A Helix-induced Oligomeric Transition of Gaegurin 4, an Antimicrobial Peptide Isolated from a Korean Frog

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Gaegurin 4 (GGN4), a novel peptide isolated from the skin of a Korean frog, Rana rugosa, has broad spectrum antimicrobial activity. A number of amphipathic peptides closely related to GGN4 undergo a coil to helix transition with concomitant oligomerization in lipid membranes or membrane-mimicking environments. Despite intensive study of their secondary structures, the oligomeric states of the peptides before and after the transition are not well understood. To clarify the structural basis of its antibiotic action, we used analytical ultracentrifugation to define the aggregation state of GGN4 in water, ethyl alcohol, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The maximum size of GGN4 in 15% HFIP corresponded to a decamer, whereas it was monomeric in buffer. The oligomeric transition is accompanied by a cooperative 9 nm blue-shift of maximum fluorescence emission and a large secondary structure change from an almost random coil to an α-helical structure. GGN4 induces pores in lipid membranes and, using electrophysiological methods, we estimated the diameter of the pores to exceed 7.3 Å, which suggests that the minimal oligomer structure responsible is a pentamer.

Keywords: Aggregation State; Analytical Ultracentrifuge; Antimicrobial Peptide; Gaegurin 4.

Introduction

In the last two decades, more than 500 antimicrobial peptides (AMPs) have been identified in host defense systems from insects to animals (Boman, 1995; Rinaldi, 2002; Zasloff, 2002). AMPs are an essential part of innate immunity for combating microbial challenges. These small peptides are multifunctional effectors of immunity on skin and mucosal surfaces and have antimicrobial activity against various bacteria, viruses, fungi, cancer cells and parasites. The skin secretions of frogs contain many different types of AMPs (Rinaldi, 2002; Zasloff, 2002). The gaegurins are six antimicrobial peptides, isolated from the frog, Rana rugosa, that can be classified into two families based on their lengths and sequence similarities (Park et al., 1994). Gaegurin 4 (GGN4) belongs to gaegurin family I and has activity against Gram negative and Gram-positive bacteria, fungi and protozoa (Park et al., 1994). Recently, a gaegurin family II peptide (gaegurin 6, GGN6) was reported to also have antitumor activity (Kim et al., 2003).

GGN4 (GILDTLKQFAKGVGKDLVKGAAQGVLST-VSCKLAKTC) consists of 37 amino acid residues with 4 net positive charges (6 K & 2 D) and a number of hydrophobic residues (Park et al., 1994). Like brevinins, ranalexins, and esculentins, all antibiotic peptides from the Rana genus, GGN4 contains a well-conserved disulfide bridge between C31 and C37 (CKLAKTC), termed a Rana box. The Rana box (heptapeptide motif) seems to be dispensable for antimicrobial (Park et al., 2000) and antitumor (Kim et al., 2003) activities, but is required for good pore-forming activity in artificial lipid membranes.

Abbreviations: GGN4, gaegurin 4; HFIP, hexafluoro-2-propanol.
(Kim et al., 1999). Truncation of GGN4 beyond the hetapeptide form causes noticeable decreases in antimicrobial activity (Kim et al., 1999a) and membrane conductance measured by a patch-clamp technique (Kim et al., 1999b).

