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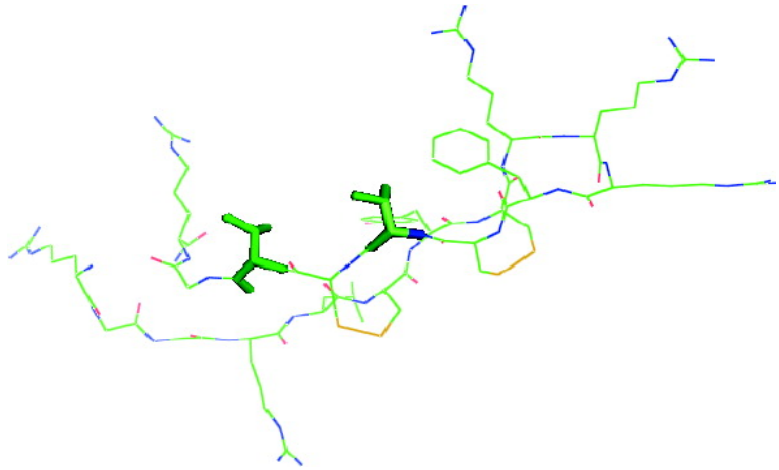

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Using Fluorous Amino Acids To Probe the Effects of Changing Hydrophobicity on the Physical and Biological Properties of the β-Hairpin Antimicrobial Peptide Protegrin-1[†]

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ABSTRACT: Protegrins are potent members of the β-hairpin-forming class of antimicrobial peptides. Key to their antimicrobial activity is their assembly into oligomeric structures upon binding to the bacterial membrane. To examine the relationship between the physicochemical properties of the peptide and its biological activity, we have synthesized variants of protegrin-1 in which key residues in the hydrophobic core, valine-14 and -16, are changed to leucine and to the extensively fluorinated analogue hexafluoroleucine. These substitutions have the effect of making the peptide progressively more hydrophobic while minimally perturbing the secondary structure. The leucine-containing peptide was significantly more active than wild-type protegrin against several common pathogenic bacterial strains, whereas the hexafluoroleucine-substituted peptide, in contrast, showed significantly diminished activity against several bacterial strains. Isothermal titration calorimetry measurements revealed significant changes in the interaction of the peptides binding to small unilamelar vesicles that mimic the lipid composition of the bacterial membrane. The binding isotherms for wild-type and leucine-substituted protegrins indicate that electrostatic interactions dominate the membrane−peptide interaction, whereas the isotherm for the hexafluoroleucine-substituted protegrin suggests a diminished electrostatic component to binding. Notably both of these substitutions appear to alter the stoichiometry of the lipid−peptide interaction, suggesting that these substitutions may stabilize oligomerized forms of protegrin that are postulated to be intermediates in the assembly of the β-barrel membrane pore structure.

The emergence of bacterial strains resistant to most of the clinically useful antibiotics has provided the impetus to develop new classes of antibiotics that may combat bacterial resistance more effectively. Antimicrobial peptides (AMPs)[1]are small peptides that show promise as therapeutic agents against bacteria, fungi, and viruses (*1*−*3*). Widely distributed in multicellular organisms, they form part of the initial line of defense in the innate immune system and are also implicated in the activation of the adaptive immune response against microbes (*4*).

Although highly diverse in sequence and structure, almost all AMPs share the property of being highly amphipathic, with one face of the peptide being hydrophobic and the other face presenting a cluster of positively charged residues (*5*−*7*). AMPs are often classified based on the structural characteristics of the peptides. These classifications include α-helical, linear, or disulfide bonded (*3*). The number of disulfide bonds range from one to four and result in the peptides adopting β-hairpin-like structures (*8*).

Whereas some AMPs have been determined to act intracellularly (*9*), most appear to function primarily by disrupting bacterial cell membranes (*10, 11*). The selectivity of AMPs for bacterial membranes arises primarily from electrostatic interactions between the positively charged peptide and the negatively charged phospholipids that predominate in bacterial cell membranes. Eukaryotic membranes, which contain predominantly neutral phospholipids, are usually less susceptible to disruption by AMPs; the presence of cholesterol in eukaryotic membranes also helps to prevent membrane disruption by AMPs (*12*). Upon association with the membrane, disruption of the bacterial membrane may proceed through a number of mechanisms, including the formation of pores, membrane thinning, and detergent-like action (*13, 14*).

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[1] Abbreviations: AMP, antimicrobial peptide; CD, circular dichroism; hFLeu, L-5,5,5,5′,5′,5′-hexafluoroleucine; ITC, isothermal titration calorimetry; MALDI-TOF MS, matrix-assisted laser desorption ionization−time-of-flight mass spectrometry; MIC, minimum inhibitor concentration; PBS, phosphate-buffered saline solution; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; PG-1, protegrin-1; SUVs, small unilamelar vesicles.

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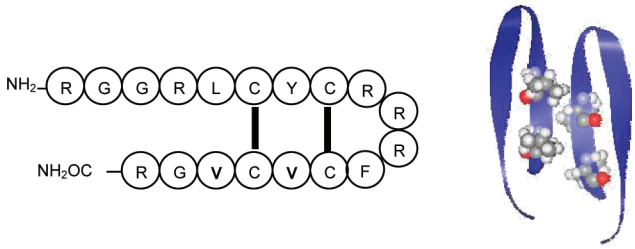

FIGURE 1: Sequence of protegrin-1 (left) showing disulfide bonding pattern; valine-14 and -16 are in bold. Right: β-Hairpin structure of protegrin dimer determined by NMR (32) with valine-14 and -16 shown in space-filling representation.

Key to the mechanism of action of AMPs is their ability to form oligomeric structures, dimers and higher oligomeric structures, in the presence of membranes (*15*−*19*). Work in our laboratory and others has shown that extensively fluorinated analogues of hydrophobic residues such as leucine and valine effectively stabilize oligomeric peptide assemblies (*20*−*26*). Among the properties conferred by fluorination are large increases in association constants, increased stability toward thermal unfolding and denaturation by organic solvents, and protection from proteolytic degradation (*27, 28*). The stabilization of such proteins may be attributed to the much greater hydrophobicity of fluorocarbons.

With this in mind, we recently designed a fluorous analogue of the potent α-helical AMP MSI-78 (pexiganan) (*28*), which we named fluorogainin-1. Incorporation of 5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu) residues in place of Leu and Ile residues in the amphipathic helix conferred increased potency toward some common pathogenic bacteria with no measurable increase in hemolytic activity. Most notably, fluorogainin-1 was far more resistant to proteolysis in the presence of lipid membranes than the parent peptide MSI-78.

Following the interesting results we observed with α-helical AMPs, we have extended our studies to investigate the effects of modulating the membrane interactions of a β-sheet AMP by fluorination. We selected protegrin-1 (PG-1) as a well-studied representative of this latter class of AMPs (*29*). PG-1 is a broad spectrum AMP that was originally isolated from porcine leukocytes (*30*). It comprises 18 residues (Figure 1A), including four cysteine residues that form two disulfide bonds that stabilize the β-hairpin structure of the peptide (Figure 1B) (*31, 32*). PG-1 is believed to form pores in lipid membranes upon oligomerization of the peptide at high concentration (*33, 34*); however, the pathway by which the peptide assembles into pores upon binding the membrane is poorly understood.

Here we describe experiments aimed at examining the effects of changing the hydrophobicity of the peptide on its biological activity and its interaction with membranes. We have systematically increased the hydrophobicity of PG-1 in a structurally conservative manner by substituting two residues, Val14 and Val16, that form part of the hydrophobic core of the peptide hydrophobic contacts at the interface of the peptide with leucine (PG-1-LL) and 5,5,5,5′,5′,5′-hexafluoroleucine (PG-1-FF). In this case, the use of the extensively fluorinated residue, hFLeu, allows the hydrophobicity of the peptide to be increased with minimal structural perturbation.

MATERIALS AND METHODS

Materials. Rink Amide resin, *t*-Boc-protected amino acids, and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophophate (HBTU) were purchased from Nova-Biochem. L-5,5,5,5′,5′,5′-Hexafluoroleucine (hFLeu) was synthesized as described previously (*35*) and converted to the *t*-Boc-protected derivative by standard procedures. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipids. Chymotrypsin and trypsin were purchased from Boehringer Mannheim GmbH. All other chemicals were purchased from Fisher and used without further purification.

Peptide Synthesis and Purification. The peptides described in this study were synthesized using *t*-Boc-protected amino acids for Merrifield manual solid-phase synthesis on MBHA resin; couplings were performed using *in situ* neutralization/HBTU protocol described by Schnolzer et al. (*36*) on a 0.25 mmol scale. The peptide was cleaved from the resin using "high"-HF conditions.

The peptides were redissolved at ~10 mg/mL in 8 M urea and 10 mM DTT, to maintain cysteine residues in reduced form, and purified by reverse-phase HPLC on a Waters semipreparative C_{18} column equilibrated in 0.1% formic acid and eluted with a linear gradient from 5% to 50% acetonitrile containing 0.1% formic acid. Acetonitrile was removed under a stream of nitrogen and the solution adjusted to pH 7.0 with 0.1 volume of PBS buffer. DMSO was then added to a final concentration of 20% (v/v), and the peptide solutions were stirred for 48 h at 4 °C open to the atmosphere to allow disulfide oxidation to occur.

The oxidized peptides were lyophilized to remove DMSO, redissolved in 5% acetonitrile and 0.1% formic acid, and repurified by reverse-phase HPLC on a Waters semipreparative C_{18} column equilibrated in 0.1% formic acid as described above. The peptides were determined to be pure by analytical HPLC and mass spectrometry. The concentration of the peptides was determined by the absorbance at 278 nm using a calculated extinction coefficient of 1520 cm^{-1} M^{-1} based on the presence of one Tyr, one Phe, and two disulfide bonds in the peptide.

The purity and identity of the peptides were confirmed by MALDI-TOF mass spectrometry. The masses obtained (PG-1, calculated 2156.58, found 2156.16; PG-1LL, calculated 2184.64, found 2184.32; PG-1-FF, calculated 2400.52, found 2400.96) were consistent with their intended sequences and indicated that the peptides contained two disulfide bonds after air oxidation.

Hydrophobicity Analysis. Peptide samples were analyzed by RP-HPLC using a C_{18} column to compare the relative hydrophobicity of the nonfluorinated and fluorinated samples. Peptides were loaded onto the column equilibrated in solvent A (5% acetonitrile with 0.1% formic acid) and eluted at 1 mL/min with a linear gradient of acetonitrile as follows: 0−30% solvent B (90% acetonitrile with 0.1% formic acid) over 0−5 min and then 30−70% solvent B from 5 to 35 min.

Circular Dichroism. To examine the secondary structure of PG-1, PG-1-LL, and PG-1-FF, 20 μL of each peptide at 680 μM was diluted 10-fold in PBS buffer, pH 7.4. POPC liposomes were freshly prepared in PBS buffer, pH 7.4, to

make a 13.6 mM solution of unilamilar liposomes by sonication with a Fisher Scientific 550 sonic dismembrator until the solution was clear. Twenty microliters of the liposome solution was added to the diluted peptide to make a solution containing 1:200 (mol/mol) peptide to lipid. The CD spectra for each peptide and peptide/liposome solution were recorded from 200 to 250 nm with an Aviv 62DS spectropolarimeter at 25 °C. Mean residue ellipticities, Θ_M, were calculated using eq 1, where Θ_{obs} is the ellipticity measured in millidegrees, c is the molar concentration, l is the cell path length in centimeters, and n is the number of residues in the protein.

$$\Theta_M = \Theta_{obs}/10lcn \quad (1)$$

Isothermal Titration Calorimetry. POPC/POPG (3:1 mol/mol) SUVs (10 mM), prepared as described for CD experiments, were titrated into peptide solutions (~55 μM) in PBS, pH 7.4. The liposomes were freshly prepared in PBS, pH 7.4. Three microliter injections of liposomes into peptide solution or PBS (as a control experiment to determine heat of dilution) were made for a total of 15 injections. Fitting was performed using the Microcal/Origin software.

Bacterial Strains and Growth. Escherichia coli D5α was obtained from Invitrogen (Carlsbad, CA). *Enterococcus faecalis* cultures were a gift from Dr. Donald B. Clewell, University of Michigan. *Bacillus subtilus* (ATCC 663), *Kocheria rhizophila (*ATCC 9341), *Earobacter aerogenese* (ATCC 13408), *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* (ATCC 25933), *Salmonella enteritis typhimurium* ATCC 14028, *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 6538), and *Shigella sonnei* (ATCC 25931) were obtained from MicroBiologics, St. Cloud, MN. All species were maintained by weekly transfer on trypticase soy agar and broth cultures grown directly from individual colonies in trypticase soy broth.

MIC Determinations. A sterile, 96-well microtiter plate was used as the platform for the assay. An overnight culture of each bacterium was diluted to 10^6 cfu/mL in sterile phosphate-buffered saline and seeded into each well (100 μL) of the plate. Doubling dilutions (200−3.13 μg/mL) of PG-1, PG-1 LL, or PG-1 FF (100 μL) in replicates of eight were then added and the cultures covered with a sterile adhesive plastic film. After centrifugation for 1 min at 800g to collect the entire inoculum to the bottom of the plate it was incubated at 37 °C overnight in air. Growth was determined by measuring the OD_{595} of each well using a microwell plate reader (GENious, Techan, Manendorff, Switzerland). The MIC for each organism was determined as the lowest dilution of each peptide without significant growth above the inoculum ($p \leq 0.01$, t test). The differences between peptides for each bacterial strain were evaluated using a t test.

Hemolytic Activity. A sterile, 96-well round-bottom microtiter plate (Costar) was used as the platform for the assay. Ovine red blood cells (2.5%) in 100 μL of PBS were added to the wells of the plate. Doubling dilutions (200−1.56 μg/mL) of PG-1, PG-1 LL, or PG-1 FF (100 μL) in replicates of eight were then added and the cultures covered with a sterile adhesive plastic film. The plate was incubated for 2 h at room temperature and examined by eye. The highest dilution of peptides without an intact cell pellet was noted.

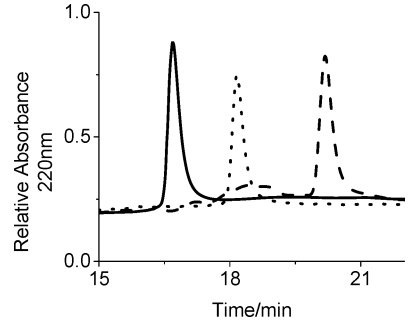

FIGURE 2: Hydrophobicity of peptides assessed by reverse-phase HPLC. Traces of PG-1 (solid line), PG-1-LL (dotted line), and PG-1-FF (dashed line). A linear gradient of acetonitrile in 0.1% TFA was used to elute peptides.

