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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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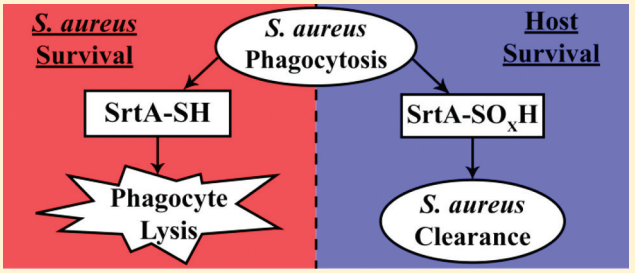
Supporting Information

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 under-appreciated, but vital, step in *S. aureus* pathogenesis. Of note, the reaction mechanism of sortases relies on a solvent-accessible cysteine for transpeptidation. Because of 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.



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 bloodstream infections, surgical site infections, prosthesis infections, skin infections, and 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 cross-bridge 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] On the basis of the importance of this residue for catalysis, the enzyme's extracellular location, and the fact

Received: June 1, 2011
Revised: July 6, 2011
Published: August 3, 2011

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 subpopulations 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

Protein and Peptide Production and Purification. Recombinant *S. aureus* $SrtA_{\Delta N24}$ was expressed and purified as previously described with minor alterations.[19] An Abz-LPETGG-Dap(DNP)-NH_2 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 (IC_{50} determination) were performed as follows: 1 μM SrtA and various concentrations of oxidant were preincubated for 2 h in 150 mM NaCl, 5 mM $CaCl_2$, 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 H_2O_2 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)-NH_2 (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 C_{18} 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 IC_{50} value using eq 1:

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

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)-NH_2 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. Because of the nonspecific 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:

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

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 $v_{uninhibited}$ and $v_{inhibited}$, 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 H_2O_2 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 H_2O_2 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:

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

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

$$k_{obs} = \frac{k_{inact}[I]}{K_I^{app} + [I]} \quad (4)$$

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 H_2O_2 and NaOCl were determined for *S. aureus* strain Newman. Tryptic Soy Broth (TSB) cultures (5 mL) were inoculated with 50 μL of an overnight stationary-phase culture. The cultures were grown to mid-log phase shaking at 37 °C until OD_{600} = 0.7. The culture (10 μL) was diluted into 190 μL of TSB in a 96-well plate with final concentrations of oxidant varying from 0 to 10 mM. Cultures were grown at 37 °C in a humidified shaker and checked for growth at 24 and 48 h. The MIC was

dx.doi.org/10.1021/bi200844h | *Biochemistry* 2011, 50, 7591−7599

defined as the lowest concentration of ROS at which no growth was observed.

LC/MS Analysis of Oxidation. Recombinant $SrtA_{\Delta N24}$ 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 $CaCl_2$, 300 mM Tris pH 7.5, and 1 mM H_2O_2 or 1 mM NaOCl and incubated at 37 °C for 2 h to oxidize Cys184. Catalase beads and equimolar $NaHSO_3$ were added at 37 °C for 30 min to remove excess H_2O_2 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 NH_4CO_3 pH 8 (AmBic), and concentrations were determined by Bradford assay (Biorad) using BSA as calibration standard. 10 μg from each sample treatment was 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 three treatments were analyzed using a nanoAcquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters Corp, Milford, MA). Approximately 5 ng of peptide material in 1 μL was first trapped at 5 μL/min for 3 min in 99.9% water with 0.1% v/v formic acid on a 20 μm × 180 mm Symmetry C_{18} column. Separations were then performed on a 75 μm × 250 mm column with 1.7 μm C_{18} BEH particles (Waters) using a 60 min gradient of 5−40% acetonitrile with 0.1% formic acid at a flow rate of 0.4 μL/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 s MS scan followed by MS/MS acquisition on the top three ions with charge greater than 1. MS/MS scans for each ion used an isolation window of 2.3 Da, a maximum of 3 s per precursor, and dynamic exclusion for 120 s 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 NCBInr 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 μm alumina, sonicated in water and isopropanol, and primed with a dilute solution of single-walled carbon nanotubes (CNT) (NanoC) in water and dihexadecyl phosphate 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/galvastat (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 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 ΔSrtA 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 antimouse 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* $SrtA_{\Delta N24}$ 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^{app} 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 5.1×10^{-2} s^{-1}, respectively. Additionally, the K_I^{app} 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

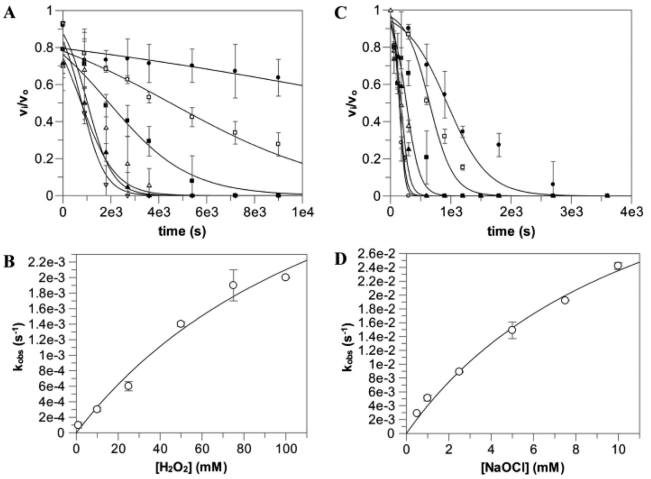
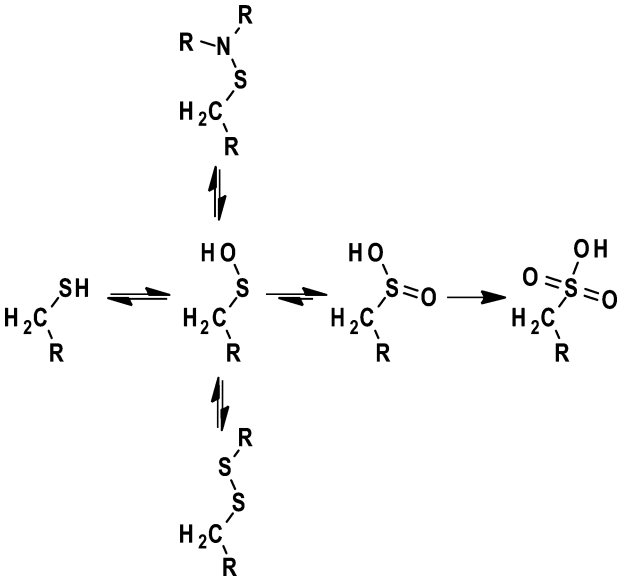


Figure 1. Time-dependent inhibition kinetics of *S. aureus* SrtA. v_i/v_0 vs time graphs were fit to obtain k_{obs} values and k_{obs} vs [ROS] graphs were fit to obtain K_I^{app} and k_{inact}. H_2O_2 concentrations were 1 mM, 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. NaOCl concentrations were 500 μM, 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.

activity even after 30 min (data not shown), indicating that inhibition was most likely entirely due to NaOCl. The K_I^{app} 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 and 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

Scheme 1. Oxidation States of a Cysteine Residue[a]



[a]Double arrows indicate physiogically 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.

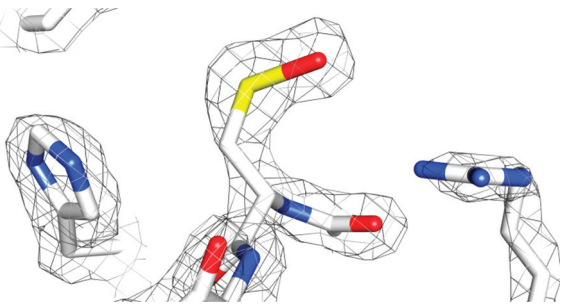


Figure 2. Active site residues (His142, Cys208, and Arg216) of *Streptococcus pyogenes* SrtA crystal structure with Cys208 oxidized to a sulfenic acid (PDB ID 3FN6).

of intermolecular Cys184-Cys184 dimerization artifacts during treatment or analysis.