In many proteins or peptides, hetero-association and self-association of protein monomers via multimerization interfaces, such as α-helical structures, to form higher order oligomers play important roles in biological activity (Marianayagam et al., 2004; Stewart, 1993). GGN4 contains two amphipathic α-helices (Park et al., 2000) (residues 2 to 10 and 16 to 32, underlined) and has been shown to form cation-selective pores in lipid membranes (Kim et al., 1999a; 2004). Despite the pore forming action and the presence of helices, only monomers were reported in the conditions for NMR analysis (Park et al., 2000; 2002; Suh et al., 1996). However, a recent NMR study of GGN5 revealed that some hydrophobic residues (Leu-5, Phe-6, Val-8, Ala-8, Ala-9, Val-12 and Val-16) underwent slow proton exchange (Park et al., 2002). Such slow exchange was also observed for some residues (Ile-17 and Ile-20) of an even shorter gaegurin, GGN6 (Suh et al., 1996), and suggested the possible existence of multimerization interfaces in gaegurins. Alamethicin (He et al., 1996), protegrin (Yang et al., 2000) magainins (Matsuzaki et al., 1995; 1996; Yang et al., 2000) melittins (Yang et al., 2001) and cecropins (Mchaourab et al., 1993) are well-known peptide antibiotics that form amphipathic helical configurations and higher-order oligomeric structures.

Even though oligomerization is important for the function of many biological macromolecules, including antimicrobial peptides, direct proof of aggregation and determination of the number of molecules in the aggregate have been difficult to achieve due to the lack of suitable high-resolution methods that can detect the higher-order structures formed by these peptides in membrane environments. In this study, we have used analytical ultracentrifugation to estimate the size of GGN4 in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) [which promotes α-helix formation in amphipathic peptides (Hirota et al., 1997)], and patch-clamp techniques to estimate the minimal pore size in artificial membranes. We also used steady-state fluorescence to follow microenvironmental changes near an introduced fluorescence probe and a CD technique to monitor overall secondary structural changes.

**Materials and Methods**

**Peptide sample** The natural GGN4 peptide was purified from the skin of *Rana rugosa* as described previously (Park et al., 1994). The purity of the peptide was greater than 99%, as confirmed by mass-spectroscopy and analytical HPLC. HFIP was obtained from Sigma (USA) and fluorescein 5-isothiocyanate (FITC) from Molecular Probes (Eugene, USA). Synthetic phospholipids such as palmitoyl-oleoyl-phosphatidylethanolamine (PE), palmitoyl-oleoyl-phosphatidylocho-line (PC), and palmitoyl-oleoyl-phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, USA). All other reagents were of the highest analytical grade from Sigma. The extinction coefficient of full-length GGN4 at 220 nm was estimated to be 30076 M⁻¹cm⁻¹ from its UV spectrum and peptide mass. The latter was determined by the Harvard Microchemical Core Facility.

**Equilibrium sedimentation** Equilibrium sedimentation studies were performed using a Beckman ProteomeLab XL-A analytical ultracentrifuge at the Analytical Ultracentrifuge Core Facility, Mokpo National University. A GGN4 sample in the absence of HFIP was measured at 25°C using a 4-hole rotor with a counterbalance and aluminum centerpiece cells, at two speeds, 40,000 rpm and 50,000 rpm, in 2.5 mM phosphate buffer containing 1 mM EDTA, at pH 3.3 or in 1× PBS buffer at pH 7.4. The data at the two speeds were fitted to the appropriate models. Experiments in the presence of HFIP were performed with the 4-hole rotor at 30,000 revs/min in the same two buffers. The peptide concentration was 25 µM (0.1 mg/ml). The time required for the attainment of equilibrium was established by running at the given rotor speed until scans were consistent over 12 h; this was achieved by at most 36 h. The distribution of the samples within the cells was determined by measuring absorbance at 220 nm or 230 nm. The samples were also scanned at 330 nm, where the peptide has no absorption, to obtain a baseline. Five scans were collected and averaged to give the final averaged data. The partial specific volume of GGN4 at 25°C was calculated to be 0.7487, from the partial specific volume of its constituent amino acids. A calculated molecular mass of 3734.7 Daltons was used for the data analysis.

