Many virulence factors in Gram-positive bacteria are covalently anchored to the cell-wall peptidoglycan by sortase enzymes, a group of widely distributed cysteine transpeptidases. The **Staphylococcus aureus** Sortase A protein (SrtA) is the archetypal member of the Sortase family and is activated by Ca$^{2+}$, an adaptation that may facilitate host colonization as elevated concentrations of this ion are encountered in human tissue. Here we show that a single Ca$^{2+}$ ion bound to an ordered pocket on SrtA allosterically activates catalysis by modulating both the structure and dynamics of a large active site loop. Detailed nitrogen-15 relaxation measurements indicate that Ca$^{2+}$ may facilitate the adaptive recognition of the substrate by inducing slow micro- to millisecond time-scale dynamics in the active site. Interestingly, relaxation compensated Carr-Purcell-Meiboom-Gill experiments suggest that the time scale of these motions is directly correlated with ion binding. The results of site-directed mutagenesis indicate that this motional coupling is mediated by the side chain of Glu-171, which is positioned within the $\beta6/\beta7$ loop and shown to contribute to Ca$^{2+}$ binding. The available structural and dynamics data are compatible with a loop closure model of Ca$^{2+}$ activation, in which the $\beta6/\beta7$ loop fluctuates between a binding competent closed form that is stabilized by Ca$^{2+}$, and an open, highly flexible state that removes key substrate contacting residues from the active site.

Surface proteins on bacteria are frequently virulence factors, promoting bacterial adhesion, resistance to phagocytic killing, and host cell invasion during infection. In Gram-positive bacteria these proteins are often covalently anchored to the cell wall by sortase enzymes, a family of novel cysteine transpeptidases (1–3). The sortase A protein (SrtA)$^2$ from **Staphylococcus aureus** has been characterized extensively (4) and anchors proteins bearing a cell wall sorting signal that consists of a conserved LP$\text{XG}$ motif (where $X$ is any amino acid), a hydrophobic domain, and a tail of mostly positively charged residues (4–6). SrtA cleaves in between the threonine and glycine of the LPXG motif (7) and catalyzes the formation of a peptide bond between the carboxyl-group of the threonine and the amine-group of the cell-wall precursor lipid II (7–9). The lipid II-linked protein is then incorporated into the peptidoglycan of the cell wall via the transglycosylation and transpeptidation reactions of bacterial cell-wall synthesis. Sortases represent an attractive target for new anti-infective agents, because they are widely distributed among a variety of bacterial pathogens (10, 11) (e.g. *Bacillus anthracis*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*), and have been shown to be required for virulence (12–16).

The catalytic domain of SrtA (SrtA$_{\text{N599}}$, residues 60–206) adopts a conserved eight-stranded $\beta$-barrel fold (17, 18). The active site is organized around the catalytically essential side chain of Cys-184, whose thiolate nucleophilically attacks the threonyl carbonyl carbon within the LPXG sorting signal, forming a thioester linkage between the enzyme and substrate (19). In addition to Cys-184, the hydrophilic side chains of His-120 and Arg-197 are absolutely required for catalysis (20–22). These residues likely participate in general acid/base catalysis, and one of them must activate the thiol for nucleophilic attack, because it is protonated at neutral pH (23). The indole ring of Trp-194 partially shields the cysteine thiol from the solvent, and its mutation to alanine reduces enzyme activity 4-fold through an unknown mechanism (20). Using NMR and crystallography, the LPXG sorting signal binding site has recently been localized to a surface formed by strands $\beta4$ and $\beta7$, and to a proximal loop that connects strands $\beta6$ to $\beta7$ (the $\beta6/\beta7$ loop) (18, 22). Substrate binding may occur through an induced-fit mechanism involving conformational changes in the $\beta6/\beta7$ loop, because it is disordered in the absence of the sorting signal substrate (17, 18).

Ca$^{2+}$ stimulates the activity of SrtA$_{\text{N599}}$ in vitro (17) and may enable *S. aureus* to increase the rate of surface protein anchoring as it encounters elevated concentrations of this ion at sites of infection. Because many surface proteins function as virulence factors, the stimulatory effect of Ca$^{2+}$ likely plays an important role in the infection process. Previously we showed that Ca$^{2+}$ bound to an ordered pocket positioned distal to the active site (hereafter referred to as the $\beta3/\beta4$ pocket) (17). However, this work did not reveal how Ca$^{2+}$ stimulated enzyme activity. Here we show using enzyme kinetic and detailed NMR nitrogen-15 measurements that a single Ca$^{2+}$ ion bound to the $\beta3/\beta4$ pocket promotes substrate binding. We provide evidence that ion binding to this distal site allosterically controls enzyme activity by altering motions in the active site $\beta6/\beta7$ loop. In particular, we show that Ca$^{2+}$ retards motions and induces slow micro- to millisecond time-scale dynamics within the loop that may promote the adaptive recognition of the substrate. The results of relaxation compensated Carr-Purcell-Meiboom-Gill (CPMG) experiments suggest that the active site motions are correlated with the rate of ion binding. The results of site-directed mutagenesis suggest that this motional coupling is mediated by the side chain of Glu-171, which is located at the C-terminal end of the $\beta6/\beta7$ loop and required for high affinity ion binding. This work represents the...
first NMR dynamics study of a sortase enzyme and reveals that complex conformational dynamics contribute to the function of these enzymes.