***S. aureus* Protein A Anchoring.** In order to examine the effect of 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).

Table 1. Inhibition Kinetics for *S. aureus* SrtA and Minimal Inhibitory Concentrations for *S. aureus* of H_2O_2 and NaOCl

	K_I^{app} (mM)	k_{inact} (s^{-1})	k_{inact}/K_I^{app} (M^{-1} s^{-1})	IC_{50} (mM)	MIC (mM)
H_2O_2	145.0 ± 74.2	$(5.2 \pm 1.7) \times 10^{-3}$	$(3.5 \pm 2.3) \times 10^{-8}$	4.3 ± 0.3	2.5
NaOCl	11.8 ± 3.1	$(5.1 \pm 0.8) \times 10^{-2}$	$(4.3 \pm 2.7) \times 10^{-6}$	$(3.6 \pm 0.3) \times 10^{-2}$	7.5

dx.doi.org/10.1021/bi200844h | *Biochemistry* 2011, 50, 7591–7599

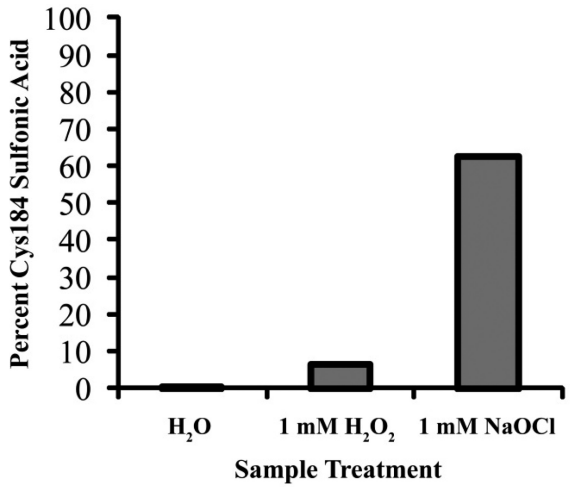


Figure 3. LC-MS/MS analysis of *S. aureus* $SrtA_{\Delta N24}$ after oxidation by H_2O_2 and NaOCl. The percent of Cys184 oxidized to the sulfonic acid form was calculated based on the sum of all peak intensities containing free Cys 184 or triply oxidized Cys184 for each of three treatment conditions (water, 1 mM H_2O_2, or 1 mM NaOCl for 2 h). Water showed no oxidation, H_2O_2 only 6%, and NaOCl 63% oxidation.

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 $SrtA_{\Delta N24}$ has a reduction potential of 1.27 V (Figure 5A). The reduction potential of $SrtA_{\Delta N24}$ 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 $SrtA_{\Delta N24}$ 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 H_2O_2 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 IC_{50} 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 H_2O_2 inhibition are not large (Table 1), NaOCl has a k_{inact} that is 10 times larger than H_2O_2. This analysis predicts that NaOCl is a more rapid oxidizer of cysteine than H_2O_2, 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] Because of 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 $SrtA_{\Delta N24}$. 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 16 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 H_2O_2 or NaOCl (Figure 3). Surprisingly, SrtA is highly resistant to inhibition by the ROS H_2O_2 and NaOCl (Table 1), with a K_I^{app} of 145.0 and 11.8 mM, respectively. The K_I^{app} 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 5.2×10^{-3} and 5.1×10^{-2} s^{-1} for H_2O_2 and NaOCl, respectively. k_{inact}/K_I^{app} is often used as a measure of irreversible time-dependent inhibitor potency. By this measure, NaOCl is a 120-fold better inhibitor than H_2O_2, at 4.3×10^{-6} M^{-1} 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 IC_{50} values are very similar (Figure S1 and Table 1). The IC_{50} 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

