Structural Transitions as Determinants of the Action of the Calcium-Dependent Antibiotic Daptomycin

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Summary

Daptomycin is a cyclic anionic lipopeptide antibiotic recently approved for the treatment of complicated skin infections (Cubicin). Its function is dependent on calcium (as Ca2+). Circular dichroism spectroscopy indicated that daptomycin experienced two structural transitions: a transition upon interaction of daptomycin with Ca2+, and a further transition upon interaction with Ca2+ and the bacterial acidic phospholipid, phosphatidyl glycerol. The Ca2+-dependent insertion of daptomycin into model membranes promoted mild and more pronounced perturbations as assessed by the increase of lipid flip-flop and membrane leakage, respectively. The NMR structure of daptomycin indicated that Ca2+ induced a conformational change in daptomycin that increased its amphipathicity. These results are consistent with the hypothesis that the association of Ca2+ with daptomycin permits it to interact with bacterial membranes with effects that are similar to those of the cationic antimicrobial peptides.

Introduction

Daptomycin (brand name Cubicin in its injectable form) is a novel lipopeptide antibiotic that recently received New Drug Approval by the US FDA for the treatment of complicated skin infections caused by gram-positive organisms, including methicillin-resistant Staphylococcus aureus [1]. Another phase 3 trial is underway to examine its efficacy in the treatment of infective endocarditis and bacteremia. In vitro studies have demonstrated that daptomycin has bactericidal activity against a wide variety of gram-positive bacteria, including vancomycin-resistant Enterococci, methicillin-resistant Staphylococcus aureus, coagulase-negative Staphylococci, glycopeptide intermediate-susceptible Staphylococcus aureus, and penicillin-resistant Streptococcus pneumoniae [2–6].

Antimicrobial peptides are ubiquitous in nature as components of host innate defense mechanisms against pathogenic microbes. However, the vast majority of these peptides are amphipathic and cationic, with charges of up to +9 conferred by excess arginine and lysine residues. In contrast, daptomycin is a negatively charged cyclic lipopeptide that is naturally produced as a fermentation end product from a strain of Streptomyces roseosporus [2]. The unique characteristic of daptomycin is that its antimicrobial activity is entirely dependent on calcium [4, 5, 7–9]. Two different mechanisms of action have been proposed for the bactericidal activity of daptomycin, involving either the inhibition of lipoteichoic acid (LTA) synthesis [10–12], which has been disputed [9, 13–15], or the dissipation of the membrane potential across the cytoplasmic membrane, leading to the disruption of several different cellular processes [14, 16]. Recently, Silverman et al. [13] proposed a multistep model for the mechanism of action of daptomycin that involves the depolarization of the cytoplasmic membrane. We were intrigued by this hypothesis as it has some similarities to a mechanism proposed by our laboratory for the cationic peptides [17]. In this study, we probed the effects of calcium on the structure of daptomycin and its interactions with membranes. Our results indicate that daptomycin undergoes two structural transitions, one of which we defined in detail through structural studies. On the basis of this structural and functional data, we propose a multistep interaction with membranes and a mechanism of action that expands on the previously proposed model by Silverman et al. [13].

Results and Discussion

The Role of Calcium in the Membrane Interaction of Daptomycin

Daptomycin is a cyclic lipopeptide that is composed of 13 amino acid residues, including three D-amino acids (D-asparagine-2, D-alanine-8, and D-serine-11), three uncommon amino acids [ornithine-6, (2S,3R)-3-methyl glutamic acid-12, and kynurenine-13], and an n-decanoyl fatty acid chain at the N-terminus [18] (Figure 1A). There are four acidic residues (three aspartic acid residues [3, 7, and 9] and one 3-methyl glutamic acid residue [12]) and one basic residue (ornithine [6]), resulting in a total molecular charge of −3 at neutral pH. The peptide is cyclic via formation of an ester bond between threonine-4 and kynurenine-13.