**MATERIALS AND METHODS**

**Protein Preparation and Purification—**Uniformly $^{15}$N-enriched SrtA$_{N59}$ (residues 60–206) was obtained as previously described (17). Three separate samples of 3 mM $^{15}$N SrtA$_{N59}$ were prepared for relaxation studies by dissolving weighed lyophilized protein in 500 µl of 50 mM Tris-HCl, 100 mM NaCl, 3 mM diethanolarnine, 7% D$_2$O, and 0.01% NaN$_3$. The pH of the sample was adjusted to 6.20 (uncorrected for the deuterium effect). Each sample differed in the amount of Ca$^{2+}$ present: 1) no Ca$^{2+}$ (apo-Ca$^{2+}$ SrtA$_{N59}$), 2) 20 µM of CaCl$_2$, or 3) 20 mM Ca$^{2+}$ (Ca$^{2+}$-bound SrtA$_{N59}$). Where needed, the water used for the samples was preconditioned with Chelex resin. The Mn$^{2+}$ and Ca$^{2+}$ titration experiments were conducted on similar samples of $^{15}$N SrtA$_{N59}$ using defined amounts of these ions. The histidine-tagged single amino acid mutants of sortase were purified using a nickel column and exchanged into the appropriate buffer for enzymatic and NMR studies.

** Modification of SrtA$_{N59}$ by the Peptidyl-Sulphydryl Compound—**A peptidyl-sulphydryl compound (benzoxaloxybenzyl-Leu-Pro-Ala-Thr with a C-terminal -CH$_2$SH group) was used to modify SrtA$_{N59}$ (the synthesis of the compound will be reported elsewhere). A 5-fold molar excess of the compound was added to two samples of SrtA$_{N59}$ (500 µl of a 20 µM protein solution) in buffer I (pH 8.0, 50 mM Tris-HCl, and 100 mM NaCl), one without Ca$^{2+}$ and one containing 20 mM CaCl$_2$. The mixture was incubated on a rotating wheel at room temperature, and samples were removed periodically and analyzed using reverse phase high-performance liquid chromatography on a C18 column (Waters, Milford, MA). The areas under the peaks corresponding to modified and unmodified SrtA in the chromatogram were integrated to calculate the percentage of modification at each time point.

**Site-directed Mutagenesis—**Wild-type SrtA$_{N59}$ plasmid containing a C-terminal 6-His tag was generated as described previously (24). Single amino acid mutations of SrtA$_{N59}$ were generated by PCR using the SrtA$_{N59}$ plasmid template and Pfu Turbo DNA polymerase (Stratagene). The Glu-108 codon was mutated to encode Ala-108 using the pGene. The Glu-171 codon was mutated to encode Ala-171 using the primers E108A-F (TAAGCTTTGCAGAAGAAAATGCATCACTACACAGATGTAGGAGTTCTAGCAGAACAAAAAGGTAAAGATAA) and E171A-R (AATTGTTTACATCATCTGT). The Asp-170 GATGATCAAAATATTT) and E108A-R (AAATATTTTGATCATCTGT) were mutated into the appropriate buffer for enzymatic and NMR studies.

**LPETG-dinitrophenyl substrate was dissolved in Me$_2$SO and added to the reaction to a final concentration between 6.25 and 25 µM, for a total reaction volume of 200 µl. The increase in fluorescence intensity was monitored at room temperature using excitation at 335 nm and recording the emission maximum at 420 nm on a SPEX spectrofluorometer (Photon Technology International, Lawrenceville, NJ). The steady-state velocities ($V_0$) from the biphasic progress curves were calculated as described previously (25). The progress curves were fit to the following equation,

$$P = \pi [1 - \exp(-k_{obs}t) + V_0t]$$

where $\pi$ is the amplitude of the burst phase. The kinetic parameters were calculated from the substrate dependence of $V_0$ as described previously (25).

**Relaxation Data Acquisition and Processing—**NMR data were acquired on Bruker Avance 500- and 600-MHz spectrometers equipped with 5-mm triple resonance cryo-probes and single axis pulsed field gradients. The chemical shift assignments have been reported previously (26–28). Ten 0.10 s $T_1$, 1.29 $T_2$ values, and if miss-identified can cause erroneous internal correlation times ($\tau_1$) and $R_2$ values (31, 32). The principal moments of the inertia tensor were calculated using the program PdbinJung, and R. T. Clubb, unpublished observation.

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addition of hydrogen atoms using the program MolMol (33), respectively. Similar moments were obtained using the program HydroNMR version 5x (34), where the proteins were assumed to be hydrated with a 3.1 Å water shell (1.00:0.80:0.84 for Ca2+-bound SrtAΔNS9 and 1.00:0.88:0.86 for apo-Ca2+-SrtAΔNS9). The latter analysis also provided an estimate of the molecular correlation time (τm) of each form of the protein: 11.03 and 9.89 ns for apo-Ca2+- and Ca2+-bound SrtAΔNS9, respectively. Because the relative moments for both apo-Ca2+- and Ca2+-bound SrtAΔNS9 vary significantly from a perfect sphere, the approach outlined by Tjandra et al. (31) was used to check the statistical significance of fitting the relaxation data to an axially symmetric model versus an isotropic model (R2R1-Diffusion program). These calculations were performed using data from residues whose R/Rt ratios were within one standard deviation from the average R/Rt ratio, and which had NOE ratios > 0.65 (35). The results from 500 MHz (and 600 MHz) data suggested a τm of 9.69 ns (9.54 ns) for Ca2+-bound SrtAΔNS9 and a τm of 10.35 ns (9.94 ns) for apo-Ca2+-SrtAΔNS9. The data also showed a statistically significant improvement when using the axial symmetric model over the simple isotropic diffusion model. Interestingly, the Ca2+-bound SrtAΔNS9 data fit best to a prolact ellipsoid, whereas the apo-Ca2+-SrtAΔNS9 data fit best assuming an oblate ellipsoid. This variation is mostly due to subtle differences in the ββ′/β′ loop orientation in the two structures. The tensor parameters were also calculated using the approach outlined by Bruschweiler et al. (36–38) using the program Quadric_Diffusion, which gave slightly elevated correlation times of 9.86 ns (9.80 ns) for Ca2+-bound SrtAΔNS9 and 10.62 ns (10.17 ns) for apo-Ca2+-SrtAΔNS9 from 500 MHz (and 600 MHz) data, but the axial symmetric model was preferred over the isotropic or fully anisotropic models. The results from this final calculation were used as an initial guess for Modelfree analysis described below.

