Mechanism of Four de Novo Designed Antimicrobial Peptides*

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As pathogenic bacteria become resistant to traditional antibiotics, alternate approaches such as designing and testing new potent selective antimicrobial peptides (AMP) are increasingly attractive. However, whereas much is known regarding the relationship between the AMP sequence and potency, less research has focused on developing links between AMP properties, such as design and structure, with mechanisms. Here we use four natural AMPs of varying known secondary structures and mechanisms of lipid bilayer disruption as controls to determine the mechanisms of four rationally designed AMPs with similar secondary structures and rearranged amino acid sequences. Using a Quartz Crystal Microbalance with Dissipation, we were able to differentiate between molecular models of AMP actions such as barrel-stave pore formation, toroidal pore formation, and peptide insertion mechanisms by quantifying differential frequencies throughout an oscillating supported lipid bilayer. Barrel-stave pores were identified by uniform frequency modulation, whereas toroidal pores possessed characteristic changes in oscillation frequency throughout the bilayer. The resulting modes of action demonstrate that rearrangement of an amino acid sequence of the AMP resulted in identical overall mechanisms, and that a given secondary structure did not necessarily predict mechanism. Also, increased mass addition to Gram-positive mimetic membranes from AMP disruption corresponded with lower minimum inhibitory concentrations against the Gram-positive Staphylococcus aureus.

All multicellular organisms such as animals and plants protect themselves against pathogenic microbes by producing antimicrobial peptides (AMPs) that selectively disrupt bacterial cell membranes (1). Although they are enormously diverse, AMPs are mainly comprised of hydrophobic and cationic amino acids that are spatially organized along the molecule. Because bacteria depend on the integrity of their anionic cell membrane, disrupting their membrane with cationic peptides could offer an alternate strategy to conventional antibiotics for killing pathogenic bacteria (2). As these pathogens become resistant to traditional antibiotics, alternate approaches such as designing and testing new potent selective antimicrobial peptides are increasingly attractive.

The proper composition of amino acids, their sequential arrangement, and peptide length are essential for effective action of AMPs (3). It has been demonstrated that AMP activity is more closely tied to amino acid composition than amino acid sequence or AMP structure (4–8). However, it has recently been shown that for the α-helical class of AMPs, ordering amino acids during AMP design into an imperfect amphipathic α-helix, a helix-barrel-stave with one hydrophobic and one hydrophilic face where the hydrophobic face is disrupted by one hydrophilic amino acid, is beneficial for increasing AMP activity (9). Understanding the mechanism behind how these peptides disrupt cell membranes could benefit future designs of potent, selective AMPs.

Various mechanisms of interaction between AMPs and microbial lipid membranes include, (i) peptide adsorption to and expansion of the headgroup region (carpet, toroidal pore formation, or sinking raft mechanisms) resulting in positive curvature strain of a supported lipid bilayer (SLB) (10–12), and (ii) peptide binding without the prerequisite positive strain (barrel-stave pore and electroporation mechanisms) (13). One instrument that can capture these interactions in real time is a quartz crystal microbalance with dissipation (QCM-D). Two main benefits to the QCM-D for studying peptide-lipid interactions over other surface measurement techniques are that (i) in addition to measuring wet-mass binding to the surface layer (i.e. the lipid bilayer) it also quantifies the energy dissipation or rigidity of the lipid bilayer in real time, and (ii) mass and dissipation changes are quantified across different penetration depths (14, 15). This information allows for analyzing differential interactions at the lipid-water interface and the internal bilayer, which provides the basis for the determination of the mode of action of AMPs.

To determine the mechanism of SLB disruption of four rationally designed AMPs for which their secondary structure is unknown (B1, ILSLRWRWKWWKK; B2, ILSLRWWRKWWKK; B3, ILSLRWWRKWWKK; B4, IRKLKSWKLRLWL (9)), the QCM-D behavior of four natural AMPs (indolicidin, 2

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2 The abbreviations used are: AMPs, antimicrobial peptides; SLB, supported lipid bilayer; QCM-D, quartz crystal microbalance with dissipation; ΔF, change in frequency; ΔΔF, change in dissipation; IND, indolicidin; LUV, large unilamellar vesicle; MIC, minimum inhibitory concentration; Fmoc, N-(9-fluorenylmethoxycarbonyl); POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); PG-1, protegrin-1.
extended/unstructured (IND), ILPWKWPWWPWRR (16); protegrin-1, β-sheet (PG-1), RGGRLCYCRRRFCVCVGR (17, 18); magainin-2, α-helix (MG-2), GIGKFLHSAKKFGKAFVGEIMNS (11); and α-defensin-1, β-sheet with disulfide bonds (selected as a non-interacting “control” peptide) (αD-1), ACYCRIPACIAGERRYGTCIYQGRLWAFCC (19)) of known secondary structures and mechanisms are used for guidance (9, 18, 20–24).

