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Background: Daptomycin forms oligomeric pores in bacterial cell membranes. Cardiolipin is a membrane lipid associated with bacterial resistance to the antibiotic.

Results: Cardiolipin makes liposomes impervious to daptomycin permeabilization, and it confines daptomycin to the outer membrane leaflet.

Conclusion: Preventing daptomycin from reaching the inner membrane leaflet inhibits pore formation.

Significance: Bacteria may become resistant to daptomycin by changing their membrane lipid composition.

Daptomycin is an acidic lipopeptide antibiotic that, in the presence of calcium, forms oligomeric pores on membranes containing phosphatidylglycerol. It is clinically used against various Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus* species. Genetic studies have indicated that an increased content of cardiolipin in the bacterial membrane may contribute to bacterial resistance against the drug. Here, we used a liposome model to demonstrate that cardiolipin directly inhibits membrane permeabilization by daptomycin. When cardiolipin is added at molar fractions of 10 or 20% to membranes containing phosphatidylglycerol, daptomycin no longer forms pores or translocates to the inner membrane leaflet. Under the same conditions, daptomycin continues to form oligomers; however, these oligomers contain only close to four subunits, which is approximately half as many as observed on membranes without cardiolipin. The collective findings lead us to propose that a daptomycin pore consists of two aligned tetramers in opposite leaflets and that cardiolipin prevents the translocation of tetramers to the inner leaflet, thereby forestalling the formation of complete, octameric pores. Our findings suggest a possible mechanism by which cardiolipin may mediate resistance to daptomycin, and they provide new insights into the action mode of this important antibiotic.

Daptomycin is a lipopeptide antibiotic that consists of 13 amino acids, several of which are nonstandard ones. The 10 C-terminal amino acids form a ring that is closed by an ester bond between a kynurenine residue and a threonine residue, whereas a decanoyl residue is attached to the exocyclic N-terminal tryptophan (Fig. 1). The antibacterial action mode of daptomycin is not yet fully understood and may be multifac-

eted. Membrane permeabilization and interference with cell division have been demonstrated, whereas inhibition of cell wall synthesis remains somewhat contentious (1). Membrane permeabilization can be observed in model liposomes and involves the formation of an oligomeric and cation- and size-selective pore. Oligomerization and pore formation require phosphatidylglycerol (PG),² which is abundant in bacterial cell membranes but not in mammalian ones; this presumably contributes to the selectivity of the drug for bacteria (2–5).

Since its approval for clinical use in 2003, daptomycin has assumed a major role in the treatment of infections with Gram-positive pathogens. Although the overall resistance situation is currently still favorable, resistant strains of *Staphylococcus aureus* and *Enterococcus* species occur. Genomic analysis of resistant strains indicate that resistance may involve changes in the lipid composition of the bacterial cell membrane. Such changes include the reduced synthesis of PG (4) and the increased conversion of PG to lysyl-phosphatidylglycerol (lysyl-PG (6)). Because lysyl-PG is positively charged, it likely detracts from the calcium-mediated membrane binding of daptomycin, which would resemble its known inhibitory effect on cationic antimicrobial peptides.

Another set of mutations implicates cardiolipin. Mutations that enhance cardiolipin synthase activity (7) were found in resistant *Enterococcus* strains (8), although replacement of the wild-type synthase with a mutant gene into a wild-type *Enterococcus faecalis* strain did not detectably change the susceptibility to daptomycin (9). Quantitative data on cardiolipin membrane levels in daptomycin-resistant *Enterococcus* strains are not available; therefore, whether or not changes in cardiolipin content can increase the daptomycin resistance *in vivo* remains to be elucidated. Here, we examined the question whether or

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² The abbreviations used are: PG, phosphatidylglycerol; PC, phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1'-glycerol); PE, phosphatidylethanolamine; CL, cardiolipin; TOCL, tetraoleyl-cardiolipin; LUV, large unilamellar vesicle(s); NBD, Nitrobenzoxadiazole; HBS, HEPES-buffered saline; ITC, isothermal calorimetry.

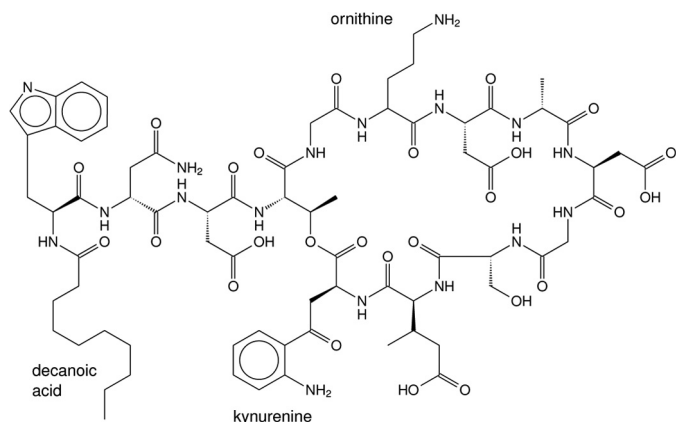


FIGURE 1. Structure of daptomycin. In NBD-daptomycin, the free amino group of ornithine is modified with NBD. In perylene-daptomycin, the N-terminally attached decanoyl residue is replaced by perylene-butanoic acid (13). Kynurenine has intrinsic fluorescence that can be used for FRET experiments in conjunction with NBD (15).

not cardiolipin can in principle affect the susceptibility of membranes to daptomycin. We found that cardiolipin may indeed protect lipid membranes from permeabilization. The inhibitory mechanism of cardiolipin is novel and surprising, and it provides further insight into the mechanism of daptomycin pore formation.