For mathematical modeling by non-linear least-squares curve fitting, the fitting function was:

\[
C_r = C_b \exp(A_p M_p (r^2 - r_0^2)) + \varepsilon \\
A_p = (1 - \rho \omega^2)/RT
\]

where \(C_r\) is the total concentration at radial position \(r\), \(C_b\) is the concentration of peptide at the bottom of the cell, \(M_p\) is the molecular weight of the peptide, \(\rho\) is the partial specific volume and \(\omega\) is the solution density. \(\omega\) is the rotor angular velocity, and \(\varepsilon\) is a baseline error term. The model was selected by examining the weighted sum of square values and weighted root mean square error values. Further data manipulation and data analysis were performed using MLAB (Knott, 1979) with the data analysis computer.

**Circular dichroism spectra** CD spectroscopy was carried out on a JASCO J-715s in Mokpo National University Central Laboratory with a 1 mm cell at 0.1 mg/ml concentration at 25°C in 2.5 mM potassium phosphate buffer, pH 3.3, or other conditions as indicated. Five accumulated scans were taken for each sample at a speed of 50 nm/min. The temperature was controlled by a Neslab RT111 circulating water bath.
Electrochemical measurements and data analysis. Planar lipid bilayers Solvent-containing lipid bilayers were formed across the tip of a patch pipette (Coronado and Latorre, 1983; Cruciani et al., 1992), and in neutral tip-dip bilayers the permeability ratio of K to Cl in GGN4-induced conductances was similar to that in painted bilayers (Kim et al., 1999a). Patch pipettes were pulled with a two-stage puller (PP-83, Narishige Scientific Instr., Tokyo, Japan) and filled with a pipette solution containing 10 mM HEPES-KOH and 100 mM KCl (pH 7.2). First, a patch pipette was lowered below the solution level in the recording chamber (1.2 ml) filled with the pipette solution, and a drop of the lipid solution composed of phosphatidylethanolamine: phosphatidylcholine: cholesterol (72:18:10), in n-decane (10 mg/ml), was layered over the pipette-water interface. Then the pipette was carefully withdrawn into the lipid drop and lowered again into the bath solution. The formation and size of the bilayer was estimated by monitoring the membrane capacitances as described for the painted bilayer. The membrane capacitance of the tip-dip bilayers was approximately 15 pF. GGN4 was added to the bath before perfusion and the bath was perfused (0.7 ml/min) with test solutions (12 ml) after channel activity was detected.

Electrical measurements and data analysis GGN4-induced conductances were obtained from the slope of the current-voltage data obtained by a ramp voltage command with a voltage clamp amplifier (CEZ2300, Nihonkoden, Japan). The amplifier headstage was connected to the bath via an agar (3%) bridge to reduce the junction potentials. Electrode asymmetry was corrected in symmetrical salt conditions before formation of the bilayer. Junction potentials produced by the differences in ion species were measured at the end of each experiment and subtracted from the command voltage (Cruciani et al., 1992). The largest junction potential (13–17 mV) occurred between KCl and LiCl (200 mM). We designated the current flow from bath to pipette as "outward current". Current-voltage relations were obtained by a ramp voltage command from -150 to +150 mV, or vice versa, for 3 s. Relative permeabilities of monovalent cations were calculated from the measured reversal potentials according to the Goldman-Hodgkin-Katz (GHK) equation. (Coronado and Latorre, 1983; Cruciani et al., 1992) GGN4-induced currents were stored on a PC using a Labmaster DMA (TL-1 125 kHz, Axon Inst. Co., USA), VTR tapes with a VR-10 digital data recorder (Instrutech Corp., USA), and a pen recorder. pClamp software (Ver. 6.03, Axon Inst. Co., USA) for electrophysiological experiments and data analysis was used for voltage clamp calculations, measurements of current amplitude and reversal potentials, and for data illustration.