It has been clearly established that calcium plays an essential role in the antimicrobial activity of daptomycin (4, 5). We confirmed this here by demonstrating that, in the absence of calcium, daptomycin had a minimal inhibitory concentration (MIC) greater than 64 μg/ml, while in 0.34 mM, 2 mM, and 5 mM Ca2+ the MICs progressively decreased from 2 to 1 to 0.625 μg/ml, respectively, confirming the dependence of daptomycin activity on Ca2+. Through fluorescence [7, 8] and fractionation [12] studies, it has been shown that daptomycin binds to membranes in a calcium-dependent manner. To see if this reflected structural transitions, we initially utilized circular dichroism (CD) spectroscopy (Figure 1B). Daptomycin in aqueous solution demonstrated an ellipticity maximum at a wavelength of 233 nm and a broad negative minimum at
The addition to daptomycin of large unilamellar liposomes composed of a one-to-one mixture of the zwitterionic phospholipid phosphatidyl choline (PC) and the acidic phospholipid phosphatidyl glycerol (PG) in the absence of Ca²⁺ similarly led to an increase (as compared to aqueous daptomycin) in positive ellipticity combined with a slight blue shift. However, the addition of PC/PG liposomes in the presence of 5 mM Ca²⁺ caused the band at 233 nm to invert from a positive ellipticity to a negative ellipticity and the region between 210 nm and 220 nm to switch from a negative ellipticity to a positive ellipticity. Large unilamellar PC liposomes in the presence of Ca²⁺ were not able to induce such an inversion of the CD spectrum (data not shown). The results indicated that daptomycin underwent a substantial conformational change when it associated with negatively charged membranes, which was dependent on the presence of Ca²⁺.

Interaction of Daptomycin with Model Membranes

A variety of model membrane studies have been developed to assess the interactions of cationic peptides with membranes [19]. We used fluorescence spectroscopy to assess the insertion of daptomycin into membranes and study membrane perturbations such as lipid flip-flop and membrane leakage.

Daptomycin contains two aromatic residues (Trp-1 and Kyn-13) that are intrinsically fluorescent. Upon the insertion of these lipophilic residues into the phospholipid membrane, their environment becomes less polar, which causes a blue shift in the fluorescence emission wavelength combined with an increase in intensity [7, 8]. In aqueous solution, daptomycin in both the presence and absence of Ca²⁺ was weakly fluorescent, with an approximate maximum emission wavelength of 465 nm (Figure 1C). Addition of neutral PC liposomes in the presence of Ca²⁺ led to an 8 nm blue shift and a 5-fold increase in fluorescence intensity. Addition of acidic PC/PG (1:1) liposomes in the presence of Ca²⁺ led to a larger 16 nm blue shift and a 9-fold increase in fluorescence intensity. The addition of either PC or PC/PG liposomes to daptomycin in the absence of Ca²⁺ caused only minor changes in fluorescence. These results indicated that daptomycin inserted into bilayers of either acidic or neutral liposomes only in the presence of Ca²⁺ and that the insertion in mixed PC/PG liposomes was deeper than in PC liposomes.

The movement of lipid molecules between the bilayer leaflets (lipid flip-flop) provides one of the most sensitive measurements of peptide-lipid interactions [19, 20]. Lipid flip-flop was monitored with the fluorescent lipid probe C6-NBD-PC. Daptomycin, in a Ca²⁺-dependent manner, was able to induce substantial lipid flip-flop in both asymmetrically labeled acidic PC/PG (1:1) and neutral PC liposomes in a concentration-dependent manner (Figure 2A). Increasing the amount of Ca²⁺ from 2 to 5 mM increased the extent of lipid flip-flop. The extent of flip-flop began to plateau at around 0.5–2 µg/ml of daptomycin, equivalent to the MIC for many bacteria.

The ability of peptides to induce membrane leakage can be assessed by measuring the release of an encapsulated dye, calcein, from unilamellar vesicles [19–21].
Daptomycin Structure

951

Figure 2. Lipid Flip-Flop and Calcein Leakage Caused by Daptomycin in Model Membranes

(A) Dose-dependent lipid flip-flop of the C6-NBD-PC asymmetrically labeled PC/PG (solid lines) and PC (dashed lines) liposomes by daptomycin in the presence of 0 mM (Δ), 2 mM (●) and 5 mM (■) CaCl₂.

(B) Dose-dependent calcein leakage from PC/PG (upper two solid lines) and PC (lower two dashed lines) liposomes caused by the addition of daptomycin with 0 mM (Δ), 2 mM (●) and 5 mM (■) CaCl₂.

At the concentrations present inside liposomes, calcein self-quenches its own fluorescence; upon disintegration of the liposome membrane, calcein is released and its fluorescence is quenched. We assessed the ability of daptomycin to induce calcein leakage from both unilamellar PC/PG (1:1) and PC liposomes (Figure 2B). Daptomycin caused only 10% calcein leakage from neutral PC liposomes even at concentrations as high as 32 μM. More significant calcein leakage was induced in acidic PC/PG liposomes, which increased with the Ca²⁺ concentration and occurred at peptide concentrations that were substantially higher than those causing lipid flip-flop. This dependence on acidic phospholipids was reminiscent of the structural transitions observed in CD studies where a major conformational change of daptomycin occurred only with PC/PG liposomes in the presence of Ca²⁺. For the calcein release studies (Figure 2B), preincubation of daptomycin with Ca²⁺ was required to observe maximal effects. If the same concentrations of daptomycin and Ca²⁺ were separately added to the calcein-loaded PC/PG liposomes, less than 10% leakage of calcein was observed even at 16 μM of daptomycin/5 mM Ca²⁺ (data not shown). No such requirement for preincubation was observed in lipid flip-flop experiments.