Modelfree Analysis—The amplitudes and effective correlation times for a protein’s internal motions can be extracted from relaxation data using the Lipari-Szabo Modelfree formalism (39, 40). The analysis considers semi-empirical forms of the spectral density function with each form composed of terms describing the motion of the N-H bond vector. This internal motion can be assumed to occur on two different, fast and slow time scales and can be characterized by effective correlation times, τ and τ (where τ < τ < τ), and the square of order parameters, S2 and S2. The square of the generalized order parameters is defined as S = S + S and corresponds to the spatial restriction of the N-H bond vector (where 0 ≤ S < 1). The analysis also accounts for line broadening due to chemical exchange, Rex. All these motional parameters were fit to the spin-relaxation data using the program Modelfree 4.01 (41, 42). For each model, 500 randomly distributed data sets were generated, and model selection was done using a statistical testing protocol described by Mandel et al. (42). Initially, models were selected at fixed diffusion tensor parameters by comparing the sum-squared error of optimal fit with the 0.05 critical value of the distribution and whenever applicable, by F-test comparisons to the 0.20 critical value of the distribution. In the next step, after a model had been assigned to each spin, both the diffusion tensor and model parameters were optimized simultaneously. We used an N-H bond length of 1.02 Å and 15N chemical shift anisotropy values of ~160 ppm in our backbone spin calculations. For the sole tryptophan (Trp-136) side-chain spin, a chemical shift anisotropy value of ~126 ppm was used (43). A minimum 3% base error is assumed in all parameters in the Modelfree analysis (44, 45). Out of 92 quantifiable residues in apo-Ca2+-SrtAΔNS9, 89 could be satisfactorily fit using Modelfree analysis (the relaxation data of Ser-70, Glu-95, and Ile-123 could not be fit). Model 1 (S2-only) was an appropriate fit for 63 residues, 3 residues fit to model 2 (S2 and τ), 13 residues fit to model 3 (S2 and τ, 2 fit to model 4 (S2, τ, and Rex), and 11 residues fit to model 5 (S2, S, and τ). In the Ca2+-bound SrtAΔNS9 data, 94 out of a total of 98 quantifiable spins could be fit satisfactorily (the exceptions were Gln-95, Arg-99, Ile-123, and Gin-172). In the Ca2+-bound SrtAΔNS9 analysis, Model 1 (S2) was selected for 75 residues, 3 residues fit to model 2 (S2 and τ), 12 residues fit to model 3 (S2 and Rex), 2 fit to model 4 (S2, τ, and Rex), and 6 residues fit to model 5 (S, S, and τ). Interestingly, in both Ca2+-bound and apo-Ca2+-SrtAΔNS9, the relaxation data from Glu-95 and Ile-123 could not be fit to any model, suggesting that they undergo more complicated motions. The results of the model-free analysis for both conditions are provided in supplemental Tables S1 and S2. The average order parameters for apo-Ca2+- and Ca2+-bound SrtAΔNS9 are 0.88 ± 0.14 and 0.89 ± 0.10, respectively. If only residues located in regions of regular secondary structure are considered, the order parameters are 0.943 ± 0.030 and 0.935 ± 0.016, for apo-Ca2+- and Ca2+-bound SrtAΔNS9, respectively.

RESULTS AND DISCUSSION

Ca2+ Promotes Substrate Binding—To identify the step(s) at which Ca2+ stimulates catalysis, we monitored the in vitro activity of SrtAΔNS9 using an internally quenched fluorescent substrate analogue (o-aminobenzoyl-L-P-E-T-G-2,4-dinitrophenyl) (25). The SrtAΔNS9 mediated cleavage of the peptide bond between the threonine and glycine residues in this substrate results in an increase in fluorescence and can be used to monitor both hydrolysis and transpeptidation. The presence of Ca2+ stimulates both the hydrolytic and transpeptidation activities of SrtAΔNS9 (data not shown). Because both reactions differ only in the nucleophile used to resolve the acyl-intermediate, these results indicate that ion binding activates an early step in catalysis (e.g. sorting signal binding, activation of the Cys-184 thiol, or resolution of the enzyme-sorting signal thioacyl intermediate). To further pinpoint the step at which it acts, we monitored the hydrolytic activity of SrtAΔNS9 in the presence of varying amounts of Ca2+. The hydrolysis reaction can be represented as in Reaction 1.

\[
\text{E} + \text{RCHO} \xrightleftharpoons[k_1]{k_4} \text{E} \cdot \text{RCHO} \xrightleftharpoons[k_5]{k_6} \text{RCHO} + \text{H}_2\text{O} \rightarrow \text{E} + \text{RCO}_2\text{H}
\]

where E, E/RCHO, and RCO₂H represent the free enzyme, the enzyme-bound to the sorting signal, and the acyl enzyme-substrate complex, respectively; RCO₂H and XH are the cleaved peptide and the free glycin e, respectively; and k₁₋₄ are the rate constants describing their interconversion (25). Lineweaver-Burk plots of the hydrolysis data recorded at varying Ca²⁺ concentrations reveal a common 1/V₅₀ intercept, indicating that the kcat of the reaction is unaffected (Fig. 1A). It can also be concluded from this data that the values of k₂ and k₄ are independent of Ca²⁺, because k₄ is defined as k₂ k₇/k₅ + k₇. In contrast, the presence of Ca²⁺ clearly alters the Michaelis-Menten constant (Kₘ) of the reaction, because the slopes of the data are inversely proportional to Kₘ kcat and the value of k₄ is independent of Ca²⁺. Because Kₘ is defined as [k₋₄ + k₋₅]/k₋₄, and k₋₄ is unchanged by Ca²⁺, we conclude that Ca²⁺ stimulates SrtAΔNS9 activity by promoting substrate binding; it either increases k₋₄ or decreases k₋₅.

The β3/β4 Pocket of SrtAΔNS9 binds One Ca²⁺ Ion with Millimolar Affinity—Previously we used NMR to localize a Ca²⁺ binding site on SrtAΔNS9 to residues within the loop connecting strands β3 to β4 (the β3/β4 pocket) (17). However, the effects of Ca²⁺ are extensive, and, in addition to the β3/β4 pocket, dramatic chemical shift changes were observed in a large adjacent loop that connects strands β6 to β7 (the
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Because many of the perturbed residues in the β6/β7 loop are far away from the β3/β4 pocket, their shift changes cannot be caused by direct effects (see Fig. 4B of Ref. 17). Instead, either additional ions bind to the β6/β7 loop, and/or ion binding to the β3/β4 pocket triggers a conformational rearrangement that perturbs the chemical shifts of residues within the β6/β7 loop. Because our previous work did not distinguish between these two possibilities, we precisely localized the divergent ion binding site(s) on SrtA_{AN59} by titrating the enzyme with Mn^{2+} and monitoring its $^{1}H$ HSQC NMR spectrum. It is expected that Mn$^{2+}$ will bind to the same site(s) as Ca$^{2+}$, because they are both divalent ions and because Mn$^{2+}$ also stimulates enzyme activity (albeit to a lesser extent) (17). Importantly, titrations with Mn$^{2+}$ enable precise definition of the ion-binding pocket, because it is paramagnetic, such that even at sub-saturating levels it selectively broadens only nearby protein resonances (46). A series of $^{1}H$ H-15N HSQC spectra of apo-Ca$^{2+}$ SrtA_{AN59} were recorded in the presence of varying concentrations of Mn$^{2+}$ (0, 25, 50, 100, 200, 500, and 1000 μM). When 50 μM Mn$^{2+}$ is added, several $^{1}H$ H-15N resonances within the β3/β4 pocket selectively disappear (Fig. 2B), whereas at higher Mn$^{2+}$ concentrations, residues adjacent to surface-exposed acidic side chains exhibit nonspecific line broadening (Fig. 2A). Only a single Ca$^{2+}$ ion binds to the β3/β4 pocket as a superposition of a series of $^{1}H$ H-15N HSQC spectra recorded with varying amounts of Ca$^{2+}$ reveals linear changes in the protein’s chemical shifts (Fig. 2C), whereas non-linear changes are expected if multiple ions were to bind (47). Moreover, an analysis of a plot of the Ca$^{2+}$ chemical shift changes versus [Ca$^{2+}$]/[SrtA_{AN59}] reveals a binding ratio of 1:1 (data not shown). Taken together, these data show that the aforementioned β3/β4 pocket binds to a single Ca$^{2+}$ ion and that it is the only high affinity ion binding site on sortase. The extensive Ca$^{2+}$-dependent chemical shift changes in the β6/β7 loop can therefore be attributed to a structural rearrangement within or nearby this region (Fig. 3A).

The affinity of the β3/β4 pocket for Ca$^{2+}$ has not been determined quantitatively and is needed for NMR dynamics and kinetic studies. Values for the dissociation constant were therefore estimated from the Ca$^{2+}$ dependence of the apparent $K_m$ (Fig. 1B) using Equation 2,

$$K_m = K_{m_{max}} \left( \frac{[Ca^{2+}]}{[Ca^{2+}] + K_d} \right)$$

(Eq. 2)

where $K_{m_{max}}$ and $K_m$ are the $K_m$ values of the enzyme in the absence and presence of Ca$^{2+}$, respectively, and $K_d$ is the dissociation constant for ion binding (48). Curve fitting of the data reveal that saturating amounts of Ca$^{2+}$ cause a 4.2-fold decrease in the $K_m$ from 121 μM to 33 μM and that the ion binds with a $K_d$ of 1.6 ± 0.3 mM. A similar binding constant of 2.2 ± 0.5 mM is obtained by directly curve fitting the Ca$^{2+}$ dependence of the chemical shift data (Fig. 2D). Because the concentration of Ca$^{2+}$ is ~2.5 mM in human serum, these results suggest that about half of the SrtA molecules on the surface of S. aureus are Ca$^{2+}$-bound during bacteremia. However, this is likely an underestimate because the binding of the substrate and ion to the protein presumably forms a closed thermodynamic cycle, which necessitates that the substrate-bound enzyme exhibit ~4-fold higher affinity for Ca$^{2+}$ as compared with the substrate-free enzyme (49).