Fluorescence measurements Due to the absence of tryptophan or tyrosine in native GGN4 we introduced an extrinsic fluorophore, fluorescein, using fluorescein 5-isothiocyanate (FITC). Labeling was carried out at pH 7.0 with a 5:1 molar ratio of dye to peptide to label the N-terminus. Free dye was removed by gel-filtration on a G-25 column (Pharmacia, Uppsala, Sweden) followed by dialysis. The labeling ratio was estimated using the extinction coefficients of the dye (72,000 M$^{-1}$cm$^{-1}$ at 494 nm) and the peptide (30,076 M$^{-1}$cm$^{-1}$ at 220 nm). We estimated that approximately 30% of the GGN4 molecules were labeled. For fluorescence emission spectra, a Hitachi F-4500 spectrofluorimeter was used.

Results

The α-helical propensity of GGN4 The solution conformation of native GGN4 in buffer and various alcohols was measured by CD (Fig. 1A). In 2.5 mM phosphate buffer, pH 3.3, the peptide existed as a random coil characterized by a typical minimum at 197 nm. Upon addition of alcohols, the helicity increased, as evidenced by the presence of double minima at 208 and 222 nm, and a positive band at 196 nm. However, the relative intensities of the mean residue ellipticity at 222 nm were in the order: methanol (MeOH) < ethanol (EtOH) < HFIP in these conditions. A similar trend was observed in a CD analysis on melittin (Hirota et al., 1997).

Figure 1B shows the CD spectra of GGN4 at different HFIP concentrations from 0 to 15% (vol/vol). The peptide conformation started to transform into a helix at approximately 6% HFIP and completed its transition near 15% HFIP. Further addition of HFIP did not cause any noticeable change. The α-helical content of the native GGN4 in 15% HFIP was estimated to be 58%, using the intensity of the minimum band at 222 nm (Scholtz et al., 1991). Along with the changes in the secondary structure, HFIP also changed the ellipticity ratio ($\theta_{222}$ nm / $\theta_{209}$ nm) from ~0.8 in the absence of HFIP to ~1.0 in the presence of HFIP. This ratio is frequently used as an indication of the inter-helical interactions yielding oligomeric structures (Lau et al., 1984).

Oligomeric state of GGN4 in buffer, EtOH and HFIP The oligomeric state of GGN4 was investigated by analytical ultracentrifugation, which has been used frequently to study the oligomeric state of peptides and proteins (Lebowitz et al., 2002). Equilibrium sedimentation was carried out in three different conditions: buffer alone, 50% EtOH, and 15% HFIP. Figure 2 demonstrates the joint fit (solid line) for the data for GGN4 in 2.5 mM phosphate buffer, pH 3.3, containing 0.1 mM EDTA at ultracentrifuge speeds of 40,000 and 50,000 rpm at 230 nm, assuming homogeneous monomers. For the analysis we used a monomeric molecular mass of 3,734.5 Da. The root-mean-square errors of the monomeric joint fit analysis at the two speeds were approximately $3.50 \times 10^{-3}$, which demonstrates the accuracy of the fit. The accuracy of these fits to the model used was such that it was appropriate to conclude that GGN4 exists as a monomer in buffer, and that thermodynamic ideality of the solutes had been
established. The actual fit is shown in the lower section, and a plot of the distribution of the residuals is shown in the upper section. GGN4 was also examined at another pH (1× PBS, pH 7.4) and again found to be a monomer (data not shown). The ultracentrifuge data in 15% HFIP are presented in Fig. 3. The presence of 15% HFIP induced significant changes in the concentration distribution of the peptide in the cell, which suggested the presence of higher order aggregates. Using the appropriate mathematical models, we determined that the actual distribution of GGN4 in 15% HFIP was best fitted to a homogeneous decamer (10-mer, 10×) (Fig. 3). The distribution of the data was close to the theoretical 10-mer line and deviated more from 9-mer or 11-mer models. Other homogeneous (1×, 2×, 4×, 8×, 12×) or interactive (1×↔2×, 1×↔4×, 1×↔6×, 1×↔10×, 1×↔12×, 1×↔2×↔4×↔8×,...) models were also tested, but they gave inferior results and were therefore discarded. In order to determine if other alcohols cause the oligomeric transition, we conducted equilibrