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***Staphylococcus aureus* sortase A contributes to the Trojan Horse mechanism of immune defense evasion with its intrinsic resistance to Cys184 oxidation[†]**

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Abstract

Staphylococcus aureus is a Gram-positive bacterial pathogen that causes serious infections which have become increasingly difficult to treat due to antimicrobial resistance and natural virulence strategies. Bacterial sortase enzymes are important virulence factors and good targets for future antibiotic development. It has recently been shown that sortase enzymes are integral to bacterial survival of phagocytosis, an underappreciated, but vital, step in *S. aureus* pathogenesis. Of note, the reaction mechanism of sortases relies on a solvent-accessible cysteine for transpeptidation. Due to the common strategy of oxidative damage employed by professional phagocytes to kill pathogens, it is possible that this cysteine may be oxidized inside the phagosome, thereby inhibiting the enzyme. This study addresses this apparent paradox by assessing the ability of physiological reactive oxygen species, hydrogen peroxide and hypochlorite, to inhibit sortase A (SrtA) from *S. aureus*. Surprisingly, we found that SrtA is highly resistant to oxidative inhibition, both *in vitro* and *in vivo*. The mechanism of resistance to oxidative damage is likely mediated by maintaining a high reduction potential of the catalytic cysteine residue, Cys184. This is due to the unusual active site utilized by *S. aureus* SrtA, which employs a reverse protonation mechanism for transpeptidation, resulting in a high pK_a as well as reduction potential for Cys184. The results of this study suggest that *S. aureus* SrtA is able to withstand the extreme conditions encountered in the phagosome and maintain function, contributing to survival of phagocytotic killing.

Keywords

Sortase; *Staphylococcus aureus*; phagocytosis; oxidation; virulence; reverse protonation; reactive oxygen species

Gram-positive bacteria are a major cause of infectious disease, posing a serious healthcare threat. *Staphylococcus aureus* is a major pathogen, responsible for pathologies such as blood stream infections, surgical site infections, prosthesis infections, skin infections, and

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Supporting Information Paragraph: Figures S1 and S2 and Table S1. Figure S1 shows IC₅₀ results for H₂O₂ and NaOCl for SrtA. Figure S2 shows Protein A anchoring by *S. aureus* strain Newman in pH 5 and pH 9. Table S1 contains the raw data from LC/MS analysis of oxidation of SrtA by H₂O₂ and NaOCl. This material is available free of charge online at <http://pubs.acs.org>.

pneumonia(1, 2). Antibiotic resistance among pathogenic bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) is surging, with few new antibacterials expected in the near future(3). As such, new antibiotic targets are needed to combat this growing epidemic. The strategy of targeting virulence factors produced by pathogens has gained interest as a way to reduce potential resistance to new antibiotics, as well as the possibility for use in combination therapies(4). The Gram-positive bacterial extracellular transpeptidase, sortase, is an attractive target for such an approach.

Sortase enzymes are localized to the outer face of the bacterial membrane. These transpeptidases recognize secreted proteins with a cell wall sorting sequence and covalently attach these proteins to cell wall peptidoglycan substrates. This includes virulence factors known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) and pilin proteins(5, 6). Sortase A (SrtA), the “housekeeping” sortase, recognizes an LPXTG sequence in its substrates where X is any amino acid. Using a reverse protonation mechanism, the peptide bond between the threonine and glycine is cleaved by nucleophilic attack from an active site cysteine thiolate(7). The resulting thioester intermediate is resolved by a deprotonated amine of the crossbridge peptide in branched lipid II, covalently attaching the protein substrate to the cell wall precursor(8). This product is then incorporated into the peptidoglycan by transpeptidases and transglycosylases such that the anchored protein is displayed to the extracellular environment.

The tertiary structures of sortases are remarkably similar despite their highly varied substrate specificity(9). There is a conserved core structure containing the substrate binding cleft and conserved active site residues cysteine, histidine, and arginine(10). It was observed in a recent crystal structure of the *Streptococcus pyogenes* SrtA that the catalytic Cys208 in the active site (Cys184 in *S. aureus* SrtA) was oxidized to a stable sulfenic acid(11). Based on the importance of this residue for catalysis, the enzyme's extracellular location, and the fact that pathogens encounter reactive oxygen species (ROS) during the host immune response to infection, we hypothesized that this modification might bear physiological relevance. Oxidation of some proteins is a normal process that regulates enzyme activity(12, 13). However, in the case of extracellular enzymes there are no redox systems to reduce oxidized residues, implying that oxidation of SrtA is a form of damage that will inhibit the enzyme to the detriment of *S. aureus*.