Ca$^{2+}$ Does Not Directly Interact with the Sorting Signal—NMR, x-ray, and targeted mutagenesis studies have localized the sorting signal binding site to a large hydrophobic surface immediately adjacent to the ion-binding pocket (18, 22), raising the possibility that direct ion-substrate interactions stimulate catalysis. An inspection of the recently determined x-ray structure of a Cys-184 → Ala mutant of SrtA_{AN59} (C184A/SrtA_{AN59}) bound to a LPETG peptide reveals that the side chain of the central glutamic acid in the peptide is nearest to the ion-binding pocket (18). However, this structure cannot reveal whether the ion directly contacts the substrate, because it was solved in the absence of Ca$^{2+}$. Because the stimulatory effect of divalent cations has only been demonstrated using a fluorogenic substrate that also has glutamic acid at this position, we wondered whether acidic residues at the central position within the substrate were needed for Ca$^{2+}$ stimulation. To answer this question, the effect of Ca$^{2+}$ on the rate of enzyme modifi-
the program CaLigator (68). The deduced Ca$^{2+}$-ion binding site(s) responsible for chelating the Ca$^{2+}$ ion, is shown in our original perturbation study (17), where $K_d = \sqrt{3K_{Ca}^{p} + \Delta \tau_{Mn}^{Ca}}$. Selected portion of two $^{1}H$-$^{15}N$ HSQC spectra acquired in the absence (red) and presence (green) of 50 $\mu$M Mn$^{2+}$. Peaks shown only in red with no trace of overlapping green disappear beyond detection and are predominantly localized to the Ca$^{2+}$-binding pocket. A small number of residues distant from the Ca$^{2+}$-binding surface also broaden outward, but these don’t show any significant changes in their chemical shifts in the presence and absence of Ca$^{2+}$ and therefore represent Mn$^{2+}$-specific interactions. C, overlay of eight $^{1}H$-$^{15}N$ HSQC spectra acquired at different Ca$^{2+}$ concentrations ranging from 0 mM (red), 1 mM (orange), 2 mM (yellow), 3 mM (green), 4 mM (light green), 5 mM (light blue), 10 mM (cyan), to 20 mM (blue). The black arrows indicate the linearity of peak movement, which is clear evidence for a single Ca$^{2+}$-ion binding event. Resonance assignments at both 0 mM and 20 mM Ca$^{2+}$ were independently verified by triple resonance experiments, and the resulting chemical shift differences are shown in A, D representative SrtA irresp$^{2+}$Ca$^{2+}$ binding profiles constructed from the backbone amide chemical shift changes observed from the series of HSQC spectra shown in panel C. Data for Asn-107 (circle and solid line); Glu-108 (square and dashed line); Asp-112 (triangle and dash-dot line); and Glu-171 (asterisk and dotted line) is shown here. The symbols represent experimental data while the lines represent curve fits performed using the program Calcium (68). The deduced Ca$^{2+}$-ion binding constant has a high precision for residues from the Ca$^{2+}$-binding surface with an average dissociation constant, $K_d = 2.21 \pm 0.51$ mM. Thirteen residues were used to estimate this $K_d$ value: Asn-107, Glu-108, Ser-109, Leu-110, Asp-112, Ile-115, Ser-116, Gly-167, Val-168, Leu-169, Asp-170, Glu-171, and Gln-172.

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FIGURE 2. A, a histogram showing residues that are broadened beyond detection due to the addition of paramagnetic Mn$^{2+}$. The magnitude of compound $^{15}N$-$^{1}H$ chemical shift changes ($\Delta \tau_{Mn}$) is shown in our original perturbation study (17), where $\Delta \tau = \sqrt{3K_{Ca}^{p} + \Delta \tau_{Mn}^{Ca}}$. B, overlay of two $^{1}H$-$^{15}N$ HSQC spectra acquired in the absence (red) and presence (green) of 50 $\mu$M Mn$^{2+}$. Peaks shown only in red with no trace of overlapping green disappear beyond detection and are predominantly localized to the Ca$^{2+}$-binding pocket. A small number of residues distant from the Ca$^{2+}$-binding surface also broaden outward, but these don’t show any significant changes in their chemical shifts in the presence and absence of Ca$^{2+}$ and therefore represent Mn$^{2+}$-specific interactions. C, overlay of eight $^{1}H$-$^{15}N$ HSQC spectra acquired at different Ca$^{2+}$ concentrations ranging from 0 mM (red), 1 mM (orange), 2 mM (yellow), 3 mM (green), 4 mM (light green), 5 mM (light blue), 10 mM (cyan), to 20 mM (blue). The black arrows indicate the linearity of peak movement, which is clear evidence for a single Ca$^{2+}$-ion binding event. Resonance assignments at both 0 mM and 20 mM Ca$^{2+}$ were independently verified by triple resonance experiments, and the resulting chemical shift differences are shown in A, D representative SrtA irresp$^{2+}$Ca$^{2+}$ binding profiles constructed from the backbone amide chemical shift changes observed from the series of HSQC spectra shown in panel C. Data for Asn-107 (circle and solid line); Glu-108 (square and dashed line); Asp-112 (triangle and dash-dot line); and Glu-171 (asterisk and dotted line) is shown here. The symbols represent experimental data while the lines represent curve fits performed using the program Calcium (68). The deduced Ca$^{2+}$-ion binding constant has a high precision for residues from the Ca$^{2+}$-binding surface with an average dissociation constant, $K_d = 2.21 \pm 0.51$ mM. Thirteen residues were used to estimate this $K_d$ value: Asn-107, Glu-108, Ser-109, Leu-110, Asp-112, Ile-115, Ser-116, Gly-167, Val-168, Leu-169, Asp-170, Glu-171, and Gln-172.