EXPERIMENTAL PROCEDURES

Preparation of Liposomes (LUV) for Fluorescence Experiments—Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used as received. For fluorescence experiments, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-rac-(1'-glycerol) (DMPG) as well as tetraoleyl-cardiolipin (TOCL) were dissolved in chloroform:methanol 3:1, mixed at the molar ratios indicated under “Results,” and dried down under nitrogen and vacuum in a round bottom flask. The lipid film was dispersed in 100 mM NaCl buffered with 10 mM Hepes (pH 7.4; Hepes-buffered saline (HBS)), and the lipid suspension was extruded 10–15 times through 100-nm polycarbonate membranes with a nitrogen-driven device (10). The liposomes were used in fluorescence experiments at a final concentration of 250 μ M total lipid. All experiments involving these liposomes were carried out at 30 °C.

Preparation of Liposomes (LUV) for ITC Experiments—LUV were prepared using the freeze-thaw and extrusion method as described previously (11). Lipid films were prepared by mixing appropriate volumes of pure DOPC, DOPG, and TOCL (Avanti) from stock solutions in chloroform and evaporating the solvent under a stream of nitrogen. The resulting films were hydrated in HBS (10 mM Hepes, 100 mM NaCl, 1 mM CaCl₂, pH 7.4) to a final total lipid concentration of 5 mM.

Measurement of Membrane Permeabilization with Pyranine-loaded Liposomes—This procedure was carried out exactly as described.³ Briefly, LUV for fluorescence experiments were prepared as above, but with an internal pH of 6.0 and entrapped pyranine (5 mM internal concentration). The liposomes were

diluted into Hepes (10 mM, pH 8.0)-buffered potassium chloride (100 mM) with 5 mM CaCl₂ and supplemented with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 5 nM, valinomycin (0.5 μ M), and daptomycin (1 μ M) as indicated. After incubation for 300 s, the samples were solubilized with Triton X-100, and the fluorescence signal recorded thereafter was used to normalize the entire data set.

Measurements of Perylene Excimer Fluorescence—Perylene-daptomycin was prepared as described before (13). The compound was added to LUV at 2 μ M. Calcium was added to 5 mM, the samples were incubated for 5 min, and the fluorescence emission were recorded with excitation at 430 nm.

Isothermal Calorimetry—Energetics of daptomycin binding to model membranes was investigated by high sensitivity isothermal titration calorimetry (MicroCal VP-ITC, GE Healthcare) as described in Ref. 11. Briefly, the sample cell (~1.4 ml) was filled with 23 μ M daptomycin prepared in HBS containing 1 mM calcium, and the reference cell was filled with identical buffer. The 250- μ l syringe was loaded with 5 mM total lipid LUV suspension. Daptomycin solutions and lipid suspensions were degassed with stirring under vacuum for 20 min prior to each titration. After system equilibration and a preliminary 1- μ l injection that was omitted from data analysis, 2.5- μ l aliquots were injected into the sample cell at 6-min intervals. Data were collected by the MicroCal customized Origin software. Baselines were normalized using Nitpic (14), and the results were analyzed with the Origin “one binding site” model.

Langmuir Monolayers—The ability of daptomycin to penetrate lipid monolayers was examined using a Langmuir surface balance and custom-built mini-trough from KSV-Nima (Biolin Scientific, Espoo, Finland). The aqueous subphase was HBS with 1 mM calcium, prepared with water purified through a Milli-Q system (Millipore, Molsheim, France). Lipid solutions were prepared by dissolving DOPC, DOPG, and TOCL in spectroscopy-grade chloroform to concentrations of ~1 mg/ml. The trough was filled with buffer, and the surface was cleaned by aspirating. Small aliquots of lipid solution were applied to the surface using a Hamilton digital syringe (Reno, NV) until an initial surface pressure of 20 mN/m was obtained. After allowing 10 min for solvent evaporation and monolayer equilibration, 50 μ l of 1 mM daptomycin, prepared in identical buffer, were injected into the stirred subphase through a side port to avoid puncturing the monolayer. Daptomycin insertion into the lipid monolayer was monitored over time and detected by surface pressure increase at constant area. Each experiment was performed at least in triplicate.

Determination of Daptomycin Oligomer Subunit Stoichiometry by FRET—Nitrobenzoxadiazole-daptomycin (NBD-daptomycin) was prepared as described in Ref. 15. Fluorescence experiments and data analysis were performed exactly as described previously (16), except that the lipid composition of the liposomes was varied as indicated under “Results.”

Dithionite Quenching of NBD Fluorescence—For the experiment shown in Fig. 8A, NBD-phosphatidylethanolamine (NBD-PE) was obtained from Avanti and incorporated at 0.5% mol/mol into LUV that otherwise consisted of equal fractions of PC and PG. The liposomes were diluted into HBS supplemented with 25 mM calcium. We found it necessary to raise the

³ T. Zhang, J. Muraih, B. MacCormick, and M. Palmer, M., submitted for publication.

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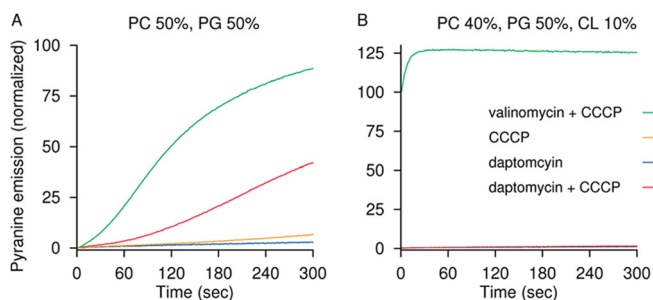


FIGURE 2. Permeabilization by daptomycin of liposomes composed of PC and PG (A) and PC, PG, and CL (B). The pH-sensitive fluorophore pyranine was entrapped in liposomes. The increase in its fluorescence by the efflux of protons was mediated by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in exchange for potassium influx mediated by valinomycin (green) or daptomycin (red). Valinomycin is active on both membranes (but acts faster on the ones containing CL), whereas daptomycin permeabilizes only the membranes devoid of CL. The fluorescence intensity is scaled to that observed after solubilization with Triton X-100, which equals 100 units.

concentration this high from the usual value of 5 mM to induce reaction between dithionite and liposomes containing PG; we assume that calcium alleviates the electrostatic repulsion between PG and dithionite. Daptomycin was added to 1 μ M where indicated, and the samples were incubated for 5 min before starting the fluorescence measurement (excitation wavelength, 465 nm; emission wavelength, 530 nm). After 1 min, dithionite was added (final concentration 10 mM), and the measurement was continued for \sim 5 min before the addition of Triton X-100 to solubilize the liposomes.

The experiments illustrated in Fig. 8B were performed analogously, except that NBD-PE was omitted from the liposomes, and daptomycin was replaced with a mixture of NBD-daptomycin and native daptomycin (molar ratio 1:2; total final concentration 1 μ M). The inclusion of native daptomycin served to reduce NBD self-quenching.

RESULTS

Cardiolipin Inhibits Membrane Permeabilization by Daptomycin—In a recent study, we showed that daptomycin forms cation-selective pores of discrete size in liposomes composed of PC and PG.³ As shown in Fig. 2, the addition of as little as 10% cardiolipin completely inhibits this pore formation.

In previous studies, we have shown that membrane permeabilization by daptomycin occurs in several successive stages, namely, monomer binding, oligomerization, and conversion of the oligomer to a functional pore (11, 13, 15, 16). The following experiments were carried out to determine which of these stages is subject to inhibition by cardiolipin.

Binding and Oligomerization of Daptomycin on Membranes Containing Cardiolipin—The binding of daptomycin to lipid membranes correlates with a strong increase in the intrinsic fluorescence of its kynurenine residue (17). Binding also requires calcium, and the calcium concentration required to induce the half-maximal extent of this fluorescence increase ($[Ca]_{50}$) can be used to compare the affinity of daptomycin for membranes differing in lipid composition. The $[Ca]_{50}$ is \sim 12 mM calcium with pure DMPC membranes, 0.2 mM with DMPC/DMPG membranes (1:1), and 2 mM with DMPC/TOCL membranes. Thus, CL binds daptomycin more avidly than PC but less avidly than PG. When CL is added to PC and PG, the

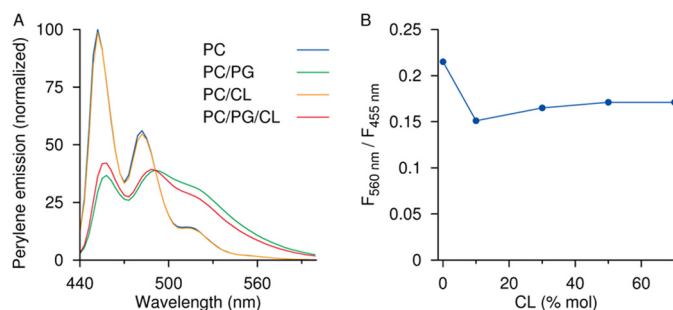


FIGURE 3. Fluorescence of perylene-daptomycin on liposomes containing PC alone or in various combinations with PG (mole fraction 30%) and CL (mole fraction 10%). A, on PC membranes as well as on PC/CL membranes, the spectrum is characteristic of the perylene monomer, indicating that oligomer formation does not occur. PC/PG membranes with or without 10% CL show a diminished monomer peak and a broad, overlapping excimer peak indicative of oligomer formation (13). The extent of excimer formation is slightly lower in the sample with CL. B, the ratio of emissions at 560 and 445 nm can be used to compare the extent of excimer formation. This ratio is shown here as a function of the CL mole fraction (0–70%). PG was constant at 30%, and the balance was made up by PC.