It is well established that sub-populations of *S. aureus* are able to survive phagocytosis, and building evidence suggests that this is important for staphylococcal pathogenesis(14-17). Intracellular survival within nonphagocytic cells as well as professional phagocytes has been demonstrated, and this provides a vehicle for *S. aureus* to remain cloaked from the immune system and antibiotics, as well as disseminate throughout the host(14, 17, 18). However, without a functioning sortase this ability is lost(14). To investigate whether oxidative modification of SrtA is important during the clearance of an infection by phagocytes, we assessed the ability of ROS to inhibit *S. aureus* SrtA *in vitro* and *in vivo*. We determined that SrtA is highly resistant to inhibition by oxidation from hydrogen peroxide and hypochlorite, prevalent ROS in the phagosome, beyond concentrations where staphylococcal growth becomes inhibited. The combination of the high reduction potential of cysteine in the thiol form and the reverse protonation mechanism provides SrtA with a mechanism of resistance to oxidation, allowing SrtA to be active in the phagosomal environment. Accordingly, *S. aureus* SrtA function *in vivo* is not observably affected by high concentrations of ROS. The data presented here demonstrates that *S. aureus* SrtA Cys184 is intrinsically resistant to oxidation, which contributes to the ability of *S. aureus* to survive phagocytotic killing.

Experimental Procedures (Materials and Methods)

Protein and Peptide Production and Purification

Recombinant *S. aureus* SrtA_{ΔN24} was expressed and purified as previously described with minor alterations(19). An Abz-LPETGG-Dap(DNP)-NH₂ peptide substrate was synthesized and purified as previously described(20), except synthesis was performed using a CEM Liberty microwave peptide synthesizer.

Inhibition Kinetics

SrtA activity was measured using a previously developed HPLC-based assay(21). Briefly, dose-response inhibition assays (IC₅₀ determination) were performed as follows: 1 μM SrtA and various concentrations of oxidant were preincubated for 2 h in 150 mM NaCl, 5 mM CaCl₂, 300 mM Tris pH 7.5 buffer (final concentrations) at 37 °C. Oxidant concentrations were determined spectrophotometrically. NaOCl has an extinction coefficient of 350 M⁻¹ cm⁻¹ at 290 nm, and H₂O₂ has an extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm(22, 23). The reaction was initiated by addition of Abz-LPETGG-Dap(DNP)-NH₂ (1 mM final concentration) and H-(Gly)₅-OH (2 mM final concentration) to obtain a final reaction volume of 100 μL. In the case of hypochlorite, sodium bisulfite (equal concentration as NaOCl) was also added to scavenge the residual hypochlorite. The reaction was quenched after 10 min by addition of half volume of 1.2 M HCl. Reactions were run on an Agilent 1200 HPLC with a Vydac reversed-phase C₁₈ column (4.6 × 50 mm, 3 μm). Cleavage product and substrate peaks were measured using UV absorption of the DNP group (λ_{max} = 355 nm). Peaks were integrated with PeakFit v4.11 (Systat Software Inc). Reaction rates were calculated and fit in GraFit 6.0.1 (Erathicus Software Limited) to obtain an IC₅₀ value using equation 1:

$$\text{percent activity} = \frac{100}{1 + \left(\frac{[I]}{IC_{50}}\right)^s} \quad (1)$$

The time-dependent inhibition kinetics experiments to determine K_I and k_{inact} were performed as follows: SrtA and various concentrations of oxidant were preincubated at 37 °C for various amounts of time and the reaction was initiated as above by addition of Abz-LPETGG-Dap(DNP)-NH₂ and H-(Gly)₅-OH (and sodium bisulfite for hypochlorite assays). Reactions were quenched after 10 min with 1.2 M HCl and analyzed by HPLC by the above method. Reaction rates were calculated and fit in GraFit 6.0.1. Due to the non-specific and complex reactions that ROS undergo with proteins, exact kinetic mechanism determination is extremely difficult. Thus, a more general approach to curve-fitting was employed. The first equation utilized is a shifted inverse logistic function:

$$\frac{v}{v_0} = \frac{\exp(-k_{\text{obs}}(t-x))}{1 + \exp(-k_{\text{obs}}(t-x))} \quad (2)$$

Where v is the velocity of the reaction, v_0 is the velocity of the uninhibited reaction, k_{obs} is the apparent first order rate constant for the interconversion of $v_{\text{uninhibited}}$ and $v_{\text{inhibited}}$, t is preincubation time, and x is a time offset. This equation was used to account for the sigmoidal shape of time-dependent inhibition of SrtA seen with H₂O₂ and NaOCl. The origin of the lag phase seen in these experiments is likely multifaceted and due to some combination of low levels of oxidation of Cys184 prior to assay initiation, conversion of Cys184 between thiol and thiolate, conversion of NaOCl between hypochlorite and hypochlorous acid, and protein dynamics inherent in the SrtA active site. The 100 mM H₂O₂

sample was instead fit to a shifted exponential decay function because the inhibition at this concentration was too rapid to observe a lag phase and accurately fit to the above logistic function:

$$\frac{v}{v_0} = \exp^{-k_{\text{obs}}(t-x)} \quad (3)$$

Where the variables are the same as above(24). The k_{obs} values obtained for each oxidant concentration were then plotted vs. oxidant concentrations and fit in GraFit 6.0.1 to:

$$k_{\text{obs}} = \frac{k_{\text{inact}} [I]}{K_i^{\text{app}} + [I]} \quad 4$$

Where k_{inact} is the rate of inactivation of the enzyme by the inhibitor, $[I]$ is the concentration of inhibitor, and K_i^{app} is the apparent concentration of the inhibitor required to reach half maximal rate of inactivation of the enzyme(24).

***S. aureus* Minimal Inhibitory Concentration Measurement**

The minimal inhibitory concentrations (MIC) of H₂O₂ and NaOCl were determined for *S. aureus* strain Newman. Tryptic Soy Broth (TSB) cultures (5 mL) were inoculated with 50 μ L of an overnight stationary-phase culture. The cultures were grown to mid-log phase shaking at 37 °C until OD₆₀₀ = 0.7. The culture (10 μ L) was diluted into 190 μ L of TSB in a 96-well plate with final concentrations of oxidant varying from 0-10 mM. Cultures were grown at 37 °C in a humidified shaker and checked for growth at 24 and 48 h. The MIC was defined as the lowest concentration of ROS at which no growth was observed.

LC/MS Analysis of Oxidation

Recombinant SrtA_{ΔN24} was treated with excess Cleland's Reductacryl™ Reagent (Calbiochem) at 37 °C for 45 min to ensure Cys184 was fully reduced. Reductacryl™ was then removed by centrifugation. Samples were adjusted to 150 mM NaCl, 5 mM CaCl₂, 300 mM Tris pH 7.5 and 1 mM H₂O₂ or 1 mM NaOCl, and incubated at 37 °C for 2 h to oxidize Cys184. Catalase beads and equimolar NaHSO₃ were added at 37 °C for 30 min to remove excess H₂O₂ and NaOCl, respectively. Free thiols were blocked with 20 mM iodoacetamide at ambient temperature in the dark for 1 h to prevent further reaction. Samples were buffer exchanged into 50 mM NH₄CO₃ pH 8 (AmBic) and concentrations were determined by Bradford assay (Biorad) using BSA as calibration standard. 10 μ g from each sample treatment were removed and volumes were normalized with AmBic. Waters RapiGest® MS compatible surfactant was added to 0.1 % w/v final for solubilization. Promega trypsin (sequencing grade) was added at 50:1 protein:trypsin and proteins were allowed to proteolytically digest at 37 °C overnight. Trifluoroacetic acid (TFA) and acetonitrile (ACN) were added to each digest the following morning to yield 1 % TFA/2 % ACN final.

LC/MS Data Collection

Peptide digests obtained from each of the 3 treatments were analyzed using a nano-Acquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters Corp, Milford, MA). Approximately 5 ng of peptide material in 1 μ L was first trapped at 5 μ L/min for 3 min in 99.9 % water with 0.1 % v/v formic acid on a 20 μ m $\times$ 180 mm Symmetry C₁₈ column. Separations were then performed on a 75 μ m $\times$ 250 mm column with 1.7 μ m C₁₈ BEH particles (Waters) using a 60-min gradient of 5 to 40% acetonitrile with 0.1 % formic acid at a flow rate of 0.4 μ L/min and 55 °C column temperature. We conducted one data-

dependent analysis (DDA) per sample in sensitivity mode (~15,000 R_s) using a 0.6 sec MS scan followed by MS/MS acquisition on the top 3 ions with charge greater than 1. MS/MS scans for each ion used an isolation window of 2.3 Da, a maximum of 3 seconds per precursor, and dynamic exclusion for 120 seconds within 1.2 Da.