FIGURE 3. A, schematic showing Ca$^{2+}$-ion coordination by SrtA irresp$^{2+}$Ca$^{2+}$-ion binding site(s) predicted previously (17) was precisely localized by Mn$^{2+}$ titration experiments monitoring its $^{1}H$-$^{15}N$ HSQC NMR spectra and by inspection of the NMR solution structure of the Ca$^{2+}$-bound form of the protein (PDB accession code: 1IJA). These new data indicate that the previously predicted $\beta3/\beta4$ pocket binds to a single Ca$^{2+}$ ion, and it is the only high affinity Ca$^{2+}$ binding site on SrtA irresp$^{2+}$. B, overlay of the NMR (pink) and crystal (gray) structures solved in the presence and absence of Ca$^{2+}$, respectively. This figure shows that only the structure of the loop connecting strands $\beta6$ to $\beta7$ in the $\beta6/\beta7$ loop is dramatically affected upon Ca$^{2+}$ binding. The side chains of Val-168 and Leu-169 (within the $\beta6/\beta7$ loop) are colored pink and gray in the NMR and crystal structures, respectively. The side chains of active site residues and Ca$^{2+}$-ligating residues are shown in blue and green, respectively. C, a ribbon diagram of the crystal structure of the complex between $^{15}N$-SrtA irresp$^{2+}$, and a peptide containing the sequence LPETG. Regions 1–3 of the $\beta6/\beta7$ loop are colored yellow, red, and green, respectively. The $\beta3/\beta4$ pocket, which is responsible for chelating the Ca$^{2+}$ ion, is shown in light brown. The LPETG peptide is shown in purple, and the side chains of Pro-163 and the catalytically important Arg-197 are shown in yellow and pink, respectively.
cation by a peptidyl-sulfhydryl compound containing the sorting signal sequence LPAT was tested (benzoyloxycarbonyl-Leu-Pro-Ala-Thr, with the C-terminal carbonyl group of Thr replaced with -CH2-SH). This compound modifies SrtA/H9004N59 by forming a disulfide bond with Cys-184 within the active site (22). As shown in Fig. 1C, the presence of Ca2+ increases the rate at which it modifies the enzyme, presumably by promoting its binding similar to the substrate. Because residues in regular secondary structure exhibited uniformly high S2 values, they are represented in blue by arrows (β sheets) and coils (α helices) of uniform size. The catalytic (His-120, Cys-184, and Arg-197) and Ca2+ binding side chains are displayed in green. The relaxation data are mapped onto the coordinates of the NMR structure of Ca2+-bound protein. E, representative histogram of the root mean square deviations of the local backbone assignments of the SrtA/H9004N59 solution structure (17). F, representative histogram of B-factors of the SrtA/H9004N59 crystal structure (18).

Nitrogen-15 Relaxation Measurements—The proximity of the β6/β7 loop to the active site and its extensive Ca2+-dependent chemical shift changes argue that it undergoes an ion-induced structural change that promotes substrate binding. Interestingly, structural data also suggest that the loop is flexible, because in both the NMR and x-ray structures, its residues exhibit elevated root mean square deviations (Fig. 4E) and B-factors (Fig. 4F), respectively (17, 18). This raises the interesting possibility that Ca2+ also modulates the flexibility of the loop to stimulate substrate binding. Because nothing is known about the conformational

4 M. T. Naik, N. Suree, U. Ilangovan, C. K. Liew, W. Thieu, D. O. Campbell, J. J. Clemens, M. E. Jung, and R. T. Clubb, unpublished observation.
dynamics of any sortase enzyme and there is no quantitative relationship between structural parameters and mobility, we rigorously defined the Ca\(^{2+}\) dependence of motions in SrtA\(_{AN59}\) by measuring the rates of longitudinal (\(R_1\)) and transverse (\(R_2\)) relaxation, as well as [\(^1\)H]-\(^{15}\)N NOE values of the backbone nitrogen-15 atoms. Two samples of SrtA\(_{AN59}\) in different states of ligation were investigated: (i) an apo-Ca\(^{2+}\) form (50 mM Tris-HCl, 100 mM NaCl, 3 mM dithiothreitol, 7% D_2O, and 0.01% NaNO\(_3\), pH 6.2) and (ii) a Ca\(^{2+}\)-bound form (conditions identical to the apo-Ca\(^{2+}\) SrtA form, but with 20 mM Ca\(^{2+}\) present). The relaxation data were then interpreted using the Modelfree formalism to gain insights into the magnitudes and time scales of motion. Graphs showing the relaxation data as a function of residue number are shown in supplemental Fig. S1 and tables listing the values of the Modelfree parameters (\(S^2\), \(\tau_m\), and \(K_m\)) are provided as supplemental Tables S1 and S2.

**Residues in the \(\beta/\beta'\) Loop Transiently Participate in Ion Binding**—The coordinates of the \(\beta/\beta'\) loop are poorly defined in both the NMR and x-ray structures of the enzyme (Fig. 4, E and F). However, its residues can be divided into three sections based on their positioning relative to the active and ion binding sites, and their dynamics properties were revealed by NMR (Fig. 3C). At its N-terminal end, residues Lys-162 to Val-166 (region 1) ascend from the body of the protein so as to position the ring of Pro-163 immediately adjacent to the catalytic side chain of Arg-197. Residues Gly-167 to Asp-170 (region 2) then form the substrate-contacting surface and are followed by residues Asp-170 to Asp-176 (region 3), which are positioned proximal to the ion-binding site in the \(\beta/\beta'\) pocket. From the relaxation data analysis, the \(S^2\) parameter is calculated, which gives a concise account of each of the mobility of N-H bond vector on the picosecond time scale; it ranges from 0 to 1, with a value of 1 indicating that the amide is completely immobilized. Inspection reveals that only the \(\beta/\beta'\) loop exhibits significant Ca\(^{2+}\)-dependent changes in its dynamics (Fig. 4, compare A and B). When the \(S^2\) data are mapped onto the structure, it is apparent that residues in regions 2 and 3 of the \(\beta/\beta'\) loop become partially immobilized when the ion is bound (Fig. 4, C and D), the thickness of the chain is correlated with increased mobility in the apo-Ca\(^{2+}\) and Ca\(^{2+}\)-bound forms, respectively. Interestingly, residual picosecond motions in the \(\beta/\beta'\) loop persist even in the presence of Ca\(^{2+}\), implying that it transiently binds Ca\(^{2+}\). This may explain the weak affinity of the protein for the ion (Figs. 1B and 2D) and the observed disorder in the \(\beta/\beta'\) loop in the NMR structure of the Ca\(^{2+}\)-bound enzyme.

**Glu-171 in the \(\beta/\beta'\) Loop Is Important for Ca\(^{2+}\) Binding**—Because the NMR data indicate that the \(\beta/\beta'\) loop becomes immobilized when the ion is present, it seems likely that it contains one or more residues that directly contact the ion in the \(\beta/\beta'\) pocket. Because the coordinates of the \(\beta/\beta'\) loop are poorly defined in both the NMR and crystal structures, it is not possible to unambiguously identify contacts from it to the ion (Fig. 4, E and F). However, as previously noted, the side chain of Glu-171 within the \(\beta/\beta'\) loop is a likely candidate for ion binding, because in several of the conformers of the NMR structure of Ca\(^{2+}\)-bound SrtA\(_{AN59}\) it is poised to interact with the ion. This is also consistent with the finding that the adjacent backbone amides of the nearby residues Leu-169 and Asp-170 experience the largest ion-dependent changes in their \(S^2\) parameters and chemical shifts, respectively.

To determine if ion contacts from Glu-171 act to immobilize the \(\beta/\beta'\) loop, the Ca\(^{2+}\) binding properties of three single amino acid mutants of SrtA\(_{AN59}\) were tested. Each mutant replaces potential ion binding acidic side chains with alanine and target residues that are located in the \(\beta/\beta'\) loop (Asp-170 \(\rightarrow\) Ala and Glu-171 \(\rightarrow\) Ala) or the \(\beta/\beta'\) ion binding pocket (Glu-108 \(\rightarrow\) Ala). All of the mutant proteins remain folded as judged by their NMR spectrum (data not shown) and retain enzymatic activity (Table 1). The Ca\(^{2+}\) binding properties of the mutants were assessed by measuring the Ca\(^{2+}\) dependence of their enzymatic activity as previously described for the wild-type protein. As shown in Fig. 1B, the \(K_m\) values for the wild-type and D170A mutant proteins show a similar dependence on Ca\(^{2+}\), indicating that Asp-170 within the \(\beta/\beta'\) loop does not bind Ca\(^{2+}\). In contrast, both the E108A and E171A mutants show reduced Ca\(^{2+}\) sensitivity (Fig. 1B). The E108A mutant serves as a positive control, because it has been shown to unambiguously interact with the ion based on Mn\(^{2+}\) titration data (Fig. 2A). The finding that it and E171A have similar effects on catalysis suggests that Glu-171 within the \(\beta/\beta'\) loop contacts the ion. Most importantly, fits of the Ca\(^{2+}\) dependence of the \(K_m\) values to Equation 2 reveal that the E108A and E171A mutants bind Ca\(^{2+}\) with 10-fold lower affinity than the wild-type or D170A proteins. To verify the relative Ca\(^{2+}\) binding affinities of the mutants, NMR was also used to directly monitor ion binding. As shown in Table 1, affinity measurements by NMR gave similar results as the kinetic analysis and indicate that Glu-108 in the \(\beta/\beta'\) pocket and Glu-171 in the \(\beta/\beta'\) loop are involved in binding the ion, whereas Asp-170 is not. These data are consistent with ion contacts from Glu-171 acting to stabilize ion binding.

**Ion Binding Redistributes Slow Motions toward the Active- and Substrate-binding Sites**—Protein motions on micro- to millisecond time scales are important for enzymatic catalysis and ligand recognition (51, 52). Because the Modelfree approach only characterizes these processes with the Modelfree approach, we only characterize these processes indirectly as a contribution to transverse relaxation in the form of the \(R_2\) term. We performed relaxation-compensated CPMG (rc-CPMG) experiments to directly investigate these motions (53, 54). Slow time-scale conformational rearrangements in SrtA\(_{AN59}\) are revealed by the data shown in Fig. 5 (A and B), which displays plots of the \(R_2\) terms derived from the Modelfree analysis (open bars) and the difference in transverse relaxation rates when long \((\tau_{cp} = 4\) ms) and short \((\tau_{cp} = 1\) ms) inter-pulse delays are used in the CPMG sequence (filled circles). Positive differences in the transverse relaxation rates are indicative of slow chemical exchange events and are in good qualitative agreement with the Modelfree data.
Dynamics of Calcium Modulated Loop Closure of Sortase A