$[Ca]_{50}$ remains close to 0.2 mM, which suggests that on such membranes, daptomycin interacts with PG rather than CL (data not shown).

Oligomerization of membrane-bound daptomycin can be observed through excimer formation of a perylene-labeled daptomycin derivative (13). Fig. 3A shows the fluorescence of perylene-daptomycin on membranes containing PC, PG, and CL in various combinations. As reported in Ref. 13, the emission spectrum observed on PC membranes is typical of perylene monomers, indicating the absence of daptomycin oligomer formation. On membranes consisting of PC and PG, monomer intensity is reduced, and an overlapping, broad-based peak excimer peak centered around 520 nm appears, indicating daptomycin oligomer formation on these membranes.

The spectrum obtained with membranes containing PC and CL, but no PG, closely resembles the one from pure PC liposomes. Therefore, unlike PG, cardiolipin does not induce oligomerization of daptomycin. In contrast, membranes that contain both PG and CL do induce formation of excimers, and hence of daptomycin oligomers.

The extent of excimer formation can be compared using the ratio of the emission at 560 nm, where monomer fluorescence is negligible, with that at 455 nm, where monomer fluorescence is dominant. In Fig. 3B, this ratio is shown as a function of CL molar fraction in the membrane. The extent of excimer formation drops noticeably from 0 to 10% CL and then increases slightly again as more CL is added. The potential significance of this finding is discussed below.

Another method for observing daptomycin oligomerization is the use of FRET between the kynurenine residue of the unlabeled molecule and the fluorescent label NBD attached to the ornithine side chain of a second daptomycin molecule; the NBD derivative retains full antibiotic activity (15). This method has also been used to estimate the number of subunits in a single daptomycin oligomer to 6–7 (16). In those previous experiments, PC/PG membranes were used, which allow the formation of functional pores (*cf.* Fig. 2A). Because the addition of CL prevents pore formation, we considered that the oligomers that form in the presence of CL might be structurally different and

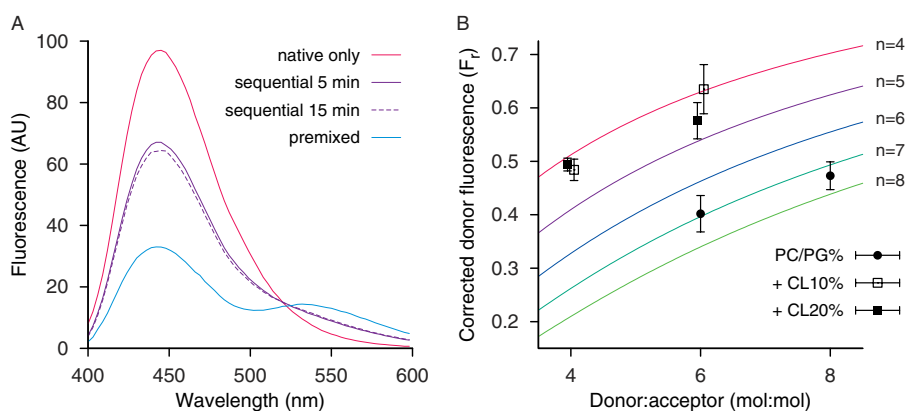


FIGURE 4. Estimation of subunit stoichiometry of the daptomycin oligomer by FRET between native daptomycin (donor) and NBD-daptomycin (acceptor). *A*, native daptomycin ($3 \mu\text{M}$ final) and NBD-daptomycin ($0.5 \mu\text{M}$ final) were added to liposomes containing PC (40%), PG (50%), and CL (10%), either sequentially, with incubation for 5 min between additions, or premixed. Emission spectra were acquired 5 and 15 min after the final addition. The peak at 450 nm represents the kynurenine emission of native daptomycin, whereas the peak at 540 nm is due to NBD. The donor emission of the sequential sample is higher than that of the premixed sample, which is due to the formation of segregated, stable oligomers. It is lower than that of donor alone, which is due to FRET between those segregated oligomers. The emission of the sequential sample remains almost the same after 15 min, which shows that the oligomers are stable on the time scale of the experiment. *AU*, arbitrary units. *B*, the two species were mixed before application to liposomes containing PG (50%), CL (0–20%, as indicated), and PC (balance to 100%) to induce the formation of hybrid oligomers. The corrected relative donor fluorescence (F_r) was obtained as described in Ref. 16. The colored lines represent theoretical F_r functions for the indicated numbers of subunits; each data point represents the means \pm S.D. of 3 or 4 repeated experiments.

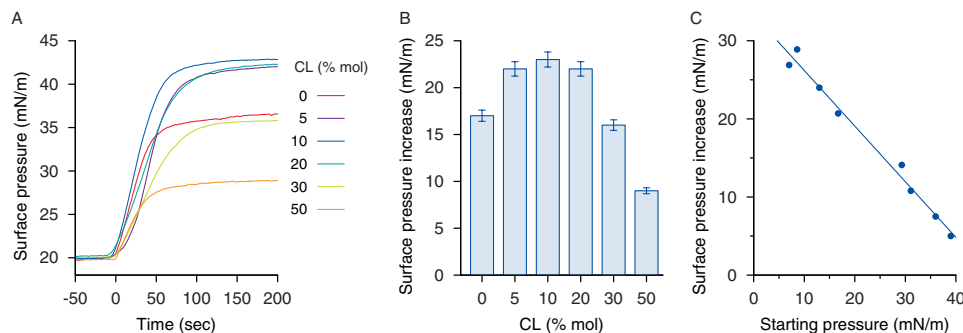


FIGURE 5. Surface pressure change of lipid monolayers consisting of CL (% mol/mol as indicated) and equal fractions of PC and PG, in response to injection of daptomycin into the subphase. *A*, time profiles; daptomycin was injected at $t = 0$ and at initial surface pressure of 20 microneutons/m. *B*, changes in surface pressure derived from the data in panel *A*. Results were reproducible to within 5% ($n = 3$ –5). Each data point represents the means \pm S.D. of 3–5 repeated experiments. *C*, surface pressure change as a function of initial pressure on PC/PG membranes without CL. The surface pressure increment drops linearly with the starting pressure. The slope of the regression line is -0.885 ; at a slope of -1 , the final surface pressure would be entirely independent of the starting pressure.

contain a different number of subunits. The method used in this experiment is fully explained in Ref. 16. Briefly, native daptomycin and NBD-daptomycin are mixed in various proportions and then applied to liposome membranes. Based on the assumptions of 1) oligomer stability on the time scale of the experiment, 2) random co-oligomerization, and 3) maximally effective FRET between the subunits of the same oligomer, and using corrections for FRET between molecules that are part of separate oligomers, the number of subunits is calculated from the residual observed donor fluorescence.

Fig. 4 shows that, indeed, the presence of cardiolipin changes the subunit stoichiometry. With cardiolipin, there were close to four subunits, whereas without CL, the number was close to seven, which is similar to the previously reported value (16). These observations will be further discussed below.

Interaction of Daptomycin with Membranes Containing CL by Surface Pressure and Isothermal Calorimetry (ITC)—To better understand the effect of CL on the membrane interaction of daptomycin, we studied a similar model system with Langmuir monolayers and with ITC. The insertion of daptomycin into

PC/PG lipid monolayers can be observed as an increase in surface pressure. The addition of CL to this lipid mixture up to a molar fraction of 10% substantially enhances this response (Fig. 5, *A* and *B*). This increase in the surface pressure differential indicates a deeper and/or more stable insertion of daptomycin molecules into monolayers containing CL. The surface pressure differential decreases again when CL is added to molar fractions of 20% or greater.

ITC was performed with LUV, which were added to a solution of daptomycin and calcium. Fig. 6 shows that, relative to liposomes containing PC and PG only, the addition of CL up to a molar fraction of 10% raised the enthalpy released by daptomycin binding significantly. This increase in negative enthalpy indicates a greater number of favorable molecular interactions between daptomycin and the lipid molecules of the membrane, which is consistent with the deeper membrane insertion of daptomycin molecules inferred from the surface pressure experiments.

The increase in negative enthalpy was accompanied by a decrease in entropy, as obtained by fitting a single-site binding

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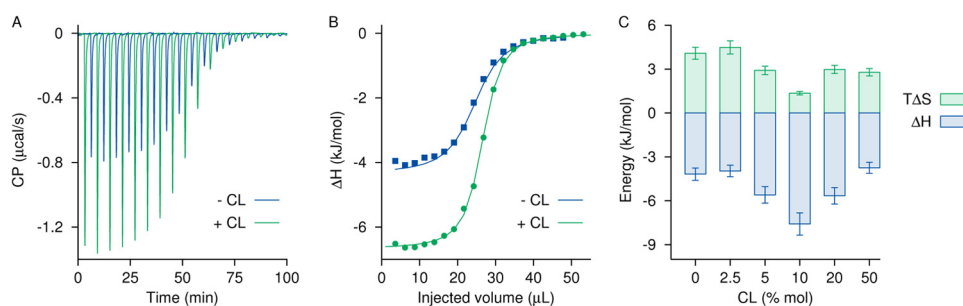