LC-MS/MS Data Interpretation

Raw LC-MS/MS data were processed in Mascot distiller v2.3.2.0 (Matrix Science, Inc) and then submitted to the Mascot v2.2 search engine. Data were searched against the NCBI nr database with *eubacteria* taxonomy with variable modifications set to carbamidomethyl (C); deamidation (NQ); single, double, and triple oxidation (C); pyroglutamic acid (N-term); and oxidation (M). Data were searched with 10 ppm precursor mass tolerance and 0.04 Da mass tolerance for product ions. The maximum number of missed cleavages was set at 2 and enzyme specificity was trypsin. Database search results and spectra have been uploaded as a Scaffold 3 file (Proteome Software, Inc) at the following link https://discovery.genome.duke.edu/express/resources/1794/1794_042011_final.sf3. Peak intensities were manually extracted from the raw data using MassLynx 4.1 (Waters) with a 20 ppm window around precursors of interest, and plots were generated within Microsoft Excel (see Table S1).

Reduction Potential Determination

Electrodes for reduction potential measurements were prepared as described previously(25). Briefly, a glassy carbon stick electrode (Bioanalytical Systems) was polished with 0.3 μ m alumina, sonicated in water and isopropanol, and primed with a dilute solution of single-walled carbon nanotubes (NanoC) in water and dihexadecylphosphate surfactant (Sigma) that increases electrode surface area, resulting in signal enhancement. Diffusional cyclic voltammetry was used to measure the reduction potential of L-cysteine. A 5 mM solution of L-cysteine in 50 mM sodium phosphate (pH 3-11) was used to fill the three-electrode electrochemical cell, utilizing Ag/AgCl as reference electrode, Pt as auxiliary electrode, and carbon nanotube-modified glassy carbon as working electrode. Electrochemical analysis was performed using a PAR 273A potentiostat/galvostat (Princeton Applied Research, Oak Ridge, TN). Buffers were degassed prior to use. Measurements were done at 4 ± 2 °C at scan rates of 50 mV/s in the oxidative direction with a scan range of 0 V to +1.3 V to observe conversion of cysteine thiol to cysteine radical. Electrode potentials were recorded vs. the reference Ag/AgCl and reduction potentials were reported relative to the standard reference of normal hydrogen electrode, +0.197 V.

For SrtA reduction potential determination, recombinant SrtA $_{\Delta N24}$ was mixed with CNTs and applied to electrodes prepared as above. SrtA $_{\Delta N24}$ has only a single cysteine, the conserved active site nucleophile, Cys184. After drying at -20 °C, electrodes were sealed with 5 % Nafion in MeOH, which does not disrupt native protein structure(26). After drying again at -20 °C, measurements were performed in a three-electrode electrochemical cell as above.

S. aureus Protein A Anchoring

S. aureus strain Newman or Δ SrtA was grown in TSB media overnight. Cultures were diluted 1:100 in TSB that was adjusted to 1 mM H_2O_2 , 1 mM NaOCl, pH 5, pH 7, or pH 9. Cultures were allowed to grow to mid-log phase, $OD_{600} = 0.6$, before harvesting. The concentration of H_2O_2 was confirmed via an Amplex Red hydrogen peroxide peroxidase assay and was not found to be significantly affected by the TSB media. Cells were pelleted, washed with PBS, and resuspended in PBS. Dilutions were plated on lysine-coated glass slides, spread, flamed, and allowed to dry. Slides were rinsed with PBS, blocked with 2 % BSA in PBS, and rinsed again with PBS. Finally, samples were incubated with 1:1000

dilution of Alexa Fluor 555 goat anti-mouse IgG (Invitrogen) in 2 % BSA for 1 h in the dark, rinsed with PBS, and allowed to dry. Slides were mounted with Dapi-Fluoromount-G (SouthernBiotech) and examined with a Zeiss Axio Imager widefield fluorescence microscope.

Results

Reactive Oxygen Species Inhibition

To assess the effects of the phagocyte oxidative burst on SrtA activity, purified *S. aureus* SrtA_{ΔN24} was examined for sensitivity to reactive oxygen species (Figure 1). The kinetic inhibition values obtained can be found in Table 1. Somewhat surprisingly, SrtA is resistant to inhibition by ROS with a K_I in the millimolar range for hydrogen peroxide and sodium hypochlorite, both found to be slow time-dependent inhibitors with k_{inact} values of 5.2×10^{-3} and $5.1 \times 10^{-2} \text{ s}^{-1}$, respectively. Additionally, the K_I value obtained for NaOCl is in the same range as its MIC of 7.5 mM for *S. aureus*, implying that *S. aureus* growth would be halted before detrimental inhibition of SrtA occurred (Table 1). Conversion of NaOCl and O_2 into NaO_3Cl could potentially contribute to inhibition seen by NaOCl. However, it was found that up to 5 mM NaO_3Cl had no effect on SrtA activity even after 30 min (data not shown), indicating that inhibition was most likely entirely due to NaOCl. The K_I for H_2O_2 is orders of magnitude higher than the MIC of 2.5 mM, as well as the micromolar concentrations that can be found in the phagosome(27). Likewise, the IC_{50} values imply that H_2O_2 is not a relevant inhibitor, while NaOCl is able to inhibit SrtA at physiological concentrations, but only after a prolonged incubation period (Figure S1, Table 1).

Cysteines can be oxidized to a number of states, including sulfenic, sulfinic, and sulfonic acid forms (Scheme 1). A recent crystal structure of *Streptococcus pyogenes* SrtA displayed a stable sulfenic acid at Cys208 (Cys184 in *S. aureus* SrtA) (Figure 2). To support our hypothesis that the inhibition of *S. aureus* SrtA is due to direct oxidation of Cys184, we incubated recombinant SrtA with 1 mM H_2O_2 or 1 mM NaOCl for 2 h and analyzed the tryptic digest by LC/MS. Sulfonic acid formation was observed at Cys184, implying oxidation at the active site cysteine can occur and is likely the cause of inhibition (Figure 3). NaOCl converted a higher fraction of Cys184 to sulfonic acid than H_2O_2 , while a control sample showed no oxidation under these conditions. This supports a low rate of oxidation of Cys184 and a model of SrtA inhibition by Cys184 oxidation in the presence of NaOCl, with H_2O_2 being a largely ineffective physiological inhibitor. No sulfenic or sulfinic acid formation was observed under these conditions. While a stable sulfenic acid modification was discovered at *S. pyogenes* SrtA Cys208(11), this was likely an artifact of crystallization, and the fully oxidized cysteine observed at *S. aureus* SrtA Cys184 in this study is expected for unprotected cysteine oxidation. Of note, we did not observe any disulfide formation, discounting the possibility of intermolecular Cys184-Cys184 dimerization artifacts during treatment or analysis.

S. aureus Protein A Anchoring

In order to examine the effect on ROS on SrtA activity *in vivo*, we investigated the ability of H_2O_2 and NaOCl to inhibit SrtA activity in *S. aureus* strain Newman. An Alexa Fluor 555 conjugated IgG was used to detect the presence or absence of protein A on the surface of *S. aureus*. NaOCl and H_2O_2 had no observable effect on protein A anchoring, supporting our hypothesis that SrtA resistance *in vitro* may be paralleled by resistance to oxidation *in vivo* during infection (Figure 4).

Reduction Potential of Cysteine

To understand why SrtA is resistant to ROS inhibition *in vitro* and *in vivo* even though Cys184 is a solvent-accessible cysteine, we measured the reduction potential. At pH 5, L-cysteine has a reduction potential of 1.18 V and SrtA Δ N24 has a reduction potential of 1.27 V (Figure 5A). The reduction potential of SrtA Δ N24 is likely in part due to Cys184, although deconvolution of the contribution to the reduction potential of different residues in SrtA is difficult. Indeed, the reduction potential of L-cysteine supports a reduction potential in this range for SrtA Δ N24 Cys184. Hydrogen peroxide has a reduction potential of 1.77 V and hypochlorous acid has a reduction potential of 1.50 V at this pH(28). Since a compound with a higher reduction potential is able to oxidize a compound with a lower reduction potential, both NaOCl and H₂O₂ should be thermodynamically capable of oxidizing cysteine at pH 5. However, inhibition assays were performed at pH 7.5, which is the pK_a of hypochlorite(29). Hypochlorite is a much less powerful oxidant than hypochlorous acid, with a reduction potential of 0.89 V(30). With that low of a reduction potential, hypochlorite would be thermodynamically unable to oxidize Cys184 of SrtA. However, inhibition assays at pH 6.5 and 8.5 showed no significant change in the IC₅₀ of hypochlorite compared to at pH 7.5, implying that this does not fully account for the slow rate of inhibition (data not shown). Accordingly, hydrogen peroxide and hypochlorous acid are both two-electron oxidants, and for this class of oxidants, the oxidation rates are more indicative of physiological oxidation potential(28). While the k_{inact} values for NaOCl and H₂O₂ inhibition are not large (Table 1), NaOCl has a k_{inact} that is ten times larger than H₂O₂. This analysis predicts that NaOCl is a more rapid oxidizer of cysteine than H₂O₂, which is consistent with published data(31, 32).

Part of the explanation of the resistance of SrtA to inhibition by oxidation may lie in the nature of cysteine. The pH dependence of the reduction potential observed in this study is expected as cysteine transitions from thiolate to thiol (Figure 5B). The rate of oxidation of a cysteine is known to depend on the protonation state of the thiol(33-35). Due to the increased reactivity of thiolates, they are more prone to oxidation than thiols. The measured reduction potentials at pH 7 were 1.13 V for L-cysteine and 1.14 for SrtA Δ N24. Free L-cysteine has a pK_a of 8.2, implying that a higher reduction potential for SrtA Cys184 is appropriate.

Discussion

Treatment of *Staphylococcus aureus* infections is complicated by issues of antimicrobial resistance and metastases in a significant portion of cases due to persistence. Persistence of *S. aureus* infections is linked to survival of intracellular subpopulations of bacteria, most notably within phagosomal compartments of phagocytes. Once intracellular, *S. aureus* is believed to undergo phenotypic switching to a small colony variant (SCV) persistent form(16, 17), which is associated with increased anchoring of fibronectin-binding proteins and clumping factors on the bacterial surface(36). These proteins are SrtA substrates, implying the requirement for an active sortase in order for a switching strategy to be effective.

Accordingly, deletion of sortase enzymes results in drastically reduced virulence in multiple Gram-positive bacterial species(37). While *S. aureus* sortase activity is already known to be important at various stages of infection, only recently has its contribution to and the importance of phagocytosis survival become apparent(14, 38). In a recent study by Kubica *et al.*, a SrtA knockout strain of *S. aureus* showed severely impaired ability to survive phagocytotic killing by macrophages(14). The anchoring of more than sixteen cell-surface substrates by SrtA in *S. aureus* appears to play a direct role in this phenomenon. Coupled with our observation of a stable Cys208 sulfenic acid in a recent crystal structure of the *Streptococcus pyogenes* SrtA enzyme (Figure 2)(11), we postulated that the interaction of

reactive oxygen species and SrtA Cys184 within the phagosome might influence the intracellular survival of *S. aureus* (Figure 6).

In this study, we investigated the effect that the hostile environment of the phagosome has on *S. aureus* SrtA activity. LC/MS analysis of ROS-treated *S. aureus* SrtA supported our hypothesis that oxidation of Cys184 can be forced to occur, confirming sulfonic acid formation at Cys184 when treated with H₂O₂ or NaOCl (Figure 3). Surprisingly, SrtA is highly resistant to inhibition by the ROS H₂O₂ and NaOCl (Table 1), with a *K_I* of 145.0 mM and 11.8 mM, respectively. The *K_I* for these ROS are significantly higher than their MIC for *S. aureus*, rendering SrtA effectively resistant to oxidation by these species at meaningful concentrations (Table 1).

In addition, inhibition by ROS is a slow reaction, with *k_{inact}* of $5.2 \times 10^{-3} \text{ s}^{-1}$ and $5.1 \times 10^{-2} \text{ s}^{-1}$ for H₂O₂ and NaOCl, respectively. *k_{inact}*/*K_I* is often used as a measure of irreversible time-dependent inhibitor potency. By this measure, NaOCl is a 120-fold better inhibitor than H₂O₂, at $4.3 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$. For comparison, these values are 4-6 orders of magnitude less potent than phenyl vinyl sulfones, irreversible inactivators of SrtA(39), while the IC₅₀ values are very similar (Figure S1, Table 1). The IC₅₀ value of 36.3 μM for NaOCl indicates that given enough time, NaOCl is capable of inhibiting SrtA at a physiologically relevant concentration. More importantly, NaOCl is the predominant ROS in the phagosome, reaching levels capable of killing *S. aureus*(27). However, *S. aureus* treated with 1 mM NaOCl showed no observable defect in SrtA activity *in vivo* (Figure 4C), while *S. aureus* treated with 7.5 mM NaOCl were unable to grow (Table 1). It is not yet clear whether phagosomal NaOCl concentrations are maintained at or beyond the timeframe required by kinetic studies to oxidize SrtA at levels that affect its function *in vivo*. In addition, we observed that H₂O₂ also had no appreciable effect on SrtA activity *in vivo* (Figure 4B). These findings are supported by LC/MS analysis, demonstrating that NaOCl was able to partially convert Cys184 to the sulfonic acid form, whereas H₂O₂ was largely unable to do so (Figure 3). Thus, we conclude that NaOCl is a possible, if inefficient, inhibitor in the phagosome, while H₂O₂ most likely is ineffectual.