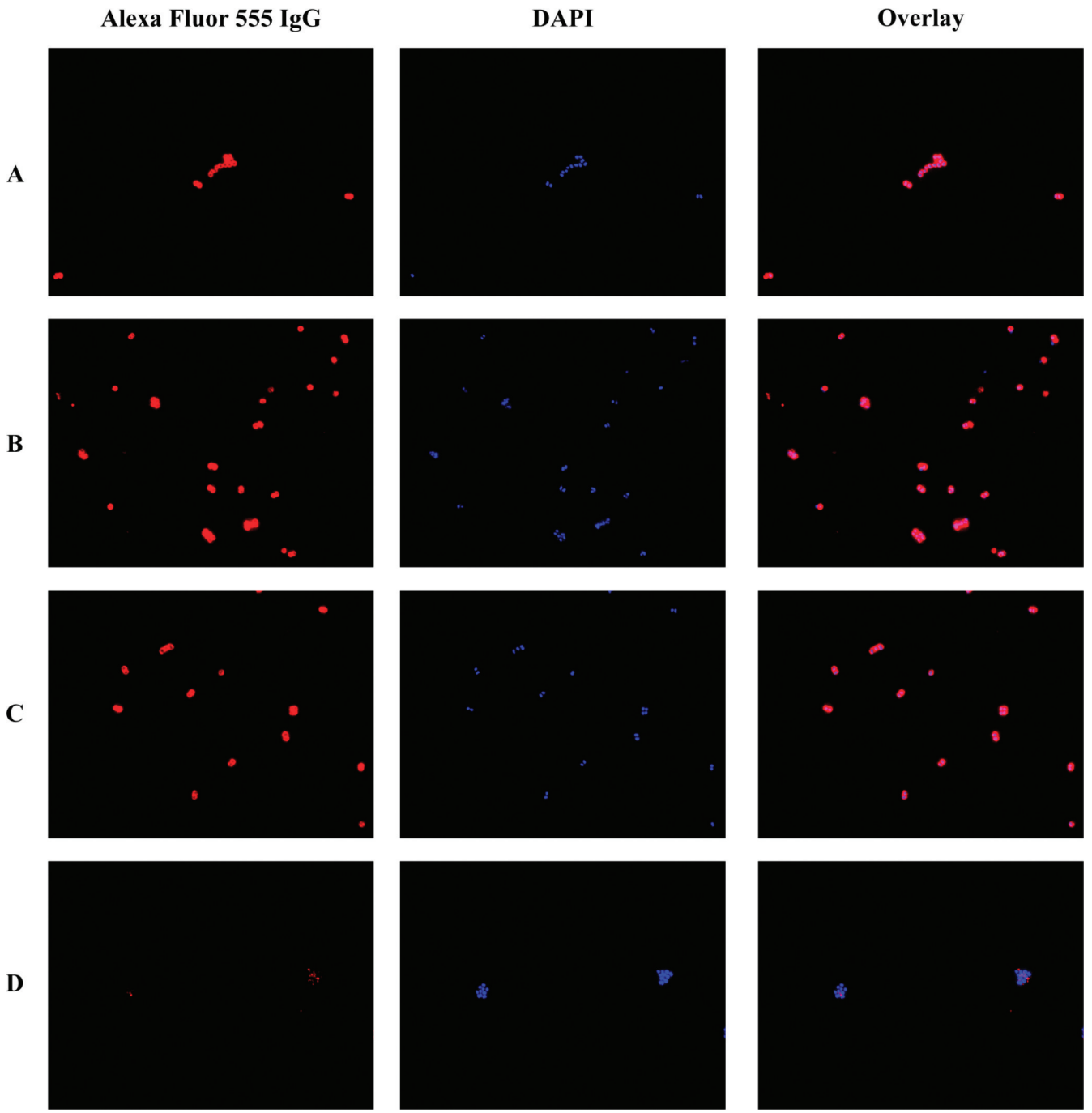


Figure 4. Analysis of Protein A anchoring in *S. aureus* strain Newman grown in TSB media at pH 7 under different conditions. Rows: (A) No ROS, (B) 1 mM H_2O_2, (C) 1 mM NaOCl, (D) *S. aureus* ΔSrtA.

concentrations are maintained at or beyond the time frame required by kinetic studies to oxidize SrtA at levels that affect its function *in vivo*. In addition, we observed that H_2O_2 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 H_2O_2 was largely unable to do so (Figure 3). Thus, we conclude that NaOCl is a possible, if inefficient, inhibitor in the phagosome, while H_2O_2 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

dx.doi.org/10.1021/bi200844h | *Biochemistry* 2011, 50, 7591–7599

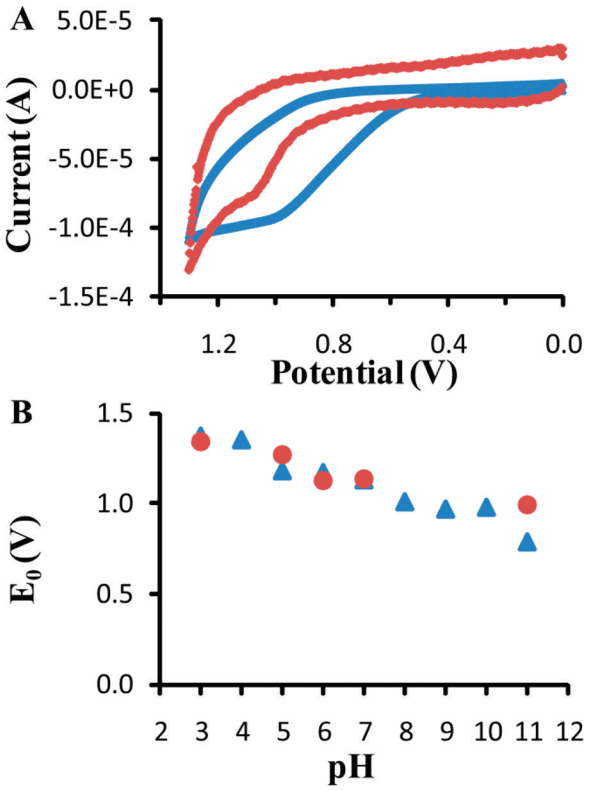


Figure 5. Cysteine reduction potential. (A) Representative cyclic voltammogram of L-cysteine (blue) and $SrtA_{\Delta N24}$ (red) at pH 5 used to determine reduction potential. (B) Pourbaix diagram showing pH dependence of the measured reduction potential of L-cysteine (blue ▲) and $SrtA_{\Delta N24}$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

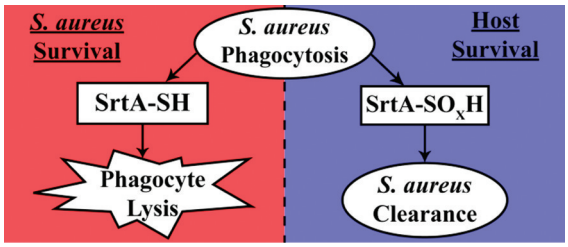


Figure 6. Model of SrtA oxidation during phagocytosis of *S. aureus*. Oxidation of SrtA (to sulfenic, sulfinic, or sulfonic acid) inside phagocytes results in inhibition of SrtA and death of *S. aureus*. To combat this, SrtA is resistant to oxidation, allowing *S. aureus* to survive within the phagocyte.

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 H_2O_2, sometimes with IC_{50} 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 $SrtA_{\Delta N24}$ (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 H_2O_2 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, viruses such as 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,

dx.doi.org/10.1021/bi200844h | *Biochemistry* 2011, 50, 7591–7599

resistance to phagocytotic killing, and putative phenotypic switching, this enzyme takes on additional importance as an antivirulence antimicrobial target.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 showing IC_{50} results for H_2O_2 and NaOCl for SrtA; Figure S2 showing Protein A anchoring by *S. aureus* strain Newman in pH 5 and pH 9; Table S1 giving raw data from LC/MS analysis of oxidation of SrtA by H_2O_2 and NaOCl. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported, in whole or in part, by National Institutes of Health Grant AI46611.

■ ACKNOWLEDGMENTS

We thank T. Prest for technical assistance with microscopy, Dr. O. Schneewind for the gift of the *S. aureus* ΔSrtA strain, and E. J. Soderblom and McCafferty lab members for critical comments and advice during preparation of this manuscript.

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