Structure of Ca²⁺-free and Ca²⁺-Conjugated Daptomycin

The addition of Ca²⁺ to the buffered daptomycin caused an increase in molecular ellipticity of the CD maximum at 233 nm (Figure 1B), and we confirmed, using fluorescence emission spectra, that membrane interaction, including neutral PC liposomes, was Ca²⁺ dependent (Figure 1C). These observations are consistent with the hypothesis that Ca²⁺ causes a structural change in daptomycin, which promotes lipid interaction. To examine this further, we analyzed the structures of daptomycin in the presence and absence of Ca²⁺ using two-dimensional homonuclear NMR spectroscopy. Resonance-specific chemical shift assignments of Ca²⁺-free daptomycin (hereafter called apodaptomycin) and its Ca²⁺-conjugated form were obtained using conventional NMR methods [22] (see Tables S1 and S2 in the Supplemental Data). Figure 3 shows the NOESY spectra at 25°C (τm = 150 ms) for apodaptomycin and Ca²⁺-conjugated daptomycin. A comparison of these spectra shows clear differences for apodaptomycin versus Ca²⁺-conjugated daptomycin. The resonances for the apopeptide were well resolved, whereas those for the Ca²⁺-conjugated peptide were broadened. The increase in resonance linewidths may be due to intermediate chemical exchange between the Ca²⁺-free and Ca²⁺-bound peptide. Alternatively, the line broadening may indicate an increase in molecular weight due to peptide oligomerization [15]. Residues Trp-1 and Kyn-13 suffered the biggest increase in linewidth. The amide resonance of Trp-1 was broadened. The increase in resonance linewidths was accompanied by some chemical shift changes. The largest chemical shift change observed was 0.1 ppm for the Kyn-13 amide resonance. The similarity of chemical shifts suggests that there were no major conformational changes between the Ca²⁺-bound and -unbound peptides. However, the NOESY spectra showed distinct differences in the pattern of NOE cross-peaks. For example, new NOE contacts were observed for Ca²⁺-conjugated daptomycin between the n-decanoic fatty acid chain and the side chains of Trp-1, D-Asn-2, and Kyn-13 (Figure 3B, boxed region). Together with the above-average increase in resonance linewidth for Trp-1 and Kyn-13, this data indicated that the interactions between these residues were enhanced in the presence of Ca²⁺. To better describe the structural transition of daptomycin in the presence of Ca²⁺, the structures of apodaptomycin and Ca²⁺-conjugated daptomycin were determined. NOE-based distance restraints were collected from the NOESY spectra at 17°C (τm = 150 ms) (Figure S1) and 35°C (τm = 150 ms) (Figure S2), respectively. The adjustments in acquisition temperature were made to optimize the spectral resolution and the signal-to-noise ratio of NOE cross-peaks. An overview of the NOE restraints used to calculate the proposed three-dimensional structures of daptomycin in the absence and presence of Ca²⁺ is shown in Figure.
The binding of Ca\(^{2+}\) to daptomycin caused the ring structure to be drawn closer together by NOE restraints connecting Asp-3 with Ala-8, Thr-4 and Gly-5 with MeGlu-12 (three and four residues apart in the ring structure), and Ala-8 with Gly-10 and Ser-11. A type IV turn was formed between Thr-4 and Ala-8 (n + 1, \(\phi = -72^\circ, \psi = -157^\circ; n + 2, \phi = -27^\circ, \psi = -168^\circ\)). The side chain of Asp-3 was “tucked under” the ring structure due to the NOE restraints between the Asp-3 and the Ala-8 side chain (Figures 4, 5C, and 5D). The arrangement of the Asp-3 side chain suggests that this residue may be involved in the coordination of the Ca\(^{2+}\)-ion [23]. As for other Ca\(^{2+}\) binding proteins [24], the binding of Ca\(^{2+}\) to daptomycin resulted in a more constrained structure (i.e., the backbone average pairwise rmsd to the mean coordinates for the Ca\(^{2+}\)-conjugated structure was approximately half of the apostructure; Table 1). The high backbone variability in the apostructure around Gly-5 may reflect a function for this region as a flexible hinge, which would provide the conformational freedom required to permit for Asp-3 to move closer to the ring structure in order to participate in the coordination of Ca\(^{2+}\) in the Ca\(^{2+}\)-conjugated structure.

Calcium-induced changes have been linked to the exposure of hydrophobic residues that are crucial in the function of calcium-dependent proteins like annexinV [25, 26] and calmodulin [27]. Figures 5E and 5F illustrate the surface structures of apo- and Ca\(^{2+}\)-conjugated daptomycin. The total charge of the Ca\(^{2+}\)-conjugated peptide (−1) is lower than that of the apopeptide (−3) because of the binding of Ca\(^{2+}\). In addition to the total charge reduction, an increase in amphipathicity was observed in the Ca\(^{2+}\)-conjugated daptomycin structure as compared to the apostructure due to the redistribution of charged side chains toward the top of the ring structure and the orientation of the Asp-3 side chain toward the center of the ring. The calcium-induced changes in the daptomycin structure led to an increase in the solvent-exposed hydrophobic surface from 943 Å\(^2\) to 987 Å\(^2\) and a decrease in the solvent-exposed hydrophilic surface from 1090 Å\(^2\) to 945 Å\(^2\), corresponding to a relative increase of the hydrophobic face of 5% (see Table S3). The clustering of the N-terminal lipid chain with Trp-1 and Kyn-13 was recognized from the NOESY spectra of Ca\(^{2+}\)-conjugated daptomycin but was not realized in the calculated structure because of the inability to assign the individual hydrocarbon chain resonances. The total charge reduction of daptomycin combined with the increase in amphipathicity and, to a lesser extent, the increase in hydrophobic surface upon binding of Ca\(^{2+}\) may promote peptide oligomerization, which may contribute to the resonance line broadening observed in the NMR spectra (Figure 3).

Membrane Depolarization Studies

Previous research into the mechanism of action of daptomycin indicated that treatment of susceptible
Table 1. Statistical Analysis for the NMR-Derived Structures of Apodaptomycin and Ca\(^{2+}\)-Conjugated Daptomycin

<table>
<thead>
<tr>
<th></th>
<th>Apodaptomycin</th>
<th>Ca(^{2+})-Conjugated Daptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of NOE restraints</td>
<td>82</td>
<td>86</td>
</tr>
<tr>
<td>Interresidue</td>
<td>35</td>
<td>44</td>
</tr>
<tr>
<td>Intraresidue</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td>No. of NOE restraint violations &gt; 0.1 (\text{Å})</td>
<td>69 ± 3</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Average highest NOE restraint violation ((\text{Å}))</td>
<td>0.26 ± 0.03</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Average Pairwise Rmsd to the Mean ((\text{Å}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backbone</td>
<td>1.95 ± 0.09</td>
<td>3.08 ± 0.13</td>
</tr>
<tr>
<td>Heavy Atoms</td>
<td>1.15 ± 0.10</td>
<td>3.04 ± 0.13</td>
</tr>
<tr>
<td>Backbone</td>
<td>1.22 ± 0.09</td>
<td>2.16 ± 0.12</td>
</tr>
<tr>
<td>Heavy Atoms</td>
<td>1.07 ± 0.06</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>2.09 ± 0.07</td>
<td>1.48 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.58 ± 0.02</td>
<td>1.29 ± 0.07</td>
</tr>
</tbody>
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Structures were calculated using the DGII program in Insight II v.97.2 (Accelrys, San Diego, CA).

\(\text{A}\) Expressed as mean ± standard deviation of 15 (apodaptomycin) and 17 (Ca\(^{2+}\)-conjugated daptomycin) accepted structures of 40 calculated.

\(\text{B}\) Calculated using MOLMOL 2K.2 [37].

**S. aureus** cells with 100 \(\mu\)g/ml daptomycin resulted in the dissipation of membrane potential [14], while daptomycin-resistant strains of **S. aureus** had an altered membrane potential [9]. More recently, a correlation between membrane depolarization and the bactericidal activity of daptomycin against growing **S. aureus** was observed.
S. aureus interactions with calcium and membranes. In this study, the viability of daptomycin-treated nongrowing S. aureus was assessed concurrently with membrane depolarization measured with the membrane potential-sensitive dye, DiSC\textsubscript{3}. Bacterial survival (○) was measured at discrete times over a period of 90 min using the membrane potential-sensitive dye, DiSC\textsubscript{3}.5. Bacterial survival (●) over the 90 min was estimated at discrete times by colony counts. Both experiments were done in parallel.

at 2-fold the minimal inhibitory concentration [13]. In this study, the viability of daptomycin-treated nongrowing S. aureus was assessed concurrently with membrane depolarization measured with the membrane potential-sensitive dye, DiSC\textsubscript{3}.5 [13]. We utilized in these studies 10-fold the MIC of daptomycin, since lower concentrations of daptomycin, closer to the MIC, showed no depolarization of nongrowing cells within the 90 min tested. When S. aureus was treated with 10 μg/ml of daptomycin/5 mM CaCl\textsubscript{2}, 99% bacterial killing but only 36% membrane depolarization was observed within 90 min (Figure 6). The majority of the killing occurred within the first 10 min, whereas membrane depolarization was not evident until 20 min. This delay between the bactericidal affects of daptomycin and the start of membrane depolarization indicated that depolarization may be a consequence rather than the cause of bacterial death.

Significance

We demonstrated here that Ca\textsuperscript{2+} is required for two distinct conformational changes, each of which separately impacted how daptomycin interacted with membranes. Recently, Silverman et al. [13] proposed a multistep model for the mechanism of action of daptomycin. In their model, Ca\textsuperscript{2+} initially binds to daptomycin that is weakly bound to the cytoplasmic membrane and causes a conformational change that leads to the insertion and subsequent oligomerization of daptomycin within the membrane. On the basis of the studies reported here, we propose significant modifications to this model. The S. aureus cytoplasmic membrane contains both neutral and acidic phospholipids. Initially, Ca\textsuperscript{2+} binds to daptomycin in solution and causes a conformational change that increases amphipathicity and decreases its charge, thus allowing daptomycin to interact with either neutral or acidic membranes (as reflected by our fluorescence and lipid flip-flop measurements). Subsequently, Ca\textsuperscript{2+} may act as a bridge between daptomycin and acidic phospholipids, leading to a second conformational change that promotes deeper insertion of daptomycin into the membrane and large membrane perturbations, as indicated by our CD spectroscopy and calcein release studies. This two-step model would explain how this anionic peptide gains access to and interacts with bacterial cytoplasmic membranes. Silverman et al. [13] have argued that the bactericidal activity of daptomycin is due to cytoplasmic membrane depolarization caused by the leakage of intracellular ions, such as K\textsuperscript{+}. Although, in our hands, daptomycin was able to induce membrane leakage in liposomes, our observation that cytoplasmic membrane depolarization (in nongrowing cells) occurred subsequently to cell death indicates that it may not be the cause of cell death. Rather, we propose that the mechanism of action of daptomycin may involve multiple targets, as proposed previously for cationic peptides [17, 28–30]. Several other calcium-dependent lipopeptide antibiotics are known, including amphomycin, calcium-dependent antibiotic (CDA), and fruulimicin [31]. It is tempting to speculate that, since these antibiotics are thematically similar, they might share similar kinds of interactions with calcium and membranes.

Experimental Procedures

Liposome Preparation

Large unilamellar liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) or an equimolar mixture of PC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-1-glycerol (PG) (Avanti Polar Lipids Inc., Alabaster, AL) liposomes were prepared by resuspension of a dried lipid film in 20 mM HEPES buffer (pH 7.4), four freeze-thaw cycles, and extrusion through 0.1 μm filters as previously described [19].

Circular Dichroism Spectroscopy

CD spectra were recorded with 6 μM daptomycin in 20 mM HEPES buffer (pH 7.4) using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). The Ca\textsuperscript{2+}-conjugated daptomycin samples were preincubated with 5 mM CaCl\textsubscript{2}. Large unilamellar liposomes were added to a final concentration of 180 μM lipid. Spectra were acquired at room temperature from 190 to 250 nm using a quartz cuvette with a 1 mm path length at a scan rate of 50 nm/min and a bandwidth of 0.1 nm; spectra were the average of ten scans. Each spectrum was baseline corrected by subtracting a blank spectrum, and ellipticities were converted to mean residue ellipticities in units of deg x cm\textsuperscript{2}/dmol using Jasco software.

Fluorescence Spectroscopy

Fluorescence spectra were recorded with 10 μM daptomycin in 20 mM HEPES buffer (pH 7.4) using a Perkin-Elmer 650-10S fluorescence spectrometer (Perkin Elmer Inc., Norwalk, CT). Spectra were acquired at room temperature from 400 to 520 nm at an excitation wavelength of 365 nm with the slit width set to 5 nm. The Ca\textsuperscript{2+}-conjugated daptomycin samples were preincubated with 5 mM CaCl\textsubscript{2}. Large unilamellar liposomes were added to a final concentration of 300 μM lipid. Each spectrum was baseline corrected by subtracting a blank spectrum.

Lipid Flip-Flop and Calcein Release

Lipid flip-flop experiments employing large unilamellar liposomes that were asymmetrically labeled with 0.5 mol% 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-caproyl]-L-α- phosphatidyl choline (C\textsubscript{18}-NBD-PC) (Avanti Polar Lipids Inc., Alabaster, AL) were performed exactly as described [19]. Calcein release experiments were done using large unilamellar liposomes with entrapped calcein exactly as described previously [19]. Daptomycin samples

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**Figure 6. Relationship between Cytoplasmic Membrane Depolarization and Killing of S. aureus Caused by Daptomycin**

The cytoplasmic membrane depolarization (●) was measured at discrete times over a period of 90 min using the membrane potential-sensitive dye, DiSC\textsubscript{3}. Bacterial survival (○) over the 90 min was estimated at discrete times by colony counts. Both experiments were done in parallel.
were preincubated with 5 mM CaCl₂ for 1 hr prior to the experiment. Lipid flip-flop and calcein release are relative to Triton X-100.

NMR Spectroscopy and Structure Calculation
Daptomycin was provided by Cubist Pharmaceuticals. The apodaptomycin NMR sample contained 2 mM daptomycin, 100 mM KCl, 0.2 mM EDTA, 1 mM EGTA, and 7% D₂O (pH 6.6). The Ca²⁺-conjugated daptomycin NMR sample contained 2 mM daptomycin, 100 mM KCl, 0.2 mM EDTA, 5 mM CaCl₂, and 7% D₂O (pH 6.7). NMR spectra were recorded on Varian Unity500 or Inova600 spectrometers operated by the UBC Laboratory for Molecular Biophysics. Homonuclear TOCSY (spin lock time = 60 ms) [32], NOESY (τₑ = 150 ms) [33], and DQF-COSY [34] spectra were initially collected at 25°C. Additional TOCSY and NOESY spectra were collected for apodaptomycin at 17°C (τₑ = 150 ms) and for Ca²⁺-conjugated daptomycin at 30°C (τₑ = 150 ms) and 35°C (τₑ = 150–250 ms).

All NMR spectra were processed using NMRPipe [35] and analyzed using NMRVIEW v.5.0.4 [36]. NOE crosspeaks for apodaptomycin were integrated in the 17°C NOESY spectrum (τₑ = 150 ms). NOE crosspeaks for Ca²⁺-conjugated daptomycin were integrated in the 35°C NOESY spectrum (τₑ = 150 ms) and further supplemented by additional NOE crosspeaks from the 25°C NOESY spectrum (τₑ = 150 ms) after both spectra were compared for any indications of conformational differences. Conversion of NOE volumes to distance restraints and pseudooatom corrections were calculated as described [29]. Structure calculations were performed using the DGIII module of Insight II v.97.2 (Accelrys Inc., San Diego, CA). Calculated structures were accepted based on the lowest NOE distance restraints violations and best convergence. The structures were further analyzed using MOLMOL v.2.0.1 [37].

Cytoplasmic-Membrane Depolarization and Bacterial Killing
The depolarization of the cytoplasmic membrane of *S. aureus* by daptomycin was determined with the membrane potential-sensitive dye 3,3'-dipropylthiacyanocarmine (DiSC₃(5) (Molecular Probes, Eugene, OR) as previously described [17]. Briefly, *S. aureus* cells were grown to mid-log phase and then washed and resuspended in 5 mM HEPES/20 mM glucose buffer to a final OD₆₀₀ of 0.05. The suspension was equilibrated in 0.1 M DisC₃(5) and 100 mM KCl until the fluorescence (excitation at 622 nm, emission at 670 nm) was completely quenched. Daptomycin (10 μM) and 5 mM CaCl₂ were added, and the increase in fluorescence due to membrane depolarization and dye release was recorded. Bacterial viability was assessed in parallel by plating aliquots withdrawn from an identical cell suspension at the same time points to final dilutions of 10⁻² and 10⁻⁴ and counting the bacterial colonies grown overnight.

Supplemental Data
Supplemental Data including two figures and three tables are available at http://www.chembiol.com/cgi/content/full/11/7/949/DC1.

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References

Accession Numbers
The NMR structures for apo and Ca^{2+}-conjugated daptomycin were submitted to the Protein Data Bank (accession numbers 1T5M and 1T5N, respectively). The chemical shifts for apo and Ca^{2+}-conjugated daptomycin were deposited in the BioMagResBank (BMRB) (accession numbers RCSB022341 and RCSB022342, respectively).