Sortase from Staphylococcus aureus Does Not Contain a Thiolate-Imidazolium Ion Pair in Its Active Site*

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Many surface proteins are anchored to the cell wall by the action of sortase enzymes, a recently discovered family of cysteine transpeptidases. As the surface proteins of human pathogens are frequently required for virulence, the sortase-mediated anchoring reaction represents a potential target for new anti-infective agents. It has been suggested that the sortase from Staphylococcus aureus (SrtA), may use a similar catalytic strategy as the papain cysteine proteases, holding its Cys184 side chain in an active configuration through a thiolate-imidazolium ion interaction with residue His120. To investigate the mechanism of transpeptidation, we have synthesized a peptidyl-vinyl sulfone substrate mimic that irreversibly inhibits SrtA. Through the study of the pH dependence of SrtA inhibition and NMR, we have estimated the pKₐs of the active site thiol (Cys184) and imidazole (His120) to be −9.4 and 7.0, respectively. These measurements are inconsistent with the existence of a thiolate-imidazolium ion pair and suggest a general base catalysis mechanism during transpeptidation.

This catalytic dyad is reminiscent of the active sites of the papain cysteine proteases (Cys185-His159-Asn175, papain numbering) (19, 20), suggesting that they are mechanistically related (21). In this model of SrtA function, the imidazole ring of His120 promotes the formation of the Cys184 thiolate, which then nucleophilically attacks the carbonyl carbon at the scissile Thr-Gly peptide bond in the LPXGTG motif (22). After covalent linkage via a thioacetyl bond to the threonine carbonyl group, the incoming amine of the cell wall precursor lipid II (23, 24) may then be deprotonated by His120 for attack on the covalent intermediate (16, 18).

Previous work left unresolved how His120 activates Cys184. In the papain cysteine proteases, the cysteine side chain is held in an active configuration through a thiolate-imidazolium ion interaction with the histidine (25). However, in the NMR structure of SrtA solved in the absence of its substrates, the side chains of Cys184 and His120 do not interact, arguing against the presence of an ion pair (16). To resolve this issue, and as a first step toward the design of a therapeutically useful anti-infective agent, we have synthesized a peptidyl-vinyl sulfone substrate mimic that inhibits SrtA. The pH dependence of SrtA inhibition and NMR studies preclude the presence of an ion pair in the active site, because His120 and Cys184 have pKₐ values of 7.0, and −9.4, respectively.

EXPERIMENTAL PROCEDURES

Reagents—Residues 60–206 of wild-type sortase (SrtAₐₙₐₑₐ) and a single amino acid mutant of the protein containing a cysteine to alanine substitution at position 184 (C184A/SrtAₐₙₐₑₐ) were overexpressed from plasmids pSRTA and pHRT45, respectively (16, 18). The expression, uniform isotopic labeling (where applicable), and purification of SrtAₐₙₐₑₐ and C184A/SrtAₐₙₐₑₐ have been described previously (16). The fluorescent substrate peptide d-QALPETGEE-e (where d is dabcyl (4-((4-dimethylamino)phenyl)azo)-benzoyl-) and e is EDANS ((2-aminoethyl)-amino)naphththlene-1-sulfonyl-)) was purchased from Synpep (Dublin, CA) and purified by HPLC. Reagents for the synthesis of the vinyl sulfone inhibitor were purchased from Aldrich.

Synthesis of Vinyl Sulfone Inhibitor—The vinyl sulfone inhibitor was synthesized using solution phase methodology (see Fig. 1). The Leu-Pro-Ala tripeptide was synthesized by standard amino acid coupling chemistry using 1-[3-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-(dimethylamino)pyridine (DMAP), starting with the carboxbenzoxyl-protected amino acid Cbz-Leu-OH and N-t-butyloxycarbonyl (N-t-Boc) methyl esters of alanine and proline. t-3-Threone was fully protected as the N-t-Boc-threone methyl ester with the alcohol protected as the t-butyldiphenylsilyl ether (2) in three steps. The ester was reduced with diisobutylaluminum hydride (DIBAL-H) to the aldehyde, which was immediately reacted without purification with diethyl phenylsulfonylmethylphosphonate (3) to give the desired vinyl sulfone functionality (4) in good yield. Removal of the Boc group with trifluoroacetic acid followed by coupling the amine with the Cbz-pro-
Fig. 1. A, molecular structure of the peptide-tert-vinyl sulfone inhibitor Cbz-Leu-Pro-Ala-Thr-SO2Ph. B, strategy for synthesis of vinyl sulfone inhibitor. 3-Threonine was fully protected as the N-t-Boc-threonine methyl ester, with the alcohol protected as the t-butyldiphenylsilyl ether (2), in three steps. The ester was reduced with diisobutyraluminum hydride (DIBAL-H) to the aldehyde, which was immediately reacted without purification with diethyl phenylsulfonylmethylphosphonate (3) to give the desired vinyl sulfone functionlity (4). Removal of the Boc group with trifluoroacetic acid (TFA) followed by coupling the amine with the Cbz-protected tripeptide (Cbz-Leu-Pro-Ala) gave the desired tetrapeptide. Removal of the t-butyldiphenylsilyl group with HF gave the vinyl sulfone inhibitor (1). OTBDPS, t-butyldiphenylsilyl; TBDPSCI, t-butyllorchlorophenylsilane; DMAP, 4-(dimethylamino)pyridine; EDCI, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

**RESULTS AND DISCUSSION**

To characterize the active site His120-Cys184 dyad, we synthesized an irreversible inhibitor that consisted of the substrate recognition motif of SrtA (-Leu-Pro-X-Thr-Gly-, where X

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**A.**

![Chemical structure](image)

**B.**

![Chemical reactions](image)
is any amino acid) but replaced the scissile Thr-Gly amide bond with a vinyl sulfone group (C = C-SO$_2$Ph) (Fig. 1A). Alanine was placed at the X position to facilitate the synthesis, and the vinyl sulfone group was used because it has previously been shown to covalently modify the active site thiol in cysteine proteases (30–34). This is relevant because mutagenesis studies have demonstrated the catalytic importance of SrtA residue Cys$^{184}$ (18), and several sulfhydryl-directed reagents block the activity of SrtA in vitro (17, 18).

The efficacy of the vinyl sulfone compound was tested in vitro by determining how it altered the SrtA-catalyzed hydrolysis of an internally quenched fluorescent substrate analogue (d-Gln-Ala-Leu-Pro-Glu-Thr-Gly-Glu-Glu-e). Hydrolysis progress curves were generated by monitoring the increase in fluorescence that accompanies the cleavage of the substrate (18, 26).

The curves were consistent with the vinyl sulfone compound acting as an irreversible inhibitor of SrtA (Fig. 2A) (35). Typical progress curves of the reaction in the presence of 100, 300, and 600 μM inhibitor are shown in Fig. 2B. Fits of these data to Equation 1 by non-linear regression analysis determined the first-order rate constant of inactivation ($k_i$) and the dissociation constant of inhibitor binding ($K_i$). At pH 7, the $K_i$ of the inhibitor is 9 × 10$^{-6}$ M, which is comparable with the measured $K_i$ of SrtA of 3 × 10$^{-6}$ M for the fluorogenic substrate analogue (data not shown). The rate constant of inactivation ($k_i$) of the vinyl sulfone inhibitor is 4 × 10$^{-4}$ min$^{-1}$. To date, only two other irreversible SrtA inhibitors have been characterized. Both contain the Cbz-Leu-Pro-Ala-Thr sorting signal mimic of the vinyl sulfone compound but utilize different reactive groups (diazomethane and chloromethane) (26). The first-order rate constants of the peptidyl-diazomethane (5.8 × 10$^{-3}$ min$^{-1}$) and -chloromethane (1.1 × 10$^{-2}$ min$^{-1}$) SrtA inhibitors are larger than the vinyl sulfone $k_i$ by 10- and 20-fold, respectively (26). This difference in reactivity is consistent with the higher electrophilicity of chloro- and diazomethane reactive groups and has been observed in inhibition studies of other cysteine proteases (36). For example, diazomethane and chloromethane inhibitors of human cathepsin L have second-order rate constants up to 100-fold higher than vinyl sulfone inhibitors (30, 36). As the transpeptidation reaction of SrtA is extremely poor in vitro ($k_{cat}/K_m = 20.6$ μM s$^{-1}$) it probably does not reflect the efficiency of the enzyme in vivo, where it must complete the anchoring of surface proteins within the doubling time of the bacterium. The development of a quantitative in vivo assay for inhibition will be necessary to evaluate the efficacy of peptidyl inhibitors of sortase as anti-infective agents.

Because the vinyl sulfone warhead of the peptide inhibitor is expected to be most reactive toward cysteine thiolates, and...
incubated with a 20-fold molar excess of inhibitor for 20 h, and the reaction products were separated by HPLC. Fig. 3, A–C shows the results of incubating SrtA with the inhibitor at pH 9, 7, and 6, respectively. Two inhibitor-modified SrtA species elute on a C18 reverse phase HPLC column (peaks 1 and 2), and mass spectrometry indicates that both peaks contain SrtA covalently modified by a single inhibitor molecule. At pH values of 6 and 7, little modification occurs within 20 h with a slight excess of species 2 being produced. However, at pH 9.0, nearly all of the SrtA protein is converted to species 1 (Fig. 3A). An analysis of the pH dependence of modification indicates that the production of species 1 is most efficient at pH values near 9, whereas maximal production of the minor species 2 occurs at pH 7 (data not shown). Because the enzyme is maximally inhibited by the vinyl sulfone compound at high pH values (Fig. 2C), where species 1 is almost exclusively present, the data strongly suggest that species 1 corresponds to the inhibitor-SrtA complex that forms during the inactivation.

To investigate whether an imidazolium ion is present in the active site of SrtA, NMR was used to determine the pKa of the His120 side chain. Because the chemical shifts of atoms within the imidazole are expected to be sensitive to the ionization state of the side chain, a series of 1H,13C heteronuclear single quantum correlation spectra were recorded using a 13C- and 15N-enriched sample of SrtA, and the chemical shifts of the 1H-1 atom of His120 were recorded at pH values 4.5 and 10. Fig. 4 shows a plot of the chemical shift of the 1H-1 atom as a function of pH (a similar curve was obtained for the 1H-2 atom). A fit of the titration data to Equation 2 (see “Experimental Procedures”) indicates that the His120 side chain has a pKa of 7.0 ± 0.1. The similarly measured pKa values of the cysteine 184 is 9.4.

To confirm that the pH-dependent inhibition of SrtA results from the covalent modification of Cys184, we tracked the modification reaction by chromatography. The SrtA protein was
thiol would be more acidic and the histidine imidazole would be more basic than their normal values of 7.5 and 6.0, respectively (39). For example, the anomalous $pK_a$ values of 3.3 (for the Cys$^{25}$ thiol) and 8.5 (for the His$^{159}$ imidazolium) support the existence of a thiolate-imidazolium ion pair in papain (40, 41). In SrtA, however, our investigation argues against the use of a thiolate-imidazolium ion pair in the reaction mechanism.

The pH dependence of modification of Cys$^{184}$ by the vinyl sulfone inhibitor (Fig. 2C) shows a dramatic increase in the first-order rate of inactivation as the pH is raised from 7.5 to 10 (at pH 10 the inhibitor is 20 times more reactive than at pH 7.5). Because the inhibitor is expected to be more reactive toward a thiolate ion, the pH dependence of inhibition can be attributed to the deprotonation of Cys$^{184}$. These data argue against the presence of an ion pair in the active site, because the $pK_a$ of Cys$^{184}$ is estimated to be $-9.4$.

NMR studies of the His$^{120}$ side chain have measured its $pK_a$ at 7.0 in the wild-type protein, which is inconsistent with the presence of an imidazolium cation at neutral pH. Moreover, our finding that the removal of the Cys$^{184}$ side chain (C$^{1184}$SrtA$^{1369}$) has only a modest effect on the ionization state of His$^{120}$ side chain argues against the presence of an ion pair, because in the papain system the $pK_a$ of His$^{120}$ is lowered by 4.5 pH units upon the methyllithiation of the Cys$^{25}$ (25).

Although the thiolate-imidazolium ion pair is a common catalytic entity of cysteine proteases, it is not universal (42, 43). The absence of an ion pair in SrtA suggests its catalytic mechanism may be similar to the viral 3C proteases (picornains), a structurally and mechanistically distinct group of cysteine proteases that perform general base catalysis (reviewed in Refs. 36 and 44). The crystal structures of the picornains of hepatitis A (45), rhinovirus (46), and poliovirus (47) show a similarity of three-dimensional structures and catalytic mechanisms to the serine proteases of the trypsin/chymotrypsin family and may be evolutionarily related (36, 43, 44). The pH-dependent alkylation of the active site cysteine of poliovirus protease 3C with iodoacetamide has measured its $pK_a$ at 8.86 (42), which is similar to the estimated $pK_a$ of the SrtA thiol. This is consistent with a reaction mechanism in which the cysteine nucleophile is uncharged at physiological pH, and the histidine functions as a general base.

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