to rotate into the active site. Studies with recombinant Spl proteins also supported the contention that even a single additional amino acid at the N terminus would be sufficient to maintain zymogen status (46).

The third family of serine proteases in this scheme is also represented in the soil bacterium Streptomyces griseus, which in addition to the Pro_AL_Protease family expresses two similar trypsin proteases, SprT and SprU (51), that have only a four-amino acid propeptide known as the activation peptide that is removed by processing at APNP ↓ VVG or APQP ↓ IVG. It is notable that the underlined N terminus of these short propeptides is identical to the N terminus of the trypsinogen activation peptide APFDGGDK ↓ IVG (35) and also that SprT, SprU, and SspA have a common property of two or three hydrophobic amino acids at the N terminus of the mature protease that is shared among the trypsin family (28). Intriguingly as shown in Fig. 8, the first Aur-dependent processing site in maturation of zSspA exposes a new N-terminal segment, LKPFLQREHAN ↓ VI, where the underlined residues are similar to the trypsinogen activation peptide (Fig. 8). Although trypsinogen activation strictly depends on enterokinase cleaving at Lys↓-Ile$^{16}$, the first molecules of active trypsin then process the same site in remaining trypsinogen through intramolecular cleavage. In a variation of this theme, activation of zSspA strictly depends on Aur processing at Leu$^{38}$ and then Val$^{69}$, but this occurs less effectively unless preceded by initial intramolecular processing at Glu$^{44}$. Autocatalytic intramolecular processing at Glu$^{43}$ also occurs after the first molecules of Val$^{69}$SspA are produced by Aur. Consequently as with trypsinogen, activation of zSspA is strictly dependent on another protease but is also assisted by autocatalytic processing.

It is apparent now that activation of zSspA most closely resembles trypsinogen with some variations. Notably although the zSspA propeptide has some similarity to the trypsinogen activation peptide, additional features place it in a class of its own. These supplementary features seem to function as a molecular fuse, which must be “burned” or processed in proper sequence for effective activation. These features together with a repetitive disordered segment of unknown function at the C terminus of zSspA are uniquely associated with S. aureus and one species of coagulase-negative Staphylococcus where SspA is in an operon and serves to activate an adjacent encoded cysteine protease. Others have found that expression and secretion of zSspB is toxic to S. aureus unless accompanied by a third gene in the ssp operon encoding a cytoplasmic protein, SspC (Staphos- tatin B), that binds to and inhibits mature SspB but reportedly has no interaction with zSspB (12, 14, 15). Our present study has described additional safety features incorporated into the zSspA propeptide that appear to have evolved to minimize the likelihood of either protease being activated prematurely during protein export.

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