FIGURE 6. ITC experiments on daptomycin binding to PC/PG membranes with or without CL. *A*, representative raw traces. Each peak is caused by the injection of 2.5 μL of liposome suspension (see “Experimental Procedures” for details). *CP*, chlorophenylhydrazone. *B*, integrals (data points) and single-site model fit curves for the traces shown in *A*. *C*, energies of interaction between daptomycin and liposome membranes (mole fraction of CL as indicated, balance of equal parts of PC and PG) obtained from a series of isothermal titrations ($n = 2\text{--}3$ at each lipid composition). Each data point represents the means \pm S.D. of 3 or 4 repeated experiments.

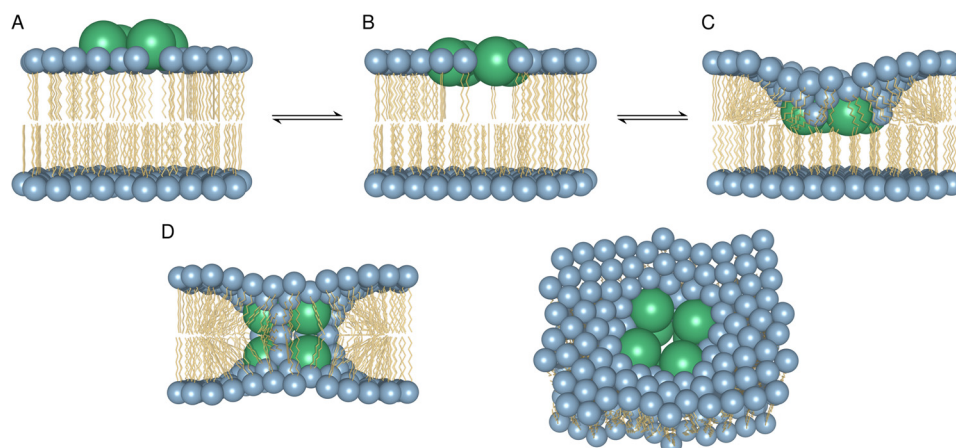


FIGURE 7. Hypothetical model of daptomycin membrane insertion, pore formation, and pore structure. *A*, a daptomycin tetramer forms in the outer membrane leaflet. *B*, its insertion into the head group layer is limited by the distension and creation of voids in the acyl chain layer; this is unfavorable in terms of enthalpy and increases disorder (entropy). If CL is present, it provides extra bulk in the acyl chain layer, which stabilizes this situation and thus allows for deeper penetration of daptomycin. *C*, if CL is not present, head group distension and voids in the acyl layer may alternatively cause the outer leaflet to cave in, forming a half-toroidal structure. *D*, a tetramer as shown in *C* may flip to the other leaflet and then combine with a second tetramer in the outer leaflet, which gives an octameric pore.

model to the raw data. At a molar fraction of 20% or more CL, the changes to both the enthalpy and the entropy were partially reversible.

It may be noted that the response to changes in CL molar fraction was similar in three different assays, namely surface pressure, ITC, and perylene excimer fluorescence, which suggests that these assays reflect the same underlying effect of CL on the membrane interaction of daptomycin. In all three assays, the maximum signal was observed at a CL mole fraction of 10%, whereas higher mole fractions caused a partial reversal. We currently have no experimental basis on which to propose an explanation for this reversal; it may be noted that molar fractions of CL higher than 10% are not common in bacterial membranes.

Cardiolipin Inhibits Membrane Translocation of Daptomycin—One question that had not been addressed in our previous studies is how daptomycin distributes between the two leaflets of the target membrane. The observation that cardiolipin both prevented pore formation and reduced the number of oligomer subunits by approximately half suggested the possibility that a functional pore might comprise subunits in both leaflets, whereas the nonfunctional oligomers that form in the presence of cardiolipin might be confined to the outer membrane leaflet only (Fig. 7). This hypothesis was tested using the

fluorescence of NBD-daptomycin and its sensitivity to quenching with dithionite.

NBD is instantly reduced, and its fluorescence is abolished, by dithionite. Because dithionite is membrane-impermeant, this reaction can be used in liposome models to detect the accessibility of membrane-associated molecules from the outside (18). A potential complication that arises here, however, is that daptomycin pores might allow dithionite to reach the liposome interior and reduce NBD-daptomycin also bound to the inner leaflet. Therefore, in a control experiment, a small amount of NBD-PE was incorporated into PC/PG liposomes, and its reduction by dithionite was monitored both with and without daptomycin. In both cases, the extent of reduction is close to 50%, which is consistent with a reduction of NBD-PE in the outer leaflet only (Fig. 8*A*). Thus, in keeping with the previously observed cation selectivity of the daptomycin pore,³ dithionite does not appreciably enter the liposomes on the time scale of the experiment. Dithionite was also excluded by liposomes containing CL in addition to PC and PG, with or without daptomycin (data not shown).

Fig. 8*B* depicts the results obtained with NBD-daptomycin; no NBD-PE was present in these experiments. When NBD-daptomycin was bound to membranes containing PC, PG, and either 10% or 20% CL, almost all NBD fluorescence was imme-

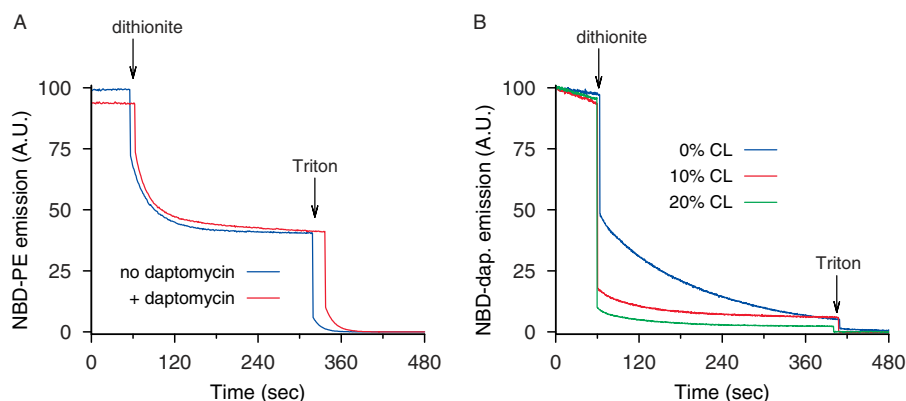


FIGURE 8. **Distribution of daptomycin across inner and outer leaflets, examined with NBD fluorescence quenching using dithionite.** *A*, control (dithionite (10 mM)) reduces only 50% of NBD-PE (0.5% in PC/PG membranes), both in the absence and in the presence of daptomycin. Therefore, dithionite continues to be excluded from membranes in the presence of daptomycin pores. *A*, U., absorbance units. *B*, in PC/PG membranes containing 10 or 20% CL, most of the NBD-daptomycin (NBD-dap.) is reduced by dithionite instantly, indicating that it is confined to the outer leaflet. In contrast, in PC/PG membranes containing no CL, immediate reduction affects only 50% of NBD-daptomycin, whereas the remainder is reduced at a slower pace, presumably rate-limited by redistribution from the inner to the outer leaflet.

diately quenched. In contrast, the extent of immediate quenching was only about 50% in membranes that contained PC and PG but no CL. The remainder of the fluorescence decayed more slowly with a half-life of ~60–90 s, which is presumably due to the redistribution of daptomycin from the inner to the outer leaflet. These findings support the notion that daptomycin is able to distribute symmetrically and reversibly between both membrane leaflets in PC/PG membranes, but unable to translocate across membranes containing 10 or 20% CL.

DISCUSSION

In this study, we have shown that cardiolipin inhibits the pore-forming activity of daptomycin in a liposome model. This study was prompted by genetic studies on daptomycin resistance, which suggest that bacteria may become more resistant to daptomycin by increasing the content of cardiolipin in their cell membranes. We found that inclusion of 10% CL in model membranes was sufficient to effectively suppress membrane translocation and pore formation in liposomes. This fractional amount of CL is at the upper end of the range reported for bacterial cell membranes (19–21), which suggests that this mechanism may be relevant for bacterial resistance to daptomycin. Formation of daptomycin pores in bacterial cell membranes, as detected by membrane depolarization, is well documented (22–24). It seems possible that an increased cardiolipin content would inhibit pore formation *in vivo*; however, this has yet to be experimentally examined. Daptomycin may also damage bacteria by more than one mechanism. If so, inhibition of pore formation may not suffice to prevent bactericidal action, and additional resistance mechanisms would then be required to counter the other antibacterial action modes of the drug.

Whether or not the effect of CL observed *in vitro* has a role in bacterial resistance, the findings of our study do provide significant new insight into the mechanism of daptomycin action. Firstly, they show that oligomers can form in the outer leaflet alone. However, these oligomers do not form functional pores, and they do not reach the full number of subunits; instead, they contain only four subunits. Pore formation correlates with daptomycin reaching the inner membrane leaflet, and with an increase in the apparent subunit stoichiometry to 6–7.

To account for the collective findings, we propose a working model of pore formation that comprises the following steps (*cf.* Fig. 7): 1) calcium-mediated binding of monomeric daptomycin to PG in the outer leaflet of the target membrane; 2) formation of a tetramer from four bound monomers (this step occurs in the outer leaflet and is preserved in membranes that contain cardiolipin); 3) translocation of tetramers from the outer leaflet to the inner leaflet (this step is disrupted on membranes containing CL); and 4) alignment of two tetramers in the opposite leaflets to form an octameric functional pore.

In such an octamer, half of the subunits are located in the outer leaflet and hence subject to reduction by dithionite right away. The observation that the remainder of the NBD fluorescence is subject to protracted but ultimately complete reduction indicates that the translocation from the outer to the inner leaflet is reversible. The reverse translocation might conceivably involve the dissociation of octamers into tetramers. However, we could find no evidence of such a dissociation in preliminary experiments, and we therefore favor the view that translocation comes about through the trading of places between individual subunits within an intact octamer.

Although this model accounts for most of the available evidence without contortions, the postulated octamer obviously is at odds with the subunit stoichiometry (n) of 6–7 that was experimentally obtained with PC/PG membranes here and earlier (16). Two effects might account for this discrepancy. Firstly, it is possible that not all tetramers assemble into octamers. In that case, the experimentally determined n would reflect an average of tetramers and octamers, weighted for abundance. However, because we could not detect dissociation of octamers into tetramers, it seems likely that the number of free tetramers is low at equilibrium.

Secondly, as was pointed out in our previous study (16), the value of n obtained from FRET experiments may underestimate the true value. The efficiency of FRET is limited by the Förster distance, which was estimated to 2.7 nm for the couple of donor and acceptor labels used here (kynurenine in unlabeled daptomycin and NBD-daptomycin, respectively). The distance between subunits that belong to the same oligomer, yet reside

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in opposite membrane leaflets, might approach or even exceed this value, which would significantly diminish FRET. Because our analysis assumes FRET to be maximally effective between any two positions within the same oligomer (16), a less than maximal FRET efficiency will cause the calculated value of n to underestimate the true value. Based on the currently available evidence, we consider this the most likely explanation.

If we assume the above model to be correct, the question arises exactly how cardiolipin may inhibit membrane translocation of daptomycin tetramers. This inhibitory effect was accompanied by several other changes. The slight drop of perylene excimer fluorescence (Fig. 2B) may correspond to the fraction of excimers that form between perylene-daptomycin molecules in two opposite tetramers that are part of the same octamer, whereas the remaining excimer fluorescence arises from proximity within single tetramers. In liposomes, CL effected a notable increase in daptomycin binding enthalpy, which suggests an increase in the number of favorable molecular contacts (25). In lipid monolayers, CL caused a greater increase in surface pressure in response to a given quantity of daptomycin. This suggests a deeper insertion of daptomycin into membranes containing moderate amounts of CL, which should increase the number of molecular contacts between daptomycin and the surrounding lipids, and therefore agrees with the findings from the ITC experiments.

A similar inhibitory effect of CL on membrane pore formation has previously been observed with the antimicrobial peptide magainin, and together with related findings has been interpreted in terms of lipid curvature strain (26). According to this explanation, the peptide claims space in the head group layer of the membrane. CL and other “cone-shaped” lipid species that provide more bulk in the hydrophobic acyl chain layer than in the head group layer accommodate the peptide, whereas lipids with larger head groups compete with it for space, promoting distension and bending of the head group layer, which leads to the formation of a toroidal pore.

The mechanism proposed for magainin may also underlie the effect of CL on daptomycin (Fig. 7). Such a model might lead one to expect that, because of the greater competition for space in the head group layer of monolayers without CL, daptomycin insertion into such monolayers should raise the surface pressure to a greater extent than without CL, which is the opposite of what we observed (Fig. 5). Possibly, the unstable situation illustrated in Fig. 7B may relax by transitioning either toward the toroidal defect (Fig. 7, C and D) or toward a less deeply inserted configuration, in which the bulk of the peptide remains outside of the lipid membrane and does not add lateral pressure to it (Fig. 7A). In any event, an improved accommodation of daptomycin within CL-containing membranes would agree with the increase in negative enthalpy reported here. In this context, it is worth noting that CL is believed to be enriched in localized domains in bacterial cell membranes (27). This might account for the inhomogeneous distribution of daptomycin on cell surfaces (28); the correlation has, however, not been established.

A tighter interaction between daptomycin and membrane lipids might also account for the decrease in entropy that was observed concomitantly with increases in negative enthalpy.

However, one should keep in mind that the entropy values were obtained through the use of a single-site binding model, and although this model is able to numerically fit the ITC titration curves rather well, we must not forget that daptomycin not only binds as a monomer but subsequently also forms oligomers, so that any model that assumes a one-step interaction can only be considered an approximation. Caution is therefore required with respect to interpreting the calculated entropy.

In sum, we have obtained evidence that supports a novel structural model of the daptomycin pore and suggests a possible mechanism of bacterial resistance to daptomycin. We presently have no way to further corroborate this hypothetical model. Nevertheless, it is interesting to note that many cation channels and pores have tetradic symmetry. Intriguingly, with the *Pseudomonas* lipopeptide tolaasin I, whose structure is not closely related to daptomycin, the very same kind of octameric symmetry spanning both leaflets has been obtained from *in silico* studies (12). We therefore feel that this structural model deserves consideration in future studies.

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