The rc-CPMG data indicate that Ca\(^{2+}\) binding induces slow biologically relevant motions in the active site. In the absence of Ca\(^{2+}\), residues exhibiting slow motional dynamics are evenly distributed throughout the primary sequence (Fig. 5D, blue spheres, and supplemental Table S1). The exception is the C-terminal portion of the \(\beta_6/\beta_7\) loop near the ion, which in addition to fast picosecond motions, is in flux on slower time scales prior to Ca\(^{2+}\) binding (residues within (Gly-174 and Asp-176) and underneath (Ala-202, Thr-203, and Val-204) this portion of the loop exhibit \(R_{\text{ex}}\) values). In contrast, in the Ca\(^{2+}\)-bound form, a spike in \(R_{\text{ex}}\) values was observed in region 2 of the \(\beta_6/\beta_7\) loop (Fig. 5D, red spheres; residues Asp-165, Val-168, and Leu-169). These motions are likely triggered by an ion-induced movement of almost the entire loop, because all of it exhibits Ca\(^{2+}\)-dependent chemical shift changes (Fig. 4B of Ref. 17). Interestingly, a general redistribution of slow motions toward the active site occurs upon ion binding, because significant \(R_{\text{ex}}\) terms are also observed in residues Thr-121, Phe-122, and Asp-124, and in Ile-199, which immediately follow the catalytically essential His-120 and Arg-197 side chains (Fig. 5D). These motions may be correlated with the structural rearrangements that are required to catalyze hydrolysis. Interestingly, residues in region 2 form the substrate binding site in the crystal structure of the complex. This suggests that Ca\(^{2+}\)-triggered slow motions in this part of the loop may facilitate the adaptive recognition of the sorting signal.

Ion Binding and Active Site Motions Occur on Similar Time Scales—Our results clearly illustrate that Ca\(^{2+}\) acts to quench motions within the \(\beta_6/\beta_7\) loop. Although the mutagenesis data suggest that ion contacts from Glu-171 immobilize the loop, we sought further support for this model by rigorously assessing the rate of slow protein motions and ion binding. If active site dynamics were coupled to Ca\(^{2+}\) binding via ion contacts from Glu-171, we reasoned that the rate of these conformational fluctuations would be similar. To investigate this issue, we quantified conformational exchange rates by performing rc-CPMG experiments on a new sample of SrtA\(_{\Delta N59}\) containing 20 \(\mu\)M Ca\(^{2+}\) (3.2 mM SrtA\(_{\Delta N59}\), 20 \(\mu\)M Ca\(^{2+}\), 50 mM Tris-HCl, 100 mM NaCl, 3 mM dithiothreitol, 7% D\(_2\)O, and 0.01% NaN\(_3\), pH 6.20). This enables a more accurate interpretation of the NMR data, because the exact populations of the ion-free and bound forms of SrtA can be determined (assuming a \(K_d = 2.2\) mM, this sample is composed of 99.2% and 0.08% equilibrium populations of the apo-Ca\(^{2+}\) and Ca\(^{2+}\)-bound SrtA\(_{\Delta N59}\), respectively). According to Ishima and Torchia (55), under conditions similar to ours, in which the populations of the interchanging conformers are dramatically skewed, the phenomenological transverse relaxation rate \(R_2(1/\tau_{\text{cp}})\) for all time scales is given by Equation 3,

\[
R_2(1/\tau_{\text{cp}}) = R_2(1/\tau_{\text{cp}} \rightarrow \infty) + \left( \frac{p_r p_c \Delta \omega^2 k_{ex}}{k_{ex}^2 + \left( \frac{p_r^2 \Delta \omega^4 + 144}{\tau_{cp}} \right)} \right) (\text{Eq. 3})
\]

where \(\Delta \omega\) is the chemical shift difference between the two exchanging populations, \(k_{ex}\) is the rate of exchange, \(\tau_{ex}\) is the delay between 180 degree pulses in the experiment, and \(p_r\) and \(p_c\) are the populations of the apo-Ca\(^{2+}\) and Ca\(^{2+}\)-bound forms, respectively. Some residues exhibit \(R_{\text{ex}}\) terms in both the presence and absence of Ca\(^{2+}\) and are also indicated by a red sphere (residues Asp-124, Ala-135, Val-168, Thr-180, Val-193, Ala-202, and Thr-203).

![Figure 5: Observed milli- to microsecond Ca\(^{2+}\)-dependent dynamics in SrtA\(_{\Delta N59}\).](image-url)
FIGURE 6. Loop closure model describing how Ca\(^{2+}\) modulates the sorting signal binding site on SrtA. The panel on the left shows SrtA in the absence of Ca\(^{2+}\), emphasizing that the substrate contacting side chains of Val-168 and Leu-169 are removed from the active site, and the loop is more flexible. The panel on the right shows how contacts from the β6/β7 loop to the Ca\(^{2+}\) ion bound in the β3/β4 site act to immobilize and reposition the loop for favorable contacts to the LPXTG sorting signal.
more complex models of motion are possible, the available structural and dynamics data are consistent with a model in which a portion of the loop is generally disordered and capable of toggling between two states (Fig. 6), a binding competent closed form, and a highly flexible open state that removes key substrate contacting residues from the active site. We propose that these conformers are in dynamic equilibrium and that a single Ca$^{2+}$ ion acts to bias the loop toward its binding competent closed form by transiently tethering the C-terminal end of the loop to the body of the protein by contacting the side chain of Glu-171. In other enzymes the dynamic behavior of active site loops has been shown to be important for catalysis (62–64). Interestingly, in SttA, the magnitude and time scales of these motions are responsive to Ca$^{2+}$, which when bound allosterically, triggers slow micro- to millisecond motions that may be ideally suited for substrate recognition. Bacteria becoming increasingly resistant to multiple antibiotics is an increasing health concern. The central role of sortases in bacterial virulence makes them an attractive target for new anti-infective agents, and an understanding of how Ca$^{2+}$ regulates their activity should aid in the ongoing development of small molecule inhibitors of this enzyme class (65–67).

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