Results

Mechanistic information regarding the natural interactions of AMPs with SLBs are determined by examining kinetic (Fig. 1A–IV)
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and equilibrium (Fig. 1B1,1v) changes in the properties of the bilayer during AMP flow and subsequent buffer wash. These changes are quantified by the change in frequency (Δf) and change in dissipation (ΔD) of an oscillating silica sensor, which is a technique commonly reported in the literature (15, 27–32). Here, kinetic information is generally used to determine the rates and number of observable stages involved in each mode of action of the AMPs, whereas equilibrium data are related to the final mechanistic changes of the SLB. The equilibrium data, calculated by the changes in Δf (ΔΔf) of the initial SLB (Fig. 1A, t = 0 s) subtracted from the final bilayer properties after a buffer wash following AMP interaction (Fig. 1A, t = 1,700 s), are plotted against overtone number where the overtone with the furthest penetration from the sensor surface, three, is plotted at the top, whereas shallower penetrating overtones are plotted below in order of penetration depth (Fig. 1B1,1v) (14). To quantify the change in bilayer properties as a parameter relating distance to the sensor, the difference in Δf for the ninth overtone (Δf9) is subtracted from Δf of the third overtone (Δf3), and that difference is normalized by the molecular mass of the AMP in kDa (ΔΔf3–9). These values are overlaid onto their corresponding panels in Fig. 1B.

To understand how Δf and ΔD relate to mass changes, the total mass (m) of the SLB was calculated using the third, fifth, seventh, and ninth overtones of Δf and ΔD and an assumed SLB density, ρf of 1,100 kg/m3 using the Voigt model (14, 26). Upon fitting the model, the mean film thickness (δ) was obtained. The total mass was then calculated using δ, ρf, and the total surface area of the crystal, 154 mm2. δ was shown to be linearly dependent on ρf (R² = 0.9687), whereas m was independent of ρf. Thus results are presented in terms of m and not δ.

Natural AMPs

Kinetic Behavior—Addition of PG-1 induced a two-step effect on the SLB. In the first 60 s of AMP flow (0.50 nmol AMP dosage), Δf3 decreased from −26.2 ± 0.39 to −28.5 ± 0.60 Hz, whereas ΔD increased to 2.25 ± 0.25 × 10⁻⁶, indicating net mass addition to the SLB with decreased rigidity (Fig. 1A1). Both Δf and ΔD plateaued for an additional 60 s following the initial decrease (1.0 nmol of AMP dosage). Subsequent PG-1 flow resulted in an additional decrease in Δf to −45.1 ± 2.8 Hz and increase in ΔD to 9.53 ± 0.34 × 10⁻⁶ (Fig. 1A1). During the following buffer wash, Δf further decreased to −48.0 ± 2.7 Hz coupled with a ΔD increase to 12.3 ± 0.26 × 10⁻⁶ indicating further mass addition to the SLB (Fig. 1A).

Applying the Voigt model to the kinetic data resulted in a change in mass (Δm) once the AMP reached a dose of 0.47 nmol of PG-1 applied to the SLB (Δm1) to 0.28 ± 0.030 μg during the first step of PG-1 (Fig. 2, A and A1). When the dosage of PG-1 reached ×10 the amount of Δm1, 4.7 nmol (Δm2), the SLB modeled mass increased to 2.12 ± 0.096 μg (Fig. 2A2, A1), corresponding to significant Δf and ΔD changes observed in Fig. 2A. Finally, during the buffer wash, the SLB mass (Δm3) ultimately increased to 3.16 ± 0.25 μg (Fig. 2, A and A1). Mechanistically, this shows that PG-1 requires an initial critical concentration of peptide before severe damage occurs to the SLB. Assuming the initial mass change calculated by the Voigt model is due purely to AMP addition, the initial PG-1 amount to reach this critical threshold is 0.13 nmol of PG-1.

IND, similar to PG-1, induced a two-step effect on the SLB, however, the initial effect on Δf and ΔD of the SLB was drastically different. After 60 s of IND flow (0.50 nmol of IND dosage), Δf and ΔD remain unchanged at −26.2 ± 0.39 Hz and 0.97 ± 0.11 × 10⁻⁶ (Fig. 1A4). Prior to a subsequent sharp decrease in Δf and increase in ΔD, IND caused slight increases in Δf up to −25.7 ± 0.29 Hz and ΔD to 1.16 ± 0.12 × 10⁻⁶ after 84 s of IND flow (0.70 nmol of IND dosage, Fig. 1A1). IND next caused a sharp decrease in Δf to −40.9 ± 0.32 Hz coupled with an increase in ΔD to 5.08 ± 0.20 × 10⁻⁶ followed by IND reaching a pseudo-equilibrium state after 250 s IND flow (dose of 2.1 nmol of IND applied). The subsequent buffer wash caused a decrease in Δf to −55.3 ± 3.66 Hz with a ΔD increase to 8.66 ± 3.11 × 10⁻⁶ (Fig. 1A4).

Modeling revealed that during the first 84 s (0.70 nmol) of IND dosage where Δf and ΔD slightly increased, there was an initial Δm of 0.63 ± 0.56 μg (Fig. 2A). The critical amount of IND moles required to bypass this first mechanistic step was 0.33 ± 0.28 nmol of IND. In the second step, IND quickly reached a pseudo-equilibrium state with the SLB at Δm = 1.33 ± 0.84 μg after 122 s of IND flow (1.02 nmol of IND, Fig. 2A). Following the buffer wash, the SLB became saturated at Δm3 = 1.74 ± 0.36 μg (Fig. 2A1), the second largest Δm3 for any AMP examined.

MG-2 was the only AMP to increase Δf permanently during AMP flow. Δf increased slightly from the −26.1 ± 0.29 Hz baseline to −25.0 ± 0.22 Hz, whereas ΔD simultaneously increased to 1.35 ± 0.28 × 10⁻⁶ during AMP cross-flow (Fig. 1A1). Following buffer wash, Δf again increased slightly to −24.5 ± 0.26 Hz, whereas ΔD decreased slightly to 1.19 ± 0.18 × 10⁻⁶ (Fig. 1A1). These slight changes in Δf and ΔD led to increased values of Δm1, Δm2, and Δm3 of 0.10 ± 0.02, 0.17 ± 0.04, and 0.05 ± 0.04 μg, respectively (Fig. 2A1). α-D exhibited very little effect on the SLB. Δf remained essentially constant at −24.2 ± 0.28 Hz before α-D-1, −24.8 ± 0.32 Hz during α-D-1 flow, and −24.5 ± 0.38 Hz during buffer wash, whereas ΔD decreased slightly from 0.79 ± 0.16 × 10⁻⁶ to 0.44 ± 0.17 × 10⁻⁶ during α-D-1 flow and increased back to 0.87 ± 0.29 × 10⁻⁶ during buffer wash (Fig. 1A1). Furthermore, Voigt modeling revealed negligible changes in total SLB mass (Fig. 2A). These slight changes in Δf and ΔD led to increased values of Δm1, Δm2, and Δm3 of −0.048 ± 0.037, −0.056 ± 0.12, and 0.096 ± 0.102 μg, respectively (Fig. 2A1).

Ultimately, α-D-1 had a non-measurable effect on the SLB.

Equilibrium Behavior—PG-1 has large negative values of ΔΔf (all overtones < −20 Hz) and a value of ΔΔf₇–₉ = 9.1 Hz kDa⁻¹, indicating significant frequency decreases, a parameter change commonly related to increasing mass (31), throughout the SLB with a preference for interaction furthest from the sensor surface (Fig. 1B1). Similarly, IND has a similar ΔΔf overtone profile to PG-1, where all overtones have large negative ΔΔfs (< −20 Hz) and a value of ΔΔf₇–₉ = 9.0 Hz kDa⁻¹ (Fig. 1B1). For MG-2, the ΔΔf overtone profile indicates a slight uniform mass decrease across the SLB (ΔΔf₇–₉ = 0.0 Hz kDa⁻¹, Fig. 1B1). Although the increase in Δf (Fig. 1B1) may not seem consistent with the increase in mass (Fig. 2), it is possible for
simultaneous increases in both ΔF and ΔD to cause an overall increase in the mass of the adsorbed layer (14). No change in signal was observed for \( /H9251D^-1 \) during the entire AMP flow and buffer wash (Fig. 1, AIV and BIV).

**Designed AMPs: Equilibrium and Kinetic Behavior**

Four de novo designed AMPs, B1–B4, which are four similar peptides with rearranged amino acid sequences that have similar secondary structures, are described (9). Here, we are interested with the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC):1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) SLB to infer the mechanism of these newly designed AMPs. The selection of 3:1 molar ratio of POPC:POPG is used to represent the cell membrane of a Gram-positive bacteria (33). The \( /H9004/F \) overtone profile for B1 (Fig. 3 BI) indicates substantial mass bound to the SLB (\( /H9004/F \) for all overtones), yet has a more uniform distribution of \( /H9004/F \) compared with PG-1 (\( /H11005/1.9 \) for B4 (Fig. 3 BIV), which is close to the values of B1–B3, the
mechanism for B4 is apparently similar to B1–B3, but due to relatively lower $\Delta F$ changes, is evidently not as effective.

**Discussion**

Previous reports characterizing the amino acid sequence, structure, mechanism of binding, and minimum inhibitory concentrations for four well studied natural AMPs allow comparison of their QCM-D results with those of four de novo designed AMPs (9, 16, 18–24). This comparison provides a basis for suggesting the mechanism of interaction of the designed de novo AMPs when the AMPs are passed in solution across a deposited SLB consisting of 3:1 POPO:POPG lipids.
Natural AMP Mechanisms—In comparison to PG-1, similar ΔΔF trends were reported for the AMP sheep myeloid antimicrobial peptide (SMAP-29), where values for ΔΔF for all overtones were $-<10$ Hz and ΔΔF$_{3–9}$ = 4 Hz kDa$^{-1}$ ($<20$ Hz and ΔΔF$_{3–9}$ = 9 Hz kDa$^{-1}$ for PG-1) (15). Wang et al. (15) proposed that SMAP-29 had a mechanism of peptide insertion across the bilayer, whereas possibly forming water channels along with adsorption to the SLB surface. Separately, PG-1 has been shown to act through toroidal pore formation where the pores consist of 8–10 monomer units for lipids with 16–18 carbon chain lengths, which are the lengths of the POPC and POPG lipid tails examined here (20). Therefore, the signature of ΔΔF for PG-1 (Fig. 1$B_i$) appears to be one for pore formation, likely toroidal pore formation, where there is also a significant amount of AMP adsorbed at the lipid-water interface.

For IND, Wang et al. (15) notably reported very different ΔΔF overtone profiles for IND at 5 and 10 μM against egg PC lipids. They concluded that IND adsorbs at the lipid-water interface without peptide or water intercalation deep into the bilayer (15). A recent molecular dynamics simulation, which is in agreement with this result, reports IND causes thinning of a POPC lipid bilayer through adsorption at the lipid-water interface (21). In contrast, the ΔΔF overtone profiles in Fig. 1$B_i$ are much more similar to the pore formation with the surface adsorption mechanism of PG-1 than the profiles reported by Wang et al. (15) for membrane thinning caused by surface adsorption (21). This is likely due to the inclusion of the anionic POPG at a 3:1 mol ratio of PC:PG in our SLBs. IND is known to partition into large unilamellar vesicles (LUVs) composed of POPG with a partition coefficient 2 orders of magnitude higher than for POPC LUVs (22). Also, IND causes complete POPG LUV leakage, whereas it only results in 20–30% leakage of POPC LUVs at the same peptide concentrations (22). Therefore, at the AMP concentration and lipid composition examined here, IND binds further into the membrane than solely through the surface adsorption mechanism previously observed with a PC SLB, and likely through water channel formation in toroidal pores combined with a large amount of surface adsorption similar to that observed for PG-1 (Fig. 1, $B_i$ and $B_{ii}$).

MG-2, which is known to operate through a pore formation mechanism (34), undergoes a transition in the lipid interaction mechanism from membrane thinning at 4 μM to pore formation at 7 μM against 1:1 DOPC:DOPG mole ratio lipids (23). Here, at an intermediate peptide concentration (5 μM) against a similar lipid system, MG-2 appears to act through a mechanism of peptide insertion across the SLB, a result that is consistent with the interaction of alamethicin, a peptide known to insert into zwitterionic lipid systems, with PC lipids (15). Alamethicin is shown to form cylindrical pores that stabilize the SLB with similar ΔΔF overtone profiles at 0.5 and 1 μM AMP (15). A water channel, which would result in mass increases similar to those seen for PG-1 and IND, is not likely to form on addition of MG-2. Therefore, the ΔΔF overtone profile for MG-2 observed here gives strong support for peptide insertion orthogonal to the SLB plane without water channel formation (Fig. 1$B_{ii}$).

No change in signal was observed for the αD-1–SLB interaction at the experimental conditions examined here. This is in agreement with a previous publication (24) detailing that the antimicrobial mechanism of αD-1 involves functional interaction with lipid II, a precursor molecule necessary for cell wall synthesis, and not LUVs. Furthermore, it was also shown that αD-1 induced minimal leakage of liposomes with similar lipid compositions at the concentration tested here (24). Thus, αD-1 should have a negligible interaction with the SLB under the currently examined conditions.

For B1, in contrast to PG-1 indicates B1 has a significantly smaller ratio of mass bound at the lipid-water interface versus inside the SLB compared with PG-1 and IND. The more even distribution of ΔΔF with n for B1 suggests barrel-stave pore formation, a mechanism that has comparatively less peptide adsorbed at the lipid-water interface, as opposed to toroidal pore formation (32). Additionally, Wang et al. (15) showed that alamethicin, a pore forming AMP, resulted in uniform ΔΔF changes to a PC SLB (ΔΔF$_{3–9}$ = 0 Hz kDa$^{-1}$), supporting the contention here that B1 is a pore-forming AMP. Therefore, the mechanistic inference for B1 is probably a barrel-stave pore formation with relatively less surface adsorption at the lipid-water interface than for a toroidal pore.

Similar to B1, B2, B3, and B4 have minimal preference for adsorption at the lipid-water interface, with ΔΔF$_{3–9}$ = 0.94, 1.9, and 2.1 Hz kDa$^{-1}$ for B2, B3, and B4, respectively (Fig. 3B), which is well below the values for AMPs with known preferences for interfacial adsorption (ΔΔF$_{3–9}$ = 9 Hz kDa$^{-1}$, Fig. 1B). Interestingly, the dynamic behavior shows B3 first decreases ΔF and increases ΔD, but then greatly increases ΔF while still increasing ΔD (Fig. 3A$_{ii}$). This is similar to the effect seen by B2 just prior to buffer wash, but much stronger and occurs much earlier during AMP flow (Fig. 3A$_{ii}$). Therefore, a large amount of mass, relative to B2 and especially the other AMPs, appears to be naturally removed from the SLB, likely due to lipid removal from the surface, during peptide flow.

For B4, the kinetic behavior reveals a slight increase in ΔF, a feature also observed throughout MG-1 flow (Figs. 1A$_{iii}$ and 3A$_{iv}$). MG-1 inserts itself into the SLB without forming water channels similar to alamethicin (15). Thus there is evidence that B4 goes through an intermediate state of peptide insertion throughout the bilayer before transitioning to pore formation with water channels. It is possible that because B4 is a less potent AMP against numerous bacteria (9), the AMPs B1–B3 also undergo this transitional state but because they are more effective, the transition state is not observed at the AMP concentration examined here.

The ΔΔF overtone profiles indicate that the four designed AMPs follow a very similar mechanism to one of lipid removal
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with barrel-stave pore formation. However, the dynamic behavior of B1–B4 is quite different: B1 has one observable step to its kinetics, B2 has two stages of $\Delta F$ and decreases with a slight increase prior to buffer wash, B3 has three completely unique stages of $\Delta F$ changes, whereas B4 has a prolonged lag phase prior to a relatively slower one-step kinetic change (Fig. 3A1IV). Overall, these behaviors are in line with one all-encompassing four-step mechanism where different stages are observed depending on the antimicrobial activity of these peptides (B3 > B2 > B1 > B4) (9). The four stages are as follows. (i) An initial lag phase of AMP binding/insertion during which a critical AMP concentration must be reached prior to significant bilayer damage. (ii) A primary AMP water-channel formation stage during which there is rapid mass addition to the bilayer to help form the pores. (iii) A secondary mass addition into a pseudo-equilibrium state where there is slower mass addition followed by a plateau in $\Delta F$, whereas $\Delta D$ continues to increase. (iv) A significant mass removal from the surface where $\Delta F$ increases and $\Delta D$ again continues to increase.

Step i of the overall mechanism is only observed by B4, the least potent designed AMP (Fig. 3A1IV) (9). The first step, which occurred during the first 300 s of B4 flow (2.5 nmol of B2 dosage), involved $\Delta F$ increasing slightly from $\pm 25.5 \pm 0.72$ to $\pm 24.7 \pm 0.86$ Hz, whereas $\Delta D$ increased from $0.21 \pm 0.068 \times 10^{-6}$ to $0.51 \pm 0.080 \times 10^{-6}$ (Fig. 3A1IV). During this period, there was very little net mass change to the SLB ($\Delta m = 0.081 \pm 0.079 \mu g$). These signals for $\Delta F$ and $\Delta D$ are indicative of AMP insertion throughout the bilayer without water channels (similar to alamethicin (15)), and not AMP adsorption to only the surface of the SLB (IND (15)).

Step ii occurs with all 4 AMPs, but it is most obvious in B4 and B1. For B4, the primary water-channel formation stage occurred the slowest of the four AMPs, consistent with its lowest antimicrobial activity (Fig. 3A1IV) (9). After the initial lag phase of B4, $\Delta F$ decreased to a final value of $-30.9 \pm 0.79$ Hz, whereas $\Delta D$ increased to $2.39 \pm 0.56 \times 10^{-6}$ over the final 300 s of B4 flow (2.5 nmol of B4 dosage) (Fig. 3A1IV). These changes are modeled by an increase in $\Delta m$ to $0.50 \pm 0.13 \mu g$ (Fig. 2B). For B1, these changes are qualitatively similar, but occur more rapidly. In the first 70 s of B1 flow (0.58 nmol of B1 dosage), $\Delta F$ decreased from $-24.8 \pm 0.16$ to $-32.0 \pm 0.39$ Hz, whereas $\Delta D$ increased to $1.84 \pm 0.29 \times 10^{-6}$ (Fig. 3A1I). The modeled mass associated with these $\Delta F$ and $\Delta D$ changes is $0.60 \pm 0.032 \mu g$, which is the same as B4 within error (Fig. 2B). For B4 and B1, this is the final stage of the mechanism that these AMPs reach. The water-channel pores are sufficient for antimicrobial activity, as evidenced by their micromolar MICs against a range of bacteria (9). However, the more effective AMPs, B2 and B3, exhibited additional mechanistic stages beyond these initial two.

Step iii involves a slower rate of mass addition followed by a plateau of mass increase to the SLB and is only seen for B2 and B3. For B2, the secondary mass addition occurred when $\Delta F$ decreased from $-35.6 \pm 0.45$ to $-43.2 \pm 0.50$ Hz and $\Delta D$ increased from $4.41 \pm 0.16 \times 10^{-6}$ to $7.70 \pm 0.60 \times 10^{-6}$ over the course of 450 s, whereas the primary mass addition occurred in the first 120 s of B2 flow (Fig. 3A1II). The net mass addition reached a maximum of $1.20 \pm 0.16 \mu g$ in this time period, a higher value than for any other designed AMP (Fig. 2B). B3 underwent a qualitatively similar process. $\Delta F$ decreased from $-31.0 \pm 1.73$ to $-35.8 \pm 1.45$ Hz, whereas $\Delta D$ increased from $2.11 \pm 0.58 \times 10^{-6}$ to $3.31 \pm 0.98 \times 10^{-6}$ over 130 s AMP flow (Fig. 3A1III). During this time, B3 had a maximum net mass addition to the SLB of $0.63 \pm 0.015 \mu g$ while under AMP flow (Fig. 2B).