In addition to the resistance SrtA displays toward oxidation, *S. aureus* has many mechanisms to deal with ROS, including catalase, alkyl hydroperoxide reductase, and superoxide dismutases to detoxify hydrogen peroxide and superoxide anion (40, 41). *S. aureus* also produces a pigment molecule, staphyloxanthin, capable of scavenging ROS(42, 43). Additionally, the thick peptidoglycan layer, especially pronounced in vancomycin-resistant strains, and the multitude of proteins in the cell wall and membrane provide many targets more susceptible to oxidation than SrtA, in essence sacrificially titrating and diluting the killing potential of ROS by reducing their concentrations within the phagosome. Collectively, these data point to the maintenance of SrtA activity in host degradative environments.

Previous oxidation studies of enzymes with solvent exposed cysteines have focused on enzymes whose functions are regulated by ROS or involved in redox homeostasis. These proteins, such as PTP1B(13) and AhpC(12, 44), are highly susceptible to oxidation but have intrinsic protection mechanisms to avoid irreversible oxidation such as forming an intramolecular sulfenyl amide or disulfides (Scheme 1). Other cysteine hydrolases (e.g. caspases) are often potently inhibited by H₂O₂, sometimes with IC₅₀ values in the low micromolar range(45). Furthermore, ROS exposure can inhibit cellular apoptosis through redox control of caspases via direct oxidation of the catalytic cysteine nucleophile(46).

In contrast, the catalytic residue Cys184 of SrtA appears to be unusually resistant to oxidation compared to related cysteine thiol-containing enzymes, even though solved

structures show Cys184 to be readily accessible to solvent(10). We believe that this resistance is partially achieved by an unusually high reduction potential of Cys184 created by the unique protein environment. The measured reduction potential at pH 7 of free L-cysteine (1.13 V) and of SrtA Δ N24 (1.14 V) are almost identical, implying that the reduction potential of Cys184 is not depressed, as is the case with active site cysteine residues in many other enzymes. While this reduction potential makes Cys184 more difficult to oxidize, it should be noted that the reduction potentials of 1.0-1.3 V of SrtA observed across the physiological pH range does not preclude oxidation by physiological oxidants, many of which have reduction potentials higher than 1.3 V. However, whereas Cys184 is thermodynamically capable of oxidation by H₂O₂ and NaOCl, it is clearly kinetically limited on meaningful physiological time scales for concentrations that do not drastically affect *S. aureus* viability.

It is interesting to note that cysteines that are easily oxidized tend to have an abnormally low pK_a. The pK_a of the catalytic cysteine of AhpC is 5.8(44), the pK_a of PTP1B is 5.6(47), and caspases appear to have pK_a around 6.5(48). This means that at physiological pH most catalytic cysteines are predominantly in the thiolate form, increasing their nucleophilicity and activity, but also making them more susceptible to oxidation(33, 34). This observation has two consequences for the current study. First, this is part of the explanation for the inverse relationship of pH and reduction potential for cysteine that predisposes cysteines to resist oxidation by ROS under normal conditions. Second, this supports our hypothesis that SrtA needs to be resistant to oxidation in order to survive in toxic host environments even though it has a solvent accessible catalytic cysteine. This is partially accomplished through the unusual reverse protonation mechanism employed by SrtA, where the catalytic cysteine (Cys184) has a pK_a of 9.4 and the conserved histidine (His120) has a pK_a of 6.2(7). Thus, Cys184 is predominantly in the thiol form and therefore has a high reduction potential (Figure 5). Incidentally, this also contributes to enzyme activity over a broad range of pH, another trait important for activity in multiple host environments including the phagosome. Indeed, *S. aureus* SrtA activity seems to be unaffected *in vivo* at pH 5 or 9 (Figure S2).

Our results provide for the first time a biochemical basis for SrtA contribution to *S. aureus* phagocytotic survival. By design, SrtA has acquired a combination of architectural and mechanistic elements that coordinately protect the enzyme from biological inhibition. Indeed, SrtA is intrinsically resistant to concentrations of ROS that approach or greatly exceed those that cause bacterial cell death *in vitro*, and *S. aureus* gains an evolutionary advantage by maintaining SrtA activity in the acidic environment of the phagosome in order to remodel the cell wall as part of an intracellular phenotypic switch. This remodeling is likely essential to the ability of *S. aureus* to persist intracellularly. It is unclear the scope to which phenotypic switching is beneficial, but nonetheless this survival behavior likely plays a role in avoidance of immune detection, protection against antibiotic exposure, and access to host cell nutrients. Certainly, many viruses such as herpes and HIV and bacteria such as *Mycobacterium tuberculosis* persist long term intracellularly within host immune defense cells, so it is not surprising that *S. aureus* may adopt a qualitatively similar persistence mechanism. With sortases like *S. aureus* SrtA playing such a critical role in Gram-positive bacterial virulence through cell wall protein anchoring of MSCRAMMs and virulence factors, resistance to phagocytotic killing, and putative phenotypic switching, this enzyme takes on additional importance as an anti-virulence antimicrobial target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Time-dependent inhibition kinetics of *S. aureus* SrtA. v/v_0 vs time graphs were fit to obtain k_{obs} values and k_{obs} vs [ROS] graphs were fit to obtain K_1 and k_{inact} . H_2O_2 concentrations were 1 mM, 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. NaOCl concentrations were 500 μ M, 1 mM, 2.5 mM, 5 mM, 7.5 mM, and 10 mM. (A) H_2O_2 kinetic data, (B) H_2O_2 k_{obs} fit, (C) NaOCl kinetic data, (D) NaOCl k_{obs} fit.

Figure 2. Active site residues (His142, Cys208, and Arg216) of *Streptococcus pyogenes* SrtA crystal structure with Cys208 oxidized to a sulfenic acid (PDB ID 3FN6). 82 × 43mm (450 × 450 DPI)

Figure 3.

LC-MS/MS analysis of *S. aureus* SrtA_{ΔN24} after oxidation by H₂O₂ and NaOCl. The percent of Cys184 oxidized to the sulfonic acid form was calculated based on the sum of all peak intensities containing free Cys 184 or triply-oxidized Cys184 for each of three treatment conditions (water, 1 mM H₂O₂, or 1 mM NaOCl for 2 h). Water showed no oxidation, H₂O₂ only 6 %, and NaOCl 63 % oxidation.

Figure 4. Analysis of Protein A anchoring in *S. aureus* strain Newman grown in TSB media at pH 7 under different conditions. Rows: (A) No ROS, (B) 1 mM H₂O₂, (C) 1 mM NaOCl, (D) *S. aureus* Δ SrtA.

Figure 5. Cysteine reduction potential. (A) Representative cyclic voltammogram of L-cysteine (blue) and SrtA Δ N24 (red) at pH 5 used to determine reduction potential. (B) Pourbaix diagram showing pH-dependence of the measured reduction potential of L-cysteine (blue $\blacktriangle$) and SrtA Δ N24 (red $\bullet$) in 50 mM phosphate buffered solution at 4 °C over pH 3-11.

Figure 6.

Model of SrtA oxidation during phagocytosis of *S. aureus*. Oxidation of SrtA (to sulfenic, sulfinic, or sulfonic acid) inside phagocytes results in inhibition of SrtA and death of *S. aureus*. To combat this, SrtA is resistant to oxidation, allowing *S. aureus* to survive within the phagocyte. 82×34mm (600 × 600 DPI)

Scheme 1. Oxidation States of a Cysteine Residue

Double arrows indicate physiologically reversible steps. Sulfinic acid formation has only been found to be reversible in a few specific cases when catalyzed by the enzyme sulfiredoxin(49). Disulfide formation is employed by enzymes like AhpC and sulfenyl amide formation by enzymes like PTP1B to protect the enzymes from irreversible oxidation.

Table 1

Inhibition Kinetics for *S. aureus* SrtA and Minimal Inhibitory Concentrations for *S. aureus* of H₂O₂ and NaOCl.

	K_I (mM)	k_{inact} (s ⁻¹)	k_{inact}/K_I (M ⁻¹ s ⁻¹)	IC ₅₀ (mM)	MIC (mM)
H ₂ O ₂	145.0 ± 74.2	(5.2 ± 1.7) × 10 ⁻³	(3.5 ± 2.3) × 10 ⁻⁸	4.3 ± 0.3	2.5
NaOCl	11.8 ± 3.1	(5.1 ± 0.8) × 10 ⁻²	(4.3 ± 2.7) × 10 ⁻⁶	(3.6 ± 0.3) × 10 ⁻²	7.5