RESULTS

Protegrin was selected for our investigation because it is a well-studied β-hairpin-forming AMP for which there are structural data from NMR studies (*37, 38*). Two valine residues, at positions 14 and 16, together with Phe12 form a hydrophobic cluster that contributes to the amphipathic character of the peptide. To modulate the hydrophobicity of the peptide with minimal perturbation of the structure, we synthesized protegrin analogues in which Val14 and Val16 were substituted with leucine (PG-1-LL) and the more hydrophobic but sterically conservative analogue hFleu (PG-1-FF). These peptides were synthesized by standard manual solid-phase techniques using *t*-BOC chemistry with cysteine in the reduced form. After air oxidation to form the disulfide bonds and repurification the identity of the peptides was confirmed by MALDI-TOF mass spectrometry.

Hydrophobicity of Peptides. The effect of the leucine and hFLeu substitutions on the hydrophobicity of protegrin was assessed qualitatively by reverse-phase HPLC using an analytical C_{18} column. The chromatograms of each peptide are shown overlaid in Figure 2. PG-1 elutes at the earliest time of ~16.8 min followed by PG-1 LL at ~ 18.2 min and then PG-1 FF at ~20.2 min. The hydrophobicity values, Π, obtained previously from water−octanol partitioning measurements, are 1.22 for valine (*39*), 1.70 for leucine (*39*), and 1.87 for hFLeu (*40*). The gradual increase in elution times of the peptides is consistent with the expected increase in hydrophobicity of the peptides as valine is substituted by the increasingly hydrophobic leucine and hFLeu side chains.

Secondary Structure of Peptides. To confirm that the amino acid substitutions did not significantly alter the β-hairpin-like secondary structure of protegrin, the CD spectra of PG-1, PG-1-LL, and PG-1-FF were recorded in PBS buffer and in either the presence or absence of small unilamellar vesicles (SUVs), freshly prepared from POPC/POPG (3:1 mol/mol). The lipid:peptide ratio in the samples was 200:1. In the absence of SUVs the spectra of all three peptides (Figure 3) exhibited minima between 195 and 205 nm ($\Theta_M = \sim -7000$ deg cm^{2}dmol^{-1} res^{-1}) and broad maxima centered around 228 nm ($\Theta_M = \sim 1000$ deg cm^2 dmol^{-1} res^{-1}). The spectra appear to represent a mixture of random coil and β-sheet backbone conformations. In the presence of membranes the spectra become significantly more β-sheet-like with the minima shifting to between 220 and 225 nm ($\Theta_M = -6000$ to -7000 deg cm^2 dmol^{-1} res^{-1}) and the maxima shifting to ~335 nm ($\Theta_M = \sim 1500$ deg cm^2 dmol^{-1} res^{-1}). Interference from the lipids meant that the CD spectra could not be

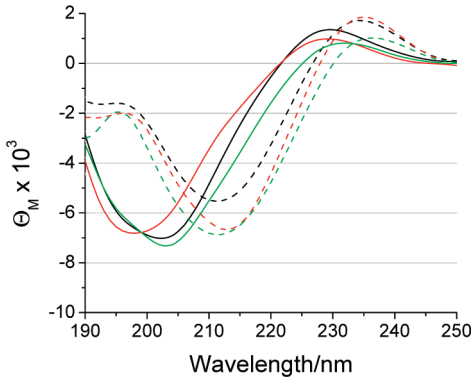

FIGURE 3: Conformational changes associated with protegrin variants binding to membranes. CD spectra of peptides: solid lines, peptides in the absence of lipid membranes; dashed lines, pepides in the presence of POPC/POPG (3:1); black lines, PG-1; red lines, PG-1-LL; green lines, PG-1-FF.

Table 1: MIC Values of PG-1, PG-1-LL, and PG-1-FF for a Panel of Common Pathogenic Bacterial Strains

			MIC values (μg/mL)		
strain name	ATCC no.	type	PG-1	PG-1-LL	PG-1-FF
B. subtilis	6633	Gram +	25	6.25	>200
K. rhizophila	9341	Gram +	25	12.5	>200
E. aerogenes	13048	Gram −	6.25	6.25	12.5
K. pneumoniae	4352	Gram −	ND	6.25	50
P. mirabilis	25933	Gram −	>200	>200	>200
S. enterica	14028	Gram −	6.25	6.25	6.25
S. aureus	UH11	Gram +	25	6.25	6.25
S. enterica		Gram −	6.25	6.25	6.25
E. faecalis	OG1 X	Gram +	ND	3.13	12.5
Y. enterocolitca		Gram −	25	1.56	12.5
S. sonnei		Gram −	12.5	3.13	12.5

reliably recorded at wavelengths below 195 nm. The presence of negatively charged POPG lipids was necessary to induce the conformational changes, as in the presence of membranes made from POPC alone little or no change in the CD spectra of the peptides was apparent (data not shown).

These results confirm the requirement for negatively charged lipids within the bilayer for the peptides to bind to the membrane and indicate that binding induces a significant conformational change in the peptide to a more β-sheet-like structure. However, the substitution of the two valine residues by either leucine or hFLeu, which have lower β-sheet propensities, does not appreciably alter the secondary structure of the peptide when bound to the membrane.

Antimicrobial Activity. Having established that the amino acid substitutions increased the hydrophobicity of the protegrin analogues without appreciably changing the secondary structure, we next investigated the antimicrobial activities of PG-1, PG-1-LL, and PG-1-FF. The activity of each peptide, as measured by its minimal inhibitory concentration (MIC), was determined against a panel of bacteria selected to include a range of common pathogenic organisms representing both Gram-positive and Gram-negative bacteria. The study was designed to include a sufficient number of bacterial strains that either an increase or decrease in the potency of a particular peptide against a particular bacterial strain would not bias the conclusions of the experiment.

The MIC data are presented in Table 1. Overall, it is apparent that PG-1-LL is more potent than the parent AMP whereas PG-1-FF is less potent. Thus PG-1-LL exhibits MICs that are significantly lower ($p < 0.05$) than PG-1 against 4 of the 11 strains tested, *B. subtilis*, *S. aureus*, *Y. enterocolitca*, and *S. sonnei*, and in no case is the MIC higher than that for PG-1. In particular, PG-1-LL exhibited excellent activity against *Y. enterocolitca*, *E. faecalis*, and *S. sonnei* with MICs of 1.56, 3.13, and 3.13 μg/mL, respectively. In contrast, PG-1-FF exhibited no detectable activity against *B. subtilis* and *K. rhizophila*, although these strains are quite susceptible to PG-1 and to PG-1-LL. The only bacterium against which PG-1-FF exhibited significantly increased potency was *S. aureus*, and no bacterial strains were found for which PG-1-FF was more potent than PG-1-LL. These results indicate that the susceptibility of bacterial strains to protegrin and similar AMPs is critically dependent upon the hydrophobicity of the peptide.

Table 2: Hemolytic Activity of PG-1, PG-1-LL, and PG-1-FF Expressed as a Percentage of Hemoglobin Released from Bovine Red Blood Cells

concn (μg/mL)	PG-1	PG-1-LL	PG-1-FF
250	100	100	100
125	100	100	100
62.5	100	100	100
31.3	100	100	100
15.6	100	100	100
7.81	44	26	100
3.91	16	0	51
1.95	0	0	0

Hemolytic Activity. The hemolytic activity of the peptides was determined for ovine red blood cells. The hemolytic activity of the peptides was quite similar, with all three eliciting hemolysis at minimum concentrations between 3.9 and 7.8 μg/mL (Table 2). Although small differences in hemolytic activity of the peptides were observed, these probably lie within the range of experimental error.

Thermodynamics of Peptide Binding to Lipids. The thermodynamic changes associated to the peptides interacting with lipid bilayers were investigated by isothermal titration calorimetry (ITC). SUVs composed of 7.5 mM POPC and 2.5 mM POPG were titrated in 3 μL increments into 55 μM peptide solutions at 303 K. The binding of peptides to SUVs was weakly exothermic for all peptides (Figure 4, top panels); binding of PG-1 ($\Delta H = -1.6$ kcal/mol) was slightly more exothermic than either PG-1-LL or PG-1-FF ($\Delta H = -1.3$ kcal/mol).

Integration of the binding energy peaks allowed the data to be fitted to a single site binding isotherm (Figure 4, lower panels) with the thermodynamic parameters listed in Table 3. The calculated free energies of binding, ΔG, were similar for the three peptides (Table 3), and it appears that the entropic contribution to lipid binding provides the major driving force for the overall free energy change. However, although the fits appear reasonable, caution must be exercised in interpreting these data, because in general, as discussed below, AMP−membrane interactions are not well described by a simple, single site binding isotherm (*41*−*43*).

To obtain further insight into the physical nature of the interactions between the peptides and SUVs, the ITC data were used to calculate the binding isotherm, following the analysis described by Seelig (*41, 42*). For each titration step, i, the concentration of free peptide remaining in bulk solution at equilibrium is defined as c^i_f, and the degree of binding (i.e., the fraction of peptide bound per mole of lipid) is defined as X^i_b. The variation of X^i_b with c^i_f constitutes the binding isotherm and does not assume single site binding.

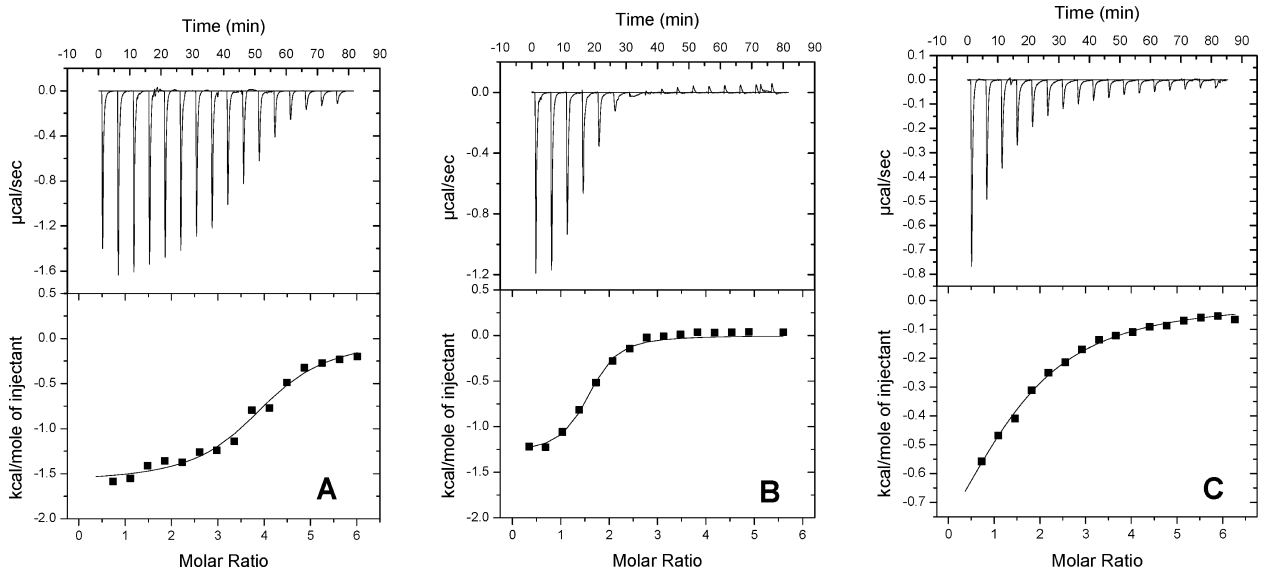

FIGURE 4: Isothermal titration calorimetry of PG-1 peptides with SUVs. Top panels: Thermograms for PG-1 (A), PG-1-LL (B), and PG-1-FF (C) titrated with SUVs. Bottom panels: Integration of corresponding thermograms showing fits to single site binding isotherms. For details see the text.

Table 3: Thermodynamic Parameters for PG-1, PG-1-LL, and PG-1-FF Binding to Small Unilamelar Vesicles Derived from ITC Data

peptide	N (mol/mol)[a]	ΔH (kcal/mol)	K_a (10^4 M^{-1})	$K_c^{(k)}$ (M^{-1}) [C_f (μM)][b]	ΔS (cal mol^{-1} deg^{-1})	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
PG-1	2.3 ± 0.1	-1.6 ± 0.05	12 ± 2.5	1.1×10^4 (49.5) 6.5×10^5 (0.6)	18 ± 0.5	5.5 ± 0.2	-7 ± 0.4
PG-1-LL	0.9 ± 0.1	-1.3 ± 0.05	27 ± 6.9	3.0×10^4 (43.6) 5.0×10^6 (0.1)	21 ± 1.2	6.4 ± 0.4	-8 ± 0.8
PG-1-FF	0.8 ± 0.1	-1.3 ± 0.16	1.7 ± 0.19	2.1×10^4 (34.6) 5.0×10^5 (0.5)	15 ± 0.7	4.5 ± 0.2	-6 ± 0.6

[a] The stoichiometry of peptide binding to lipid, N, was calculated making the standard assumption that only 60% of the lipid molecules in the SUVs are available to bind peptide. [b] $K_c^{(k)}$ were determined from the binding isotherms shown in Figure 5. $K_c^{(k)}$ is concentration dependent and is reported for representative high and low concentrations of free peptide, C_f (concentration in parentheses).

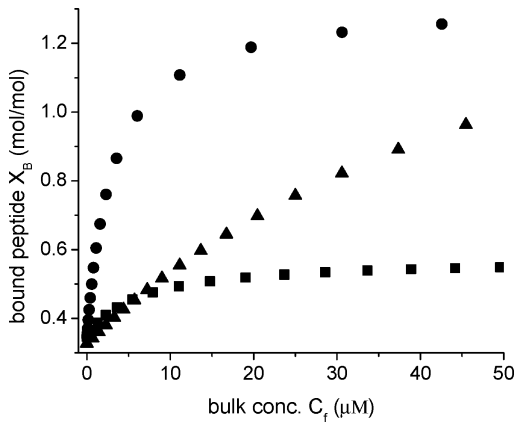

FIGURE 5: Binding isotherm as deduced from the cumulative heat of reaction obtained from ITC. The mole fraction of bound peptide to lipid, X_B, is plotted versus the equilibrium free peptide concentration in bulk solution, C_f, for PG-1 (squares), PG-1-LL (circles), and PG-1-FF (triangles).

The asymptote of each curve represents the maximal binding capacity, B_{max}, i.e., the number of peptide molecules bound per lipid with the peptide in large excess over lipid. The binding isotherm for each peptide is plotted in Figure 5.

A purely hydrophobic interaction between lipid and peptide is expected to yield a linear relationship between X^i_b and c^i_f which represents the simple partitioning of the peptide between the aqueous and lipid phases. Electrostatic interactions between the lipid bilayer surface, which carries a negative charge, and the peptide, which carries a positive charge, result in the curved plots seen in Figure 5. This is because with increasing amounts of peptide bound to the lipid membrane surface the electrostatic attraction decreases. In the initial stages of binding, i.e., at high c^i_f, X^i_b decreases gradually. This is followed by a drop in X^i_b, which is characterized by a downward turn of the binding isotherm at lower c^i_f. A result of the electrostatic interactions between peptide and lipid is that the equilibrium constant for peptide binding to lipid, K^i_c, is concentration-dependent and for a given concentration of free peptide c^i_f is given by $K^i_c = X^i_b/c^i_f$. The range of K^i_c values measured for each peptide is given in Table 3.

It is evident that the binding isotherms (Figure 5) for PG-1 and PG-1-LL exhibit similar, high degrees of curvature suggesting a strong electrostatic component to binding. In contrast, the binding isotherm for PG-1-FF exhibits far less curvature, suggesting that fluorination increases the hydrophobic component of binding and may potentially interfere with electrostatic interactions between the peptide and membrane.

Most interestingly, the substitution of valine by either leucine or hFLeu dramatically changes the stoichiometry of peptide binding to lipid. PG-1 binds to the surface of the SUVs with a stoichiometry of ~2 lipid/peptide. However, PG-1-LL binds only about half as many lipids per peptide, with a stoichiometry of ~0.8 lipid/peptide. It is evident from the binding isotherm for PG-1-FF that peptide binding does not saturate in the experimentally accessible concentration

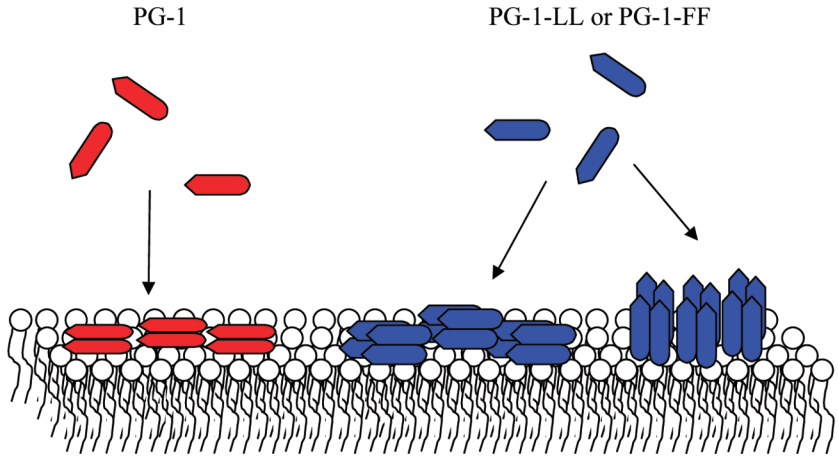

FIGURE 6: Cartoon illustrating how a change in the oligomerization state of PG-1 upon association with lipid membrane could alter the stoichiometry of lipid binding. PG-1 (red) forms dimers on initial contact with the membrane. A change in oligomerization state to a tetramer, illustrated for PG-1-LL or PG-1-FF (blue), could result in altered mode(s) of membrane binding that result in approximately twice as many peptide molecules binding per unit area of membrane surface.

range; however, the binding curve tends toward a similar lipid to peptide stoichiometry as PG-1-LL. We note that the stoichiometries estimated from the binding isotherms in Figure 5 are in good agreement with those calculated in Table 3. Both estimates are derived from the same experimental ITC data shown in Figure 4; however, the stoichiometries calculated in Table 3 assume single site binding, whereas the binding isotherms in Figure 5 do not make this assumption.

In some cases the binding of peptides to lipid membranes is approximated by a single site binding model (*44*) which is described by eq 2, where K is the apparent dissociation constant.

$$X^i_b = \frac{B_{max} c^i_f}{K + c^i_f} \quad (2)$$

However, attempts to fit the binding isotherms of the PG-1 peptides to eq 2 resulted in very poor fits. In each case the experimental curve is too shallow to be fitted by the rectangular hyperbola described by eq 2. Thus the values for ΔG, ΔS, and K in Table 3, which were calculated directly from the thermograms assuming a single site model, must be treated with some caution.

DISCUSSION

Previous studies that investigate the relationship between structure and function for the protegrin family of AMPs have focused on the roles of charged residues, the disulfide cross-links (*45*−*48*), and the amphiphilicity of the peptides (*49, 50*). In our work we have taken a fine-grained approach to modifying the structure by systematically increasing the hydrophobicity of PG-1 without significantly perturbing the structure. Fluorinated amino acids, such as hFLeu, are ideally suited for this application; they provide increased hydrophobicity with minimal structural changes. We note that whereas very extensively fluorinated molecules often exhibit unusual phase segregation behavior that actually prevents them from integrating into membranes (*51*), in the present case we have introduced relatively few fluorine atoms into the AMPs, so self-segregating behavior is not predicted.

The protegrin variants we synthesized, in which valine was conservatively substituted for the increasingly hydrophobic residues leucine and hFLeu, showed the expected increase in hydrophobicity as judged by reverse-phase HPLC. These substitutions did not significantly alter the secondary structure of the peptides in free solution as judged by CD spectroscopy, nor did they appear to perturb the conformation of the peptide bound to lipid membranes: all of the peptides exhibited a characteristic shift in their CD spectra indicative of an increase in their β-sheet content upon binding to the membranes.

The MIC data indicate that even structurally conservative changes to an AMP such as protegrin, which achieve their antimicrobial activity through nonspecific disruption of the bacterial membrane, can result in pronounced changes in the potency of the AMP. Although differences in the MIC of the peptides against a single bacterial strain may not be very informative, a sufficient number of strains were screened that a reliable trend is apparent. In general, substitution of valine by leucine increases the potency of PG-1 whereas substitution by hFLeu decreases the potency of PG-1. The most striking difference in antimicrobial activities is apparent for *B. subtilis*; PG-1-LL exhibits a 4-fold decrease in MIC, whereas PG-1-FF is completely inactive. These results are in contrast to our previous study (*28*) with the α-helical MSI-78 peptide, in which we found that substitution of leucine and isoleucine by hFLeu conferred markedly improved activity against several bacterial strains.

It should be noted that many factors may influence the potency of a particular AMP against a particular bacterial strain. In particular, the ratio of negatively charged to neutral lipid headgroups can vary quite widely from strain to strain (*52*). In addition, it is quite likely that the outer leaflet plays some role in modulating the activity of AMPs; for example, magainin-2 has been shown to bind to lipopolysaccharide-containing vesicles (*53*). Thus if PG-1-FF bound more strongly (or more weakly) to lipopolysaccharide (Gram-negative strains) or lipoteichoic acid (Gram-positive strains), that could significantly alter the potency of the AMP.

The PG-1-LL and PG-1-FF peptides, although more hydrophobic, are not noticeably more hemolytic, in contrast to other studies that noted a correlation between hydrophobicity and hemolytic activity (*54*). We suggest that this may be because we have been careful to make rather conservative substitutions that are designed to incrementally increase

hydrophobicity while preserving, as far as possible, the structure of the peptide. We suspect that it is the overall structure of protegrin, rather than hydrophobicity *per se*, which is important for hemolytic activity, as there would be evolutionary pressure to minimize this harmful side effect in animals that produce protegrin and other AMPs. Substitution of much larger hydrophobic residues is expected to be disruptive to the structure of the peptide and hence increase hemolytic activity. The ITC data for the peptides binding to anionic SUVs indicate that the enthalpy of binding is slightly decreased by substituting valine with either leucine or hFLeu. However, the substitutions appear to significantly alter the mechanism of binding. Whereas both PG-1 and PG-1-LL exhibit sharply curved binding isotherms (Figure 5), indicative of electrostatic interactions dominating binding, the isotherm for PG-1-FF is much less curved. This suggests that the effective positive charge on this peptide is lower, which might provide a reason for the decrease in the antimicrobial activity of PG-1-FF.

A most interesting observation to emerge from the ITC studies is the change in the stoichiometry of lipid binding resulting from the amino acid substitutions. The peptides bind to the membrane surface with a very high ratio of peptide to lipid, with one molecule of PG-1 being bound for every two molecules of lipid, after accounting for the fact that only the outer faces of the SUVs are available to the peptide. This is consistent with the "carpeting" model (*2*) that describes the initial association of AMPs with the membrane surface prior to membrane disruption; essentially the membrane surface is saturated with peptide. About twice as many molecules of PG-1-LL are bound per unit area of membrane surface than are molecules of PG-1, suggesting that PG-1-LL binds in a different mode. We suggest that this result is most readily explained if PG-1-LL adopts a different oligomerization state upon binding the lipid membrane.

The oligomerization of AMPs on contact with their target membranes is known to be crucial to their biological action (*15*−*19*). NMR studies (*34, 38, 55*)have established that protegrin is a monomer in solution and that on binding to the membrane it initially forms dimers comprising parallel β-sheet structures, as illustrated in Figure 1. Ultimately, the peptide is believed to assemble into higher order β-barrel-like structures that cause the membrane to form pores; the pathway for assembly, however, remains unclear. To account for the apparent doubling of the number of peptides binding to the membranes indicated by the stoichiometry measurements, we propose the following model, illustrated in Figure 6.

We speculate that the additional hydrophobicity of the PG-1-LL and PG-1-FF variants may result in the peptides forming tetramers upon membrane binding, possibly through a dimer-of-dimers in which two dimers associate through "back-to-back" hydrophobic interactions. This would allow more peptides to associate with the membrane if (a) only half of the peptides in the tetramer make direct contact with the membrane surface or (b) each peptide in the tetramer contacts only half the number of lipid molecules. There is evidence from NMR studies for tetrameric forms of protegrin binding to POPC/cholesterol membranes (*38*), although these experiments were conducted with very high concentrations of peptides (millimolar), whereas our experiments were conducted at more physiologically realistic concentrations (micromolar).

The PG-1-LL and PG-1-FF variants of protegrin retain the antimicrobial activity of PG-1, although there are, as discussed above, significant differences in their potency against individual bacterial strains. One explanation for the different membrane-binding stoichiometries we observe for these protegrin variants, that would be consistent with retaining biological activity, is that we have stabilized higher order intermediates in the assembly pathway leading to the bactericidal form of this AMP. As such, these peptides may prove useful as probes to study the assembly and mechanism of protegrin. High-resolution solid-state NMR studies are planned to elucidate the structural details of the interaction of these peptides with membranes.

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