The final step (step iv) is significant lipid removal from the SLB. Both B2 and B3 have final stages where there is a $\Delta F$ increase coupled with a $\Delta D$ increase. For B2, it occurs in the final 30 s of AMP dosage, where $\Delta F$ increases from a minimum of $-43.2 \pm 0.50$ to $-41.8 \pm 0.48$ Hz (Fig. 3A1II). For B3, this stage occurs over a more prolonged period where $\Delta F$ increases from a minimum of $-35.8 \pm 1.45$ to $-30.5 \pm 1.03$ Hz and $\Delta D$ increases from $3.31 \pm 0.98 \times 10^{-6}$ to $4.45 \pm 0.15 \times 10^{-6}$ (Fig. 3A1III). The changes for B3 are coupled with a very slight total mass decrease from a maximum of $0.63 \pm 0.015$ to $0.58 \pm 0.073 \mu g$, although these numbers only reflect the wet plus dry mass change to the surface, and not necessarily just the dry lipid mass that remains on the sensor (Fig. 2B). B2 does not result in a significant mass decrease due to the large error associated with the modeling of the B2 mass change. However, the $\Delta F$ and $\Delta D$ changes seen here for B2 and B3 are typical for an AMP with a mechanism of lipid removal from an SLB, as seen for chrysophacin-3 (32). Thus, the four stages of the designed AMP mechanism are observed throughout the dynamic behavior of the four AMPs, where the initial stages are visible for the less potent AMPs, and the more potent AMPs proceed into the final two stages of the mechanism (Fig. 3). Representative schematics for the mechanisms of all natural and designed AMPs are shown in Fig. 4.

Experimental Procedures

AMPS

Natural Peptides—The four natural AMPs were synthesized using standard Fmoc/-butyl chemistry by Dr. Haydn Ball from the University of Texas Southwestern (Dallas, TX) and were purified to over 95% purity using HPLC in Dr. Ball’s laboratory. All peptides with the exception of Magainin II were synthesized on a Protein Technologies Inc. Symphony peptide synthesizer using standard Fmoc/tetrabutylammonium chloride (TBA) activation protocols. Magainin II was synthesized on a Applied Biosystems 433A using HCTU activation. Cleavage of the peptides was achieved using 95% TFA with thioanisole and 1,2-ethanedithiol scavengers between 1.5 and 2.5 h at room temperature. The crude peptides were precipitated in cold diethyl ether and the pellet was washed 3 times with fresh ether. The peptides were analyzed and purified by Waters RP-HPLC using Vydac C4 columns. The purified fractions were characterized using either Micromass MALDI-TOF or Agilent ESI-MS mass spectrometers. Peptides were received as lyophilized powders and were dissolved in 10 mM phosphate-buffered saline, pH 7.4 (Sigma, catalog number P4417), prior to use.

Synthetic Peptides—The synthetic AMPs were produced using an automated Multiprep RS synthesizer (Intavis AG, Germany) in the laboratory of Dr. Pankaj Karande. Fmoc solid-
phase chemistry was used to synthesize the peptides from their C termini to N termini on a TentaGel rink amide resin (0.25 mmol/g) (Intavis Inc., Chicago, IL). Pre-synthesis, the resin was swollen in a dimethylformamide:dichloromethane (2:1) solution. Post peptide-chain assembly, the resin was washed with dichloromethane and the peptides were cleaved off using a TFA:TIS:H2O (88:6:6) mixture. Bulk TFA was removed by precipitating the peptides in ice-cold methyl tert-butyl ether (MTBE) followed by centrifugation and a second MTBE wash. Peptides were air dried and dissolved in acetonitrile:H2O (1:5) for lyophilization. Lyophilized peptides were stored at $-20^\circ$C.

**Mass Spectrometry Analyses**—LC-MS/MS experiments were performed on Thermo LTQ XL Orbitrap mass spectrometers (Thermo, Bremen, Germany). Samples were injected using an Agilent 1200 C18-HPLC system (Agilent, Palo Alto, CA) using an Agilent 1200 autosampler. Thermo Biobasic column (150 × 2.1 mm; particle size 5 μm) was used for separation. The injection volume was 3 μl and a flow rate of the mobile phase was 250 ml/min. The mobile phase consisted of 0.2% formic acid (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The mass spectrometers operated in ESI mode with detection of positively charged ions using the Orbitrap as detector in m/z range 300–2,000. The resolution was ~30,000 and mass accuracy was better than 3 ppm. LC-MS/MS gave the measured masses of (M+H)$^+$ as m/z (observed) 1885.1247, 1885.1212, 1885.1210, and 1812.1253 for B1, B2, B3, and B4, respectively. These results confirm that all four peptides were amidated at the C termini and is in agreement with the theoretical mass, m/z 1885.1224 (B1-B3) and 1812.1272 (B4). Finally, we also ran tandem MS/MS and all amino acid sequences as listed above.

**Lipid Vesicle Preparation**

15 μmol of total lipids, 75% by mole of POPC (Avanti Lipids®, Alabaster, AL, catalog number 850457) (8.55 mg) and 25% by mole of POPG (sodium salt) (Avanti Lipids®, catalog number 840457) (2.89 mg), were dissolved in chloroform (Sigma, catalog number 528730) in a round bottom flask. Chloroform was evaporated under a N2 stream in a 45 °C water bath. Lipids were lyophilized overnight to remove residual chloroform. Lipid film was rehydrated in 3 ml of MilliQ™ water. Lipid vesicles were extruded (Mini-extruder kit, Avanti Lipids®, catalog number 610000) 21 times through a 30-nm polycarbonate membrane (Avanti Lipids®, catalog number 610002) at 42 °C. 300 μl of lipid vesicle solution was added to 10 ml of phosphate buffer saline, pH 7.4, containing an additional 140 mg of NaCl.

**Silica Crystal Preparation**

50-nm SiO2 crystals (Biolin Scientific, Linthicum Heights, MD, catalog number QSX 303) were initially washed with MilliQ™ H2O, EtOH (Sigma, catalog number 792780), and dried with N2. Atmospheric plasma (ATOMFLO, Surfex Technologies LLC, Culver City, CA) using 30 liters/min of helium, 0.2 liter/min of O2, and 120 W power was used to clean the crystal surface. Crystals were then placed inside a flow module for use (Q-Sense Flow Module, QFM 401). After experimentation, crystals were washed with H2O, EtOH, dried with N2, and stored in air for further use.

**QCM-D**

Changes in frequency ($\Delta f$) and change in dissipation ($\Delta D$) at overtones 3, 5, 7, and 9 were obtained from the quartz crystal microbalance with dissipation (QCM-D, Biolin Scientific/Q-Sense, Q-Sense E4 Auto, Västra Frölunda, Sweden). These correspond to changes in mass and rigidity, respectively, for a rigid layer above the crystal. Lipid bilayer deposition was performed following a protocol from Cho et al. (25) AMP interaction with the newly formed SLB was carried out as follows: 5 μM AMP flow for 10 min at 100 μl/min followed by PBS flow for 20 min
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at 100 μl/min. The SiO₂ surface was then regenerated with a wash of H₂O for 10 min at 500 μl/min, 2% SDS (powder purchased from Sigma, catalog number 436143) for 10 min at 500 μl/min, H₂O for 10 min at 500 μl/min, and finally PBS for 10 min at 100 μl/min to re-equilibrate the sensor.

Model

For non-rigid layers, which the SLBs become after interaction with the all AMPs except MG-2 and αD-1, changes in frequency (Δf) and dissipation (ΔD) are related to changes in mass and viscoelastic changes to the SLB using the Voigt model, which is briefly reviewed here (14). For consistency, the Voigt model is used here to model all AMP-SLB interactions. Δf and ΔD are related to mass and viscoelastic properties by,

\[ \Delta f = \frac{\eta_l}{2\pi\delta_l m_q} - f_0 m_s \]

\[ \Delta D = \frac{\eta_l}{m\pi\delta_l m_q} + \frac{m_l}{m_q} \left[ 4 \frac{\eta_l}{\rho_l \delta_l} \frac{G'}{G' + G''} \right] \]

where \( \eta_l \) is the liquid viscosity (1,000 kg/m³), \( \delta_l \) is the acoustic wave decay length, \( m_s \) is the film mass per unit area, \( m_q \) is the mass of the quartz crystal, \( \rho_l \) is the film density (1,100 kg/m³) (26), \( G' \) and \( G'' \) are the storage and loss moduli, respectively, and \( n \) is the overtone number (14).

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