Osmoprotection of Bacterial Cells from Toxicity Caused by Antimicrobial Hybrid Peptide CM15†

Hiromi Sato and Jimmy B. Feix*

Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

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ABSTRACT: Antimicrobial peptides exist ubiquitously as a host defense system in a broad range of species, including insects, amphibians, and mammals. The binding of these peptides is followed by the disruption of cytoplasmic membranes, leading to bacterial cell death; however, the precise mechanism of membrane destruction has remained controversial. In this study, we have examined the mechanism of action for the antimicrobial peptide, CM15 (KWKLFKKIGAVLKVL), a chimeric peptide of cecropin and mellitin. We find that the cytotoxicity of CM15 against either *E. coli* or *Pseudomonas aeruginosa* can be mitigated by the addition of sugar or poly(ethylene glycol) osmolytes to the extracellular media. The dependence of osmoprotection on solute size suggests the formation of pores with an effective diameter of 2.2−3.8 nm. In contrast, no osmoprotection was observed for cell killing by the cationic detergent dodecyltrimethylammonium bromide. Osmolytes also protected cells against the cytotoxicity of CM15 expressed intracellularly as a *C*-terminal extension of the carrier protein ketosteroid isomerase (KSI). Osmoprotection against the intracellularly produced peptide was also dependent on osmolyte size, in a manner that was in agreement with that observed for extracellularly added synthetic CM15. These data indicate that the formation of discrete pores in the cytoplasmic membrane is a key factor in the mechanism of bacterial killing by CM15.

Antimicrobial peptides (AMPs[1]) comprise an essential part of the innate immune system. These peptides, typically composed of between 12 and 50 amino acids, act rapidly to combat the invasion of potential pathogens and thus serve to limit the extent of infection prior to induction of the adaptive immune response provided by antibodies and cytotoxic lymphocytes. AMPs are widely distributed and display remarkable sequence diversity (*1*−*5*). However, despite this lack of sequence homology, most AMPs are cationic, and almost all either have a native amphipathic structure or fold into amphipathic structures in the presence of membranes (*2*, *5*, *6*), suggesting that they may share a common mechanism of action. These factors, along with the fact that synthetic peptides composed of D-amino acids retain activity equivalent to the natural L-amino acid enantiomers (*7*−*10*), have indicated the cell membrane as the likely target of AMP activity. Because of their apparently novel mode of action, AMPs are of great interest as lead compounds in the development of new antimicrobial agents for the treatment of emerging drug-resistant infections (*3*, *5*, *11*).

Although the ultimate processes involved in AMP-mediated cell killing remains subject to investigation and debate (*5*, *12*, *13*), peptide interaction with cellular membranes is clearly an important aspect of AMP activity, and for most AMPs, the disruption of the membrane permeability barrier appears to be the primary mechanism of action. Cationic AMPs have been shown to increase bacterial membrane permeability (*14*−*18*), dissipate transmembrane ionic potentials (*17*−*24*), form ion-selective channels (*25*, *26*), and release entrapped contents from liposomes (*23*, *27*−*29*). A variety of models have been proposed to describe the molecular events involved in AMP-mediated membrane disruption. AMPs may compromise membrane permeability through the formation of barrel-stave peptide channels (*19*, *25*, *26*, *30*−*34*), by the induction of peptide−lipid toroidal pores (*30*, *31*, *35*−*42*), or by carpeting the membrane surface at high concentrations and acting as amphipathic detergents (*27*, *43*, *44*). These mechanisms need not be mutually exclusive: a given peptide may interact with the target bilayer in different ways depending on the peptide/lipid ratio or membrane composition.

Our studies have focused on a small 15-residue synthetic hybrid peptide, first described by Andreau et al. (*45*), composed of the first seven residues of the silk moth AMP cecropin A and residues 2−9 from the bee venom peptide, mellitin. This peptide (which we designate CM15, Table 1) displays potent broad-spectrum antimicrobial activity but lacks the hemolytic properties associated with mellitin (*45*). The small size of CM15 greatly facilitates solid-phase synthesis relative to that of the parent 37-residue peptide. Both CM15 (*26*) and cecropin A (*25*) form ion channels

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* To whom correspondence should be addressed. Tel: 414-456-4037. Fax: 414- 456-6512. E-mail: jfeix@mcw.edu.

[1] Abbreviations: AMP, antimicrobial peptide; PEG, poly(ethylene glycol); DTAB, dodecyltrimethylammonium bromide; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; MTSL, 1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate; PBS, phosphate-buffered saline; LB medium, Luria−Bertani medium; MHB, Mueller−Hinton broth, OD, optical density; MIC, minimum inhibitory concentration; CFU, colony forming unit; KSI, ketosteroid isomerase; IPTG, isopropyl-beta-D-thiogalactopyranoside; LUV, large unilamellar vesicles; EPR, electron paramagnetic resonance; CMC, critical micelle concentration.

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Table 1: Amino Acid Sequences of the Cecropin A(1−7), Mellitin(2−9) Hybrid Peptide CM15, and a Single Cysteine Mutant CM15-C10.

CM15-wild type	K W K L F K K I G A V L K V L
CM15-C10	K W K L F K K I G *C* V L K V L

detectable by patch-clamp experiments, suggesting the formation of discrete membrane pores. In contrast, it has been suggested on the basis of studies examining the release of liposome-entrapped fluorophores that a related 26-residue cecropin−mellitin hybrid peptide disrupts membranes via a detergent-like carpet mechanism (*29*). A similar proposal was made for full-length cecropin A on the basis of calculations suggesting that at the lethal concentration the peptide was present in sufficient amount to cover the bacterial cell surface (*27*). Thus, the mechanism of membrane disruption by cecropins and cecropin−mellitin hybrid peptides has not been well established.

In the present studies, we have examined the ability of osmotically active solutes to protect bacterial cells against the cytotoxic effects of CM15. Osmoprotection is a well-established experimental approach that has been used successfully to elucidate the size of a variety of transmembrane channels and protein secretion apparatus (*46*−*49*), including those produced by a number of bacterial pore-forming toxins (*50*−*52*). These studies utilize either sugars or poly(ethylene glycol)s (PEGs) of varying dimensions to provide osmotic balance for intracellular proteins and nucleic acids. Those osmoprotectants too large to pass through a given transmembrane pore thus protect cells against osmotic rupture, whereas those small enough to pass through the pore redistribute across the bilayer and provide no osmotic protection. Our results show significant protection against CM15-mediated bacterial cell killing by solutes with a hydrated diameter of 3.8 nm or larger and little or no protection with solutes smaller than 2.2 nm. Osmoprotection was observed both for synthetic CM15 added extracellularly and for CM15 expressed intracellularly from a plasmid as part of a fusion protein. The osmolytes tested had no effect on peptide binding to lipid bilayers. In contrast, osmolytes provided no protection against the cytotoxic effects of the cationic detergent, dodecyltrimethylammonium bromide (DTAB). To our knowledge, this is the first report utilizing osmoprotection to examine pore formation by AMPs in intact, viable bacterial cells. These results demonstrate that the primary mechanism of CM15-mediated bacterial cell killing is through the formation of membrane pores and suggest an effective pore diameter in the range of 2.2−3.8 nm.

MATERIALS AND METHODS

Materials. POPE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine) and POPG (1-palmitoyl-2-oleoyl-phosphatidylglycerol) were purchased from Avanti Polar Lipids (Alabaster, AL). The methanethiosulfonate spin label, MTSL (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate), was obtained from Toronto Research Chemicals (North York, ON, Canada). For osmoprotection studies, we employed sucrose, raffinose, poly(ethylene glycol) (PEG) 1000, PEG 1450, PEG 3350, PEG 8000 (Sigma, St. Louis, MO), maltoheptaose (Boehringer Mannheim GmbH, Germany), and dextranT10 (Amersham Biosciences Corp, Piscataway, NJ).

Peptide Synthesis. Peptides were synthesized using Fmoc-based solid-phase methods and purified by semipreparative reverse-phase HPLC on a C8 column as described previously (*53*). The purified peptide was lyophilized and stored at −20 °C. Peptides were reconstituted in Dulbecco's phosphate-buffered saline (PBS), stored at 4 °C, and used within one week.

96-Well Broth-Dilution Assay. E. coli BL21(DE3) and *P. aeruginosa* PAO1 were grown in LB (Luria−Bertani) medium overnight, then diluted 1:100 into MHB (Mueller−Hinton broth), and grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.8 (approximately 3 h at 37 °C). The culture was then diluted to an OD_{600} of 0.00025 to obtain approximately 2×10^5 cells/mL. Purified synthetic CM15 in PBS was diluted 1:1 in 2× MHB, followed by serial 2-fold dilutions into a 96-well plate containing MHB/0.1% human serum albumin to give a volume of 100 μL in each well. For osmoprotection studies, osmolytes were added to cell suspensions to a concentration of 30 mM, and aliquots (100 μL) were then added to each well to give a final volume of 200 μL and a final concentration of 15 mM osmoprotectant. (Concentrations of osmolytes greater than 15 mM could not be used because it was not possible to shake the small volumes sufficiently to maintain aeration and dispersal of the cells in the 96-well format without spilling samples between wells.) The plate was incubated at 37 °C overnight or as indicated, and optical densities at 600 nm (OD_{600}) were measured with a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). A minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration for which there is no growth. Typical OD_{600} values for controls were approximately 0.6 for *E. coli* and 1.5 for *P. aeruginosa*, and OD_{600} values at or above the MIC were <0.05.

Colony Forming Unit (CFU) Assay. The 96-well broth-dilution assays were set up as described above, except that each well contained a final concentration of 10^8 cells/mL. At desired time points, aliquots were removed from the wells and diluted in LB medium in 10-fold increments, and 100 μL of each diluted sample was spread on LB agar plates. Plates were incubated at 37 °C overnight, and bacterial colonies were counted to determine the CFU/mL. All data shown are the average of at least three experiments.

Construction of pET31b(+)-cm15. The peptide coding sequences 5′-P-AAATGGAAACTGTTTAAAAAAATTG-GCGCGGTGCTGAAAGTGCTGATG-3′ and 5′-P-CAG-CACTTTCAGCACCGCGCCAATTTTTTTAAACAGTT-TCCATTTCAT-3′ were synthesized and PAGE purified by Integrated DNA Technologies (Coralville, IA). We used the pET31b(+) vector for cloning, which contains a gene encoding KSI to facilitate the purification of target proteins (Novagen, Madison, WI). The oligonucleotides were denatured at 98 °C for 2 min and then annealed at 30 °C for 30 min, ligated into the pET31b(+) plasmid (*Alw*NI-digested and dephosphorylated) with T4 DNA ligase (USB Corporation, Cleveland, OH), and transformed into *E. coli* BLR-(DE3) pLysS (a *recA*$^-$ derivative of BL21) as described by the manufacturer. As anticipated, this resulted in a series of products with a varying number of *cm15* insertions. The insertion of *cm15* and its tandem repeats were confirmed by DNA sequencing (Protein and Nucleic Acid Core Facility, Medical College of Wisconsin, WI) using a T7-terminator

primer (Novagen). We selected plasmids containing one (monomer, KSI−CM15) and seven (heptamer, KSI−$(CM15)_7$) copies of the CM15 coding sequence for further study.

Expression of a KSI−CM15 Fusion Protein. E. coli transformants were grown in LB medium containing 100 μg/mL of ampicillin and 30 μg/mL of chloramphenicol. An overnight culture was diluted and further grown to reach an OD_{600} of 0.4 or higher, and the cell number was normalized to an OD_{600} of 0.4 prior to isopropyl-beta-D-thiogalactopyranoside (IPTG) induction. The KSI, KSI−CM15, and KSI−$(CM15)_7$ fusion proteins were expressed by a *lac* operator system in the presence of 1 mM IPTG. For osmoprotection and time course studies, protein induction was performed in the presence or absence of 30 mM osmoprotectants at 35.5 °C with rotary shaking (250 rpm), and cells were harvested after various incubation times as indicated.

Spot-Dilution Assay. Bacterial cells were grown to an exponential phase, and KSI, KSI−CM15, and KSI−$(CM15)_7$ were induced as described above. After 1.5 h of IPTG induction, the culture was diluted in LB medium in a series of 10-fold increments. One μL of each dilution was spotted on a LB agar plate containing 100 μg/mL of ampicillin and 30 μg/mL of chloramphenicol and incubated at 37 °C overnight.

Liposome Preparation. Chloroform stock solutions of POPE and POPG were mixed at a molar ratio of 8:2, dried under nitrogen flow, and then placed under vacuum for at least 2 h. The dried lipid film was hydrated with PBS to give a final concentration of approximately 50 mM phospholipid. To prepare large unilamellar vesicles (LUVs), the hydrated lipid solution was subjected to five freeze−thaw cycles (liquid N_2, 42 °C water bath), and then extruded sequentially through 400-nm and then 100-nm polycarbonate membrane filters (Osmonics, Minnetonka, MN) using a mini-extruder (Avanti Polar Lipids, Alabaster, AL). Final phospholipid concentration was measured by the Stewart assay (*54*).

Electron Paramagnetic Resonance (EPR) Measurement of Membrane-Bound Peptide. A single-cysteine analogue of CM15, CM15-C10 (Table 1), was used for the attachment of the sulfhydryl-specific methanethiosulfonate spin label, MTSL, as described previously (*53*). For quantitation of membrane-binding, MTSL-labeled CM15-C10 was mixed with 5.25 mM LUVs in the presence or absence of an osmoprotectant (15 mM final concentration) to give the desired peptide-to-lipid ratio. To determine the fraction of membrane-bound peptide, the EPR signal amplitude of the high-field ($M_I = -1$) line, $A(-1)x$, was measured and compared to the signal amplitude of the free peptide in the absence of liposomes, $A(-1)f$. The fraction of membrane-bound peptide (f_b) is given by

$$f_b = \frac{[A(-1)f - A(-1)x]}{[A(-1)f - A(-1)b]}$$

where $A(-1)b$ is the amplitude of the high-field line under conditions where the peptide is fully bound (*53*, *55*).

RESULTS

Antimicrobial Activity of Synthetic CM15. CM15 was first identified by Andreau et al. as a significantly shortened version of the cecropin−mellitin hybrid peptide that retained strong antimicrobial activity without the hemolytic properties associated with mellitin (*45*). Using a 96-well broth-dilution assay, we verified that CM15 possesses significant activity against both *E. coli* and *Pseudomonas aeruginosa*, with MIC values at cell concentrations of 10^5 cells/mL on the order of 1 μg/mL (~0.5 μM) and 4 μg/mL (~2 μM), respectively (Table 2). These values are in good agreement with those reported by Andreau et al. using an agarose plate diffusion assay (*45*). We also examined the effect of bacterial cell number on the MIC of CM15. MIC values increased slightly at higher cell concentrations (Table 2) but remained relatively low. A 10-fold increase in cell concentration caused only a 2-fold increase in the MIC, and a 1000× increase in cell concentration increased the observed MICs by factors of 8 for *E. coli* and 16 for *P. aeruginosa*.

Table 2: Minimum Inhibitory Concentration (MIC) of CM15 as a Function of Bacterial Cell Number

	MIC (μg/mL) of CM15		
bacterium	10^5 cells/mL	10^6 cells/mL	10^8 cells/mL
E. coli BL21	1	2	8
P. aeruginosa PAO1	4	8	64

MIC values were determined by 96-well broth-dilution assays ($n \geq 3$).

The activity of synthetic CM15 at early time points was quantitated by a CFU assay. As shown in Figure 1, a 10-min incubation at CM15 concentrations corresponding to the MIC reduced the number of viable cells (CFU/mL) by 4 and 5 logs for *P. aeruginosa* and *E. coli*, respectively. The results were dose-dependent, with a 10-minute incubation at half the MIC resulting in a 2- to 2.5-log reduction in viable cells (Figure 1). Time courses for cell survival in the presence of various concentrations of synthetic CM15 both above and below the MIC confirmed the rapid kinetics of cell killing (Figure 2). This data is in agreement with previous studies of a somewhat larger cecropin−mellitin hybrid (*16*) and full length cecropin A (*17*). Starting with a relatively high bacterial cell density of 10^8 CFU/mL, a peptide concentration of 1−2× the MIC reduced viable cell numbers by almost 6 orders of magnitude in 30 min and to below the detectable limit within 1−4 h for *E. coli* and *P. aeruginosa*, respectively (Figure 2, (○),and (♦), respectively). Even at half the MIC (▲), viable cell numbers were decreased by approximately 5 log units in 1 h. At peptide concentrations below the MIC, the bacteria were able to recover over extended growth periods, reflecting proliferation of surviving cells, whereas no recovery was observed when the peptide concentration was greater than or equal to the MIC (Figure 2).

Size-Dependent Osmoprotection against CM15-Mediated Toxicity. The use of osmoprotectants has proven to be a valuable approach for determining the size of pores produced by a number of hemolytic toxins (*50*−*52*), peptides (*56*), and channel-forming proteins (*47*, *48*). Solutes too large to pass through a transmembrane pore protect cells against osmotic rupture, whereas solutes small enough to pass through the channel redistribute across the bilayer and offer no protection against osmotic lysis. A list of the osmoprotectants used in this study and their effective diameters is given in Table 3.

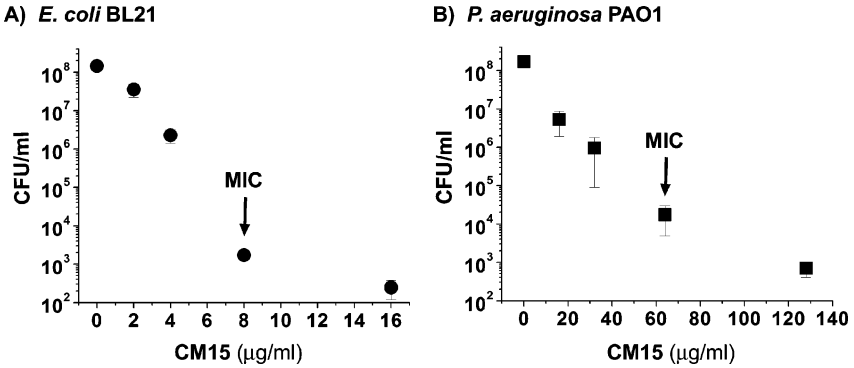

FIGURE 1: Dose−response of the rapid, initial cell killing by synthetic CM15. *E. coli* BL21 (A) and *P. aeruginosa* PAO1 (B) at an initial cell concentration of 10^8 CFU/mL were incubated with different concentrations of CM15 for 10 min at 37 °C, and aliquots were removed and plated for determination of surviving cell numbers. The results are average (± SEM) for a minimum of three experiments. For comparison, MIC values determined by overnight broth-dilution assays using a starting concentration of 10^8 cells/mL are indicated.

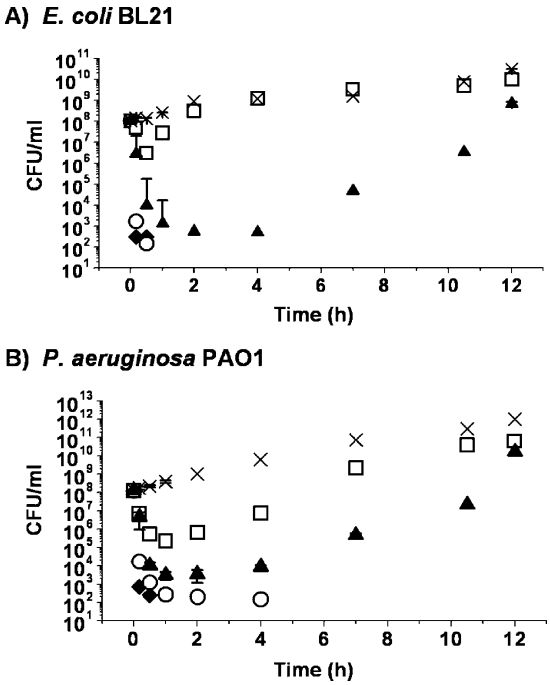

FIGURE 2: Kinetics of cell killing by synthetic CM15. *E. coli* BL21 (A) and *P. aeruginosa* PAO1 (B) at an initial cell concentration of 10^8 CFU/mL were incubated for the times indicated with bacteria only (×) and at peptide concentrations equal to $^1/_4$ (□), $^1/_2$ (▲), 1× (○), or 2× (◆) the MIC (determined from overnight broth-dilution experiments), and aliquots were removed and plated for determination of surviving cell numbers. At this cell concentration, the MICs for CM15 are 8 and 64 μg/mL for *E. coli* and *P. aeruginosa*, respectively. For peptide concentrations at or above the MIC, all cells are killed within 30 min−4 h. At peptide concentrations below the MIC, an initial decrease in cell numbers is followed by the proliferation of surviving cells. The results are the average of three experiments. Error bars indicating ±SEM are shown for bacteria only (×) and at 1/2 MIC (▲) and are smaller than the symbol for most points. Similar errors were observed for other CM15 concentrations and are omitted for clarity.

We examined the effects of osmoprotectants on cell killing by extracellularly added synthetic CM15. None of the osmolytes used in this study affected cell growth at a concentration of 15 mM, although growth was inhibited at higher concentrations of the larger osmolytes, presumably due to viscosity effects on aeration (data not shown). The results from a standard 96-well broth dilution assay for the killing of *P. aeruginosa* PAO1 by synthetic CM15 in the presence of various osmoprotectants are shown in Figure 3A.

Table 3: Osmoprotectants Used in This Study[a]

osmoprotectant	M.W.	diameter (nm)
glucose	180	0.72
sucrose	342	0.92
raffinose	504	1.14
PEG 1000	1000	2.0
maltoheptaose	1153	2.2
PEG 1450	1450	2.4
PEG 3350	3350	3.8
dextran T10	10000	4.6
PEG 8000	8000	6.4

[a] Osmoprotectant effective diameters are based on mean molecular weights, determined by either vapor pressure osmometry or gel filtration, as reported by Scherrer and Gerhardt (*46*).

The MIC for the control sample was 4 μg/mL. No osmoprotection was observed with either glucose (0.72 nm diameter) or sucrose (0.92 nm) (data not shown). Relatively small osmoprotectants, such as raffinose (1.14 nm diameter) and PEG 1000 (2.0 nm), offered little protection. PEG 3350 (3.8 nm diameter) increased the MIC 4-fold, whereas larger solutes, such as dextran T10 (4.6 nm) and PEG 8000 (6.4 nm), provided the most significant osmoprotection, up to 16× and 32× the MIC, respectively. Many of the osmoprotectants used in this study exist as somewhat heterogeneous mixtures with a distribution of sizes (*46*). Consequently, some osmotic protection can be achieved with solutes having a mean diameter smaller that that of the membrane pore, as observed previously for red cell lysis by the RTX family of toxins (*51*, *52*), the type III insertion complex from *P. aeruginosa* (*48*), and the hemolysin from *Serritia marcescens* (*50*). In addition, although the optimal solute concentration for osmoprotection of red cells is 30 mM (*52*), we were unable to use concentrations greater than 15 mM because of the effects of viscosity of the larger osmolytes on cell growth in a 96-well plate. Thus, osmoprotection may have been incomplete, even for those solutes larger than the pore size. To obtain an estimate of the limiting pore dimensions, we followed the method used previously for the analysis of bacterial cell wall permeability (*46*), plotting MIC values against the logarithm of osmolyte size and extrapolating to the solute diameter that provided no osmoprotection. This suggested the formation of a pore in the *P. aeruginosa* membrane with an effective diameter of ∼3.5 nm (Figure 3B). With *E. coli* BL21, no osmoprotection was observed for solutes with diameters <2.2 nm, whereas solutes with

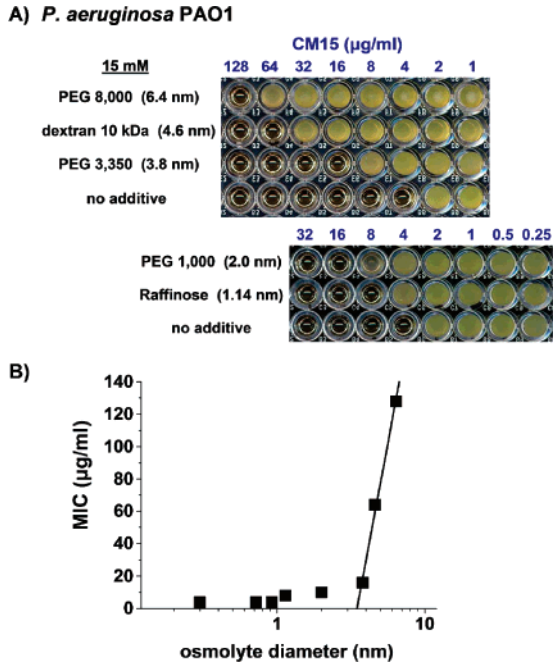

FIGURE 3: Osmoprotection against cell killing of *P. aeruginosa* PAO1 by synthetic CM15. (A) *P.* aeruginosa PAO1 at a final concentration of 10^5 cells/mL was incubated overnight at 37 °C with serial dilutions of CM15 in the presence or absence of 15 mM (final concentration) osmoprotectant. The MIC is the lowest concentration of peptide for which there is no growth (e.g., 4 μg/mL with no additive). Below the MIC, surviving cells proliferate to give a high optical density. (B) MIC values observed for *P. aeruginosa* PAO1 in overnight broth-dilution assays in the presence of 15 mM osmolytes are plotted as a function of osmolyte diameter (*46*). Extrapolation of the linear region of the curve indicates an effective pore diameter of 3.48 nm. The results are from one of three similar experiments.

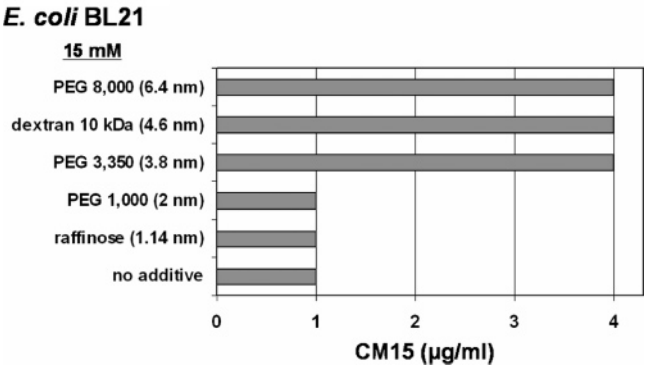

FIGURE 4: Osmoprotection against the cell killing of *E. coli* BL21 by synthetic CM15. The MIC values determined from overnight broth-dilution assays were determined in the presence or absence of the indicated osmolytes (15 mM final concentration). Data for *E. coli*, which are not as readily visible as that for *P. aeruginosa*, are based on optical densities measured at 600 nm. The same results were obtained from at least three experiments.

diameters >3.8 nm caused a 4-fold increase in the MIC (Figure 4). Thus, we conservatively estimate pore size to be in the range of 2.2−3.8 nm. The ability of osmotically active solutes to protect cells from the cytotoxic effects of CM15 strongly supports a discrete-pore mechanism for membrane disruption by this peptide.

Osmolytes Do Not Reduce Peptide Binding. To ensure that osmoprotection was not due to a decrease in AMP binding, we used an EPR binding assay to quantitate the interaction of a spin-labeled CM15 analogue (CM15-C10, Table 1) with liposomes in the presence of the various osmoprotectants. The EPR spectrum is highly sensitive to the mobility of the spin-labeled peptide and undergoes a distinct change in amplitude upon membrane binding (*53*, *55*). Liposomes composed of POPE/POPG (8:2) were utilized to mimic the composition of the bacterial inner membrane (*57*). On the basis of the change in the EPR signal amplitude of the high-field ($M_I = -1$) line, approximately 94.8 (± 1.17) % of spin-labeled CM15-C10 was membrane-bound in the absence of osmoprotectant at a peptide/lipid ratio of 1/117 (Table 4). As shown in Table 4, this value was unchanged for raffinose, PEG 1000, PEG 3350, and dextrose T-10. Although a very slight decrease (to 91.0 ± 5.45%) was observed in the presence of PEG 8000, this change was not statistically significant ($P > 0.56$) and could not account for the marked effect of PEG 8000 on cell viability. Thus, osmoprotection is not due to the inhibition of peptide binding to the membrane.

Table 4: Binding of CM15-C10-MTSL to POPE/POPG Liposomes

osmolyte	% binding (±SEM)[a]
no additive	94.8 (±1.17)
raffinose	95.3 (±0.99)
PEG 1000	95.7 (±0.99)
PEG 3350	95.5 (±0.99)
dextrose T10	95.1 (±1.86)
PEG 8000	91.0 (±5.45)

[a] The *P* values (student's *t*-test) relative to that of the control were >0.56 for all osmolytes tested.

Production of KSI−CM15 Inclusion Bodies and Toxicity of KSI−CM15 Fusion Constructs. To facilitate the large-scale production and purification of CM15, we sought to recombinantly express the peptide in *E. coli.* We constructed clones containing one or more copies of *cm15* fused to a gene encoding ketosteroid isomerase (KSI) using a pET31b plasmid. KSI-fusion proteins generally form inclusion bodies in *E. coli* because of the strong hydrophobicity of this carrier protein, which can allow the production of peptides normally toxic to the host (*58*, *59*). As anticipated, following IPTG induction, we observed high levels of inclusion body production. However, initial studies of fusion peptide production in *E. coli* indicated that constructs containing CM15 were cytotoxic. Cell density, as measured by absorbance at 600 nm, did not increase following IPTG induction for those constructs containing one or more copies of CM15, whereas the control plasmid expressing KSI alone (no insert) continued to grow. This was observed regardless of the *E. coli* strain employed (BL21 or BLR, with or without pLysS). To examine this toxic effect further, we chose clones containing either one or seven copies of *cm15* (KSI−CM15 and KSI−$(CM15)_7$, respectively). A spot-dilution analysis of cell growth and viability following IPTG induction of cells containing pET31b/KSI, pET31b/KSI−CM15, and pET31b/KSI−$(CM15)_7$ is shown in Figure 5. Constructs containing only KSI showed detectable cell growth at dilutions as large as 10^6 and maintained viability following IPTG induction (Figure 5). In contrast, cells expressing the KSI−CM15 and KSI−$(CM15)_7$ constructs exhibited significantly reduced viability after only 30 min of induction, and the viability continued to decrease at longer induction times (Figure 5).

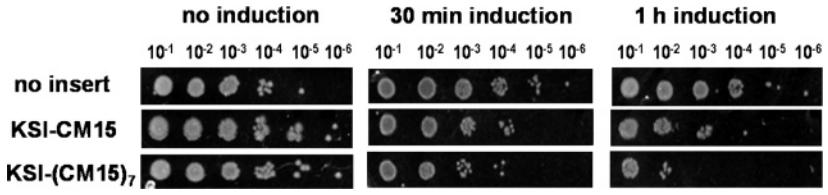

FIGURE 5: Spot-dilution assay indicating toxicity of CM15 fusion peptides. BLR(DE3)pLysS cells transformed with pET31b plasmids containing the indicated constructs were grown to an OD_{600} of 0.4 and protein expression induced by the addition of IPTG to 1 mM final concentration for an additional 30 min−1 h. Serial 10-fold dilutions of the cell suspensions were then made in LB and 1 μL aliquots spotted on LB/ampicillin/chloramphenicol agar plates and grown overnight at 37 °C. No insert indicates cells harboring pET31b without peptide, and no induction indicates aliquots taken prior to the addition of IPTG. The viability of cells expressing KSI−CM15 and KSI−$(CM15)_7$ were reduced approximately 2−4 orders of magnitude after 1 h of induction, respectively.

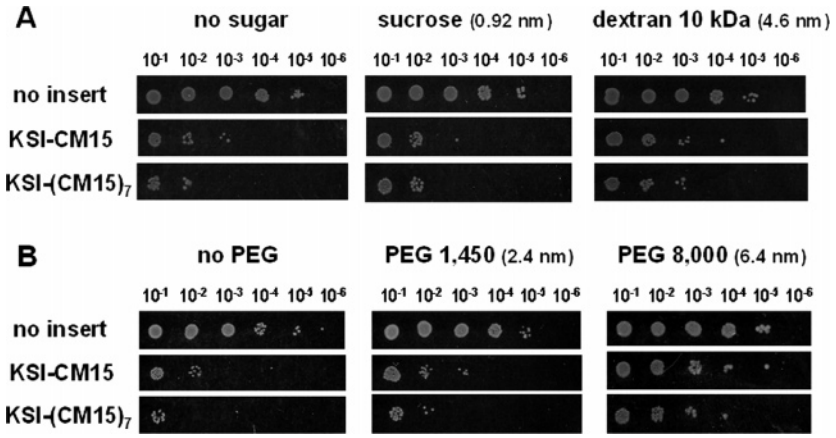

FIGURE 6: Osmoprotection against cell killing by intracellularly expressed KSI−CM15 fusion peptides. *E. coli* BLR(DE3)pLysS cells transformed with pET31b plasmids containing the indicated constructs were diluted to an OD_{600} of 0.4 in LB medium containing various osmoprotectants (30 mM final concentration) and protein expression induced by addition of IPTG, followed by incubation at 37 °C for an additional 1.5 h. Serial 10-fold dilutions of the cell suspensions were then made in LB and 1 μL aliquots spotted on LB/ ampicillin/ chloramphenicol agar plates and grown overnight at 37 °C. The degree of osmoprotection by sugars (A) and poly(ethylene glycol)s (B) increased with increasing size of the osmolyte.

Osmoprotection against Intracellularly Produced KSI−CM15 Fusion Constructs. When cells intracellularly expressing KSI−CM15 constructs from pET31b plasmids were grown in the presence of osmoprotectants, we observed enhanced cell survival that was dependent on osmolyte size (Figure 6). Following 90 min of IPTG induction, cells expressing KSI only (no insert) gave detectable colonies up to dilutions of 10^5−10^6-fold, and cell growth/viability was not affected by any of the sugars or PEGs (top row, Figure 6A and B, respectively). In contrast, cells expressing KSI−CM15 or KSI−$(CM15)_7$ gave viable colonies in the absence of osmoprotectant only at dilutions of 10^1−10^3. The addition of sucrose, raffinose, PEG 1000, or PEG 1450 had little or no protective effect (Figure 6 and data not shown). Intermediate-size solutes (maltoheptaose and PEG 3350) appeared to give some slight protection, and larger solutes (dextran T10, PEG 8000) provided 1 to 3 log protection (Figure 6 and data not shown).

These results were verified by CFU assays (Figure 7). For transformants expressing the CM15 monomer, 3 h of induction resulted in ~2.5 log reduction in CFU. We observed no osmoprotection with raffinose, 0.5 log protection with PEG 1000 and PEG 3350, 1 log protection with dextran T10 and 2 log protection with PEG 8000 (Figure 7A). The expression of the CM15 heptamer was more cytotoxic, resulting in greater than 3 log reduction in CFU after 3 h of induction (Figure 7B). Again, no osmoprotection was observed with raffinose, 0.5−1 log protection with PEG 1000, PEG 3350, and dextran T-10, and ~3 log protection with PEG 8000. The fact that extracellular solutes can protect cells even when the peptide is generated intracellularly strongly supports the concept that damage to the cytoplasmic membrane is the cytotoxic event.

Osmolytes Do Not Protect against Detergent-Mediated Cell Killing. To gain insight into how osmoprotectants might influence cell killing by a proposed detergent mechanism, we carried out studies with a known detergent, dodecyltrimethylammonium bromide (DTAB). DTAB was chosen because, like CM15 and most other known AMPs, it is cationic at physiological pH. Preliminary studies showed that MICs for DTAB were roughly 1.3 mM for *P. aeruginosa* PAO1 and 0.13 mM for *E. coli* BL21 (data not shown). These concentrations are well below DTAB's reported CMC (critical micelle concentration) of approximately 15 mM (*60*), indicating that this detergent partitions into the bacterial membrane as monomers, which accumulate and eventually destroy the permeability barrier. In marked contrast to the results obtained with CM15, only PEG 8000 had any effect on DTAB toxicity, a slight 2-fold increase in the MIC for *P. aeruginosa* (Figure 8A), and none of the osmolytes tested afforded any protection against DTAB-mediated cell killing of *E. coli* (Figure 8B). These results indicate that the osmolytes tested in this study do not protect against cell killing by a detergent-mediated mechanism, strengthening our conclusion that CM15-mediated cytotoxicity occurs through the formation of membrane pores.

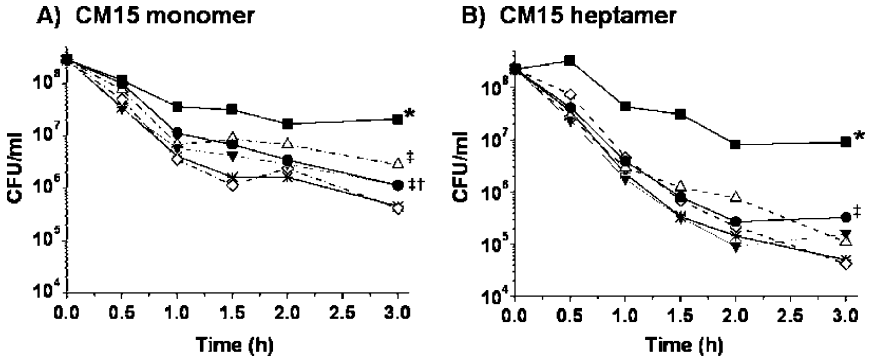

FIGURE 7: Quantitative analysis of osmoprotection against cell killing by intracellularly expressed KSI−CM15 as a function of osmolyte size and time after induction. *E. coli* BLR(DE3)pLysS transformants with pET31b plasmids containing CM15 (A) monomer or heptamer (B) were grown to an OD_{600} of 0.4 and then induced in the presence of 30 mM osmoprotectant at 37 °C for an additional 0.5 to 3 h. Serial 10-fold dilutions of the cell suspensions were then made in LB, and 100 μL aliquots were spread on LB/ ampicillin/chloramphenicol agar plates and grown overnight at 37 °C. Viable cells were quantitated as CFU/mL as indicated with osmolytes PEG 8000 (−■−, diameter 6.4 nm), dextran 10 kDa (−△−, diameter 4.6 nm), PEG 3350 (−●−, diameter 3.8 nm), PEG 1,000 (−▼−, diameter 2.0 nm), raffinose (−◇−, diameter 1.14 nm), or no osmolyte (−*−). The results are mean values from at least three independent experiments with (*) $p < 0.001$, (‡) $p < 0.01$, and (†) $p < 0.05$ (determined by student's *t*-test) compared to those of the no osmolyte control.

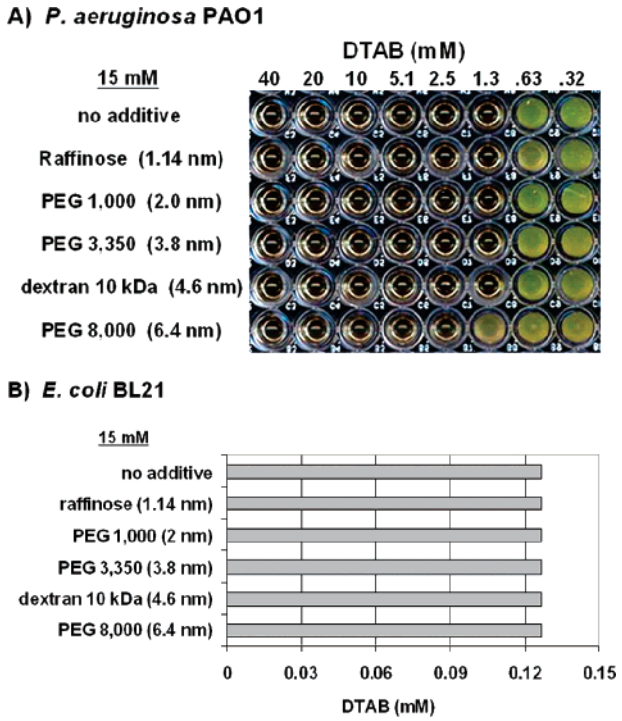

FIGURE 8: Absence of osmoprotection against cell killing by the cationic detergent, DTAB. *P.* aeruginosa PAO1 (A) and *E. coli* BL21 (B) at a final concentration of 10^5 cells/mL were incubated overnight at 37 °C with serial dilutions of DTAB in the presence or absence of 15 mM (final concentration) of the indicated osmoprotectants. Data for *E. coli*, which are not as readily visible, are based on optical densities measured at 600 nm. Other than the small, 2-fold inhibition observed for PEG 8000 with *P. aeruginosa*, none of the osmolytes had any effect on DTAB-mediated cell killing.

DISCUSSION

The mechanisms involved in AMP-mediated cell killing have been extensively studied. Nonetheless, two important issues continue to be debated: the potential role of intracellular targets as opposed to membrane disruption and the precise mechanism(s) of membrane destruction. As with most AMPs, the antimicrobial activities of both full-length cecropins and the cecropin−mellitin hybrid CM15 are nonsterospecific (*7*, *10*), suggesting the cytoplasmic membrane as a likely cellular target. This is supported by a considerable body of research demonstrating membrane damage by AMPs. Prevailing models for the mechanism of membrane disruption include barrel-stave, toroidal-pore, and detergent-like carpet mechanisms (*31*, *36*, *40*, *44*). All of these proposed mechanisms involve the accumulation of peptide at the membrane surface up to some critical concentration, with the first two models invoking the subsequent formation of a transmembrane channel. In this study, we report that extracellular osmoprotectants are able to mitigate the cytotoxic effects of a cecropin−mellitin hybrid AMP in a size-dependent manner. This protective effect was not due to inhibition of peptide binding and was observed both for peptides added extracellularly and for peptides expressed intracellularly from a plasmid carrying *cm15* or seven repeats of *cm15* fused to the noncytotoxic carrier protein, KSI. In contrast, osmoprotection was not observed for cell killing mediated by a cationic detergent, DTAB. These studies strongly support membrane pore formation as a key cytotoxic event in the antimicrobial activity of CM15.

Our results are in agreement with previous studies showing a close correlation between the threshold level of membrane permeabilization and bactericidal activity for the parental peptide, cecropin A (*17*), dissipation of ionic gradients at low peptide concentrations (*17*,*23*), and demonstrations of ion channel formation by cecropin A (*25*) and CM15 (*26*) using patch-clamp methodology. In contrast, other studies have not found good correlation between the dissipation of the cytoplasmic membrane potential and cytotoxicity (*24*), leading to the suggestion that targets other than the cell membrane may be important (*12*). Some AMPs clearly target intracellular processes (*61*, *62*), and the inhibition of macromolecular synthesis at sublethal concentrations of AMPs has been observed (*63*). In the present studies, however, the ability of extracellular osmolytes to protect against cell killing by peptides produced intracellularly clearly implicates membrane damage and a loss of the permeability barrier in the antimicrobial activity of CM15.

The rapid kinetics observed for cell killing at peptide concentrations at or above the MIC (e.g., Figures 1 and 2) are also consistent with a central role for membrane disruption in the bactericidal activity of CM15. The osmotic

shock associated with loss of the cytoplasmic membrane permeability barrier is a relatively rapid process, occurring on a much shorter time scale than would be expected for the loss of viability due to a disruption of metabolic processes. This is not to say that other, subsequent processes could not also contribute to CM15-mediated cell killing, particularly at time points later than the initial 15−30 min of rapid cytotoxicity (Figure 2). Indeed, it is expected that an irreversible loss of the cytoplasmic membrane permeability barrier will have additional deleterious effects on cellular respiration and metabolism, and these effects may well contribute to the observed decrease in viability. Such processes could be especially important at concentrations below the MIC, where the number and size of transmembrane pores may be limited.

Our studies suggest that CM15 produces pores with diameters in the range of 2.2−3.8 nm. For *P. aeruginosa*, glucose (0.72 nm) and sucrose (0.92 nm) afforded no osmoprotection, raffinose (1.14 nm) and PEG 1000 (2 nm) provided weak osmoprotection, PEG 3350 (3.8 nm) provided moderate protection, and strong osmoprotection was observed with dextran T-10 (4.6 nm) and PEG 8000 (6.4 nm). A plot of MIC as a function of osmolyte size (*46*, *51*) indicated a pore diameter of approximately 3.5 nm in the *P. aeruginosa* membrane (Figure 3B). The addition of CM15 to *E. coli* resulted in a different pattern of osmoprotection, with solutes 3.8 nm diameter (PEG 3350) and larger providing protection, whereas no osmoprotection was observed for solutes with diameters less than or equal to 2.2 nm. These differences may reflect the formation of channels that differ in size for the two organisms or the differences in the susceptibility of the two organisms to a loss of the permeability barrier. As discussed below, *E. coli* is more susceptible to CM15 (Table 2), and the cell killing of *P. aeruginosa* proceeds more slowly (Figure 2). In addition, pore size and composition may be somewhat heterogeneous, with a range of diameters between 2.2 and 3.8 nm. This might well be expected if channels are formed by the toroidal pore mechanism, which invokes membrane lipids as an essential part of the pore structure (*31*, *36*, *40*). Toroidal pores formed by the α-helical AMP magainin appear to be somewhat heterogeneous, containing between five and nine peptides (*30*, *31*, *35*, *38*). CM15 initially binds to membrane bilayers as an α-helix, with its helical axis aligned parallel to the membrane surface (*53*), and may subsequently form toroidal pores or similar transient membrane defects as the concentration of membrane-bound peptide is increased. This mechanism would allow channel formation even though CM15 is too short to span the unperturbed bilayer. A significant body of data indicate toroidal pore formation by both magainins (*40*, *64*) and LL37 (*65*), two other linear amphipathic α-helical AMPs that also initially bind parallel to the membrane surface (*65*−*67*). In addition, a computational study has indicated that channels formed by highly charged peptides (such as CM15, with a formal net charge of +5) are most likely toroidal pores because the lipid headgroups that participate in pore formation are essential for the reduction of electrostatic repulsion between peptides (*68*).

This is the first report of utilizing osmoprotection to assess pore formation and dimensions by AMPs in intact, viable bacterial cells. Classically, osmotic protection has been used to examine channels formed in intact red blood cells (*50*, *51*, *56*, *69*, *70*), mammalian cultured cells (*47*, *48*), or liposomes (*71*). In studies of hemolytic toxins, the kinetics of hemoglobin release is measured as a function of osmoprotectant size. Because many of the solutes used for osmoprotection, particularly the PEGs and larger dextrans, exist as a heterogeneous mixture with a distribution of molecular sizes, one typically does not observe a sharp all or none cutoff in the dependence of lysis on solute size (*46*, *51*, *72*). Rather, one observes a logarithmic relationship between the hydrated radius and the rate constant for lysis, with extrapolation to zero lysis used to estimate pore size (*46*, *51*). The theoretical basis for this has been examined (*72*). We observe a similar dependence for cell killing of *P aeruginosa* (Figure 3). Because *P. aeruginosa* is relatively resilient to the cytotoxicity of CM15, we hypothesize that the rate of cell killing in the presence of osmoprotectants may be reduced so that it is comparable to or longer than the doubling time. This introduces a kinetic component into the effects of osmoprotectants on cell survival in which the rate of cell killing must be considered relative to the rate of reproduction and may explain, for example, why some osmoprotection is observed even in the presence of solutes such as raffinose that are smaller than the dimensions of the proposed pore. Similarly, raffinose (1.1 nm diameter) delayed but did not prevent hemolysis by *E. coli* hemolysin HlyA, which has an estimated pore diameter of 2.2 nm (*51*). In contrast, *E. coli*, which is significantly more susceptible to cell-killing by CM15 (Table 2), evidently loses viability at a rate that is short compared to its doubling time and consequently exhibits a clear delineation between solutes that are too large to pass through the putative membrane pores. A close examination of the cell survival curves shown in Figure 2 indicates that at a peptide concentration equal to the MIC, in the absence of osmoprotectant, no viable *E. coli* cells are detected beyond 30 min, whereas for *P. aeruginosa*, surviving cells could be detected up to 2−4 h. In addition, technical complications prevented the use of the larger osmoprotectants at concentrations greater than 15 mM because of the effects of solution viscosity on cell growth in the 96-well broth-dilution assay (see Materials and Methods). In hemolysis experiments, sugar/PEG concentrations of 30 mM are optimal for providing osmotic balance (*51*, *52*). Thus, even for solutes too large to pass through the putative membrane pores, osmoprotection may have been incomplete.

In addition to their effects on cell survival in the presence of externally added, synthetic CM15, we also found that osmoprotectants reduced cytotoxicity due to CM15 produced intracellularly as part of a fusion to the carrier protein, KSI. It is remarkable that CM15 remains toxic even when expressed as a KSI fusion. KSI is highly insoluble and has been used successfully to direct toxic peptides into inclusion bodies (*58*, *59*). A number of laboratories have reported the recombinant expression of antimicrobial peptides; however, these constructs have typically included leader peptides containing multiple anionic residues to balance the charge of the cationic AMP (*73*−*75*). Although we observed robust production of inclusion bodies containing KSI, cell viability declined rapidly following induced expression of the KSI−CM15 fusion protein. The molecular basis for this remaining toxicity is not clear, and unfortunately, their poor solubility precludes testing the antimicrobial activity of the isolated

fusion proteins. We hypothesize that a small fraction of the fusion protein may remain soluble and that this soluble population disrupts the cytoplasmic membrane. Alternatively, inclusion bodies may accumulate near the membrane interface allowing the insertion of the *C*-terminal CM15 peptide. Nonetheless, we observe that osmoprotectants do help maintain cell viability and that the size dependence for osmoprotection parallels that observed for extracellularly added CM15. Although the relationship between the mechanism of membrane disruption by KSI−CM15 fusion proteins and extracellular CM15 is uncertain, the protection afforded by extracellular osmoprotectants clearly implicates the membrane as the cellular target. Further studies on the cytotoxicity of intracellularly expressed CM15, without the confounding presence of KSI, are in progress.

The methods described in this work should be generally applicable for the analysis of putative membrane channels formed by antimicrobial peptides in native bacterial cell membranes. Most previous attempts to determine pore dimensions by membrane-active peptides have utilized the differential release of fluorescent markers entrapped in liposomes. For example, on the basis of the preferential release of a 4 kDa fluorescent dextran, the 26-residue peptide mellitin was found to form pores in POPC liposomes with a diameter of ~2.5 nm (*76*), in general agreement with estimates based on osmoprotection against red cell hemolysis (*56*). However, the mellitin-induced release of fluorophores from POPG vesicles was nonselective (*77*). A 26-residue cecropin−mellitin hybrid was proposed to disrupt bilayers by a detergent-like mechanism based on the similar release of different sized fluorescent dextrans from liposomes composed of either pure PC or pure PS (*29*). Although liposomes are extremely valuable model systems that allow control of lipid composition and peptide/lipid ratios, they do not precisely replicate native membranes in that they lack integral membrane proteins, a possibly heterogeneous distributions of lipid species, and a peptidoglycan layer that might influence cell membrane stability. Consequently, the ability to use viable bacterial cells as a target makes osmoprotection a highly useful approach for studying the mechanism of membrane disruption by antimicrobial peptides.

It has been suggested that toroidal pore formation may occur as an early step in AMP-mediated membrane disruption via the carpet mechanism (*22*, *44*). In our studies, sufficiently high peptide concentrations were able to overcome the osmoprotection afforded by even the largest solutes, suggesting that the pore formation mechanism that predominates near the MIC may transition to a detergent-like carpet mechanism at higher peptide concentrations. It has previously been noted that the 37-residue AMP cecropin A dissipates membrane potentials in *E. coli* at low concentrations but that only high peptide concentrations are able to release β-galactosidase (*17*). Thus, it seems likely that membrane composition, the concentration of membrane-bound peptide (i.e., peptide/lipid ratio), and the structure of the AMP under investigation all play a role in the precise mechanism of membrane disruption.

In summary, these studies demonstrate the ability of osmoprotectants to promote cell survival during cell killing mediated by the linear cationic antimicrobial peptide, CM15, and provide an estimate of pore size in the range 2.2−3.8 nm. Osmoprotectants promote cell survival even though peptide binding is not altered, which is inconsistent with a carpet mechanism for cytotoxicity. Osmoprotection is not observed for cell killing by a cationic detergent. Bacterial cell killing by CM15 occurs within minutes, consistent with cytotoxicity due to the rapid loss of the membrane permeability barrier. Although additional processes may contribute to the antibiotic activity of CM15, we conclude that pore formation in the cytoplasmic membrane is the primary mechanism underlying the bactericidal effects of this hybrid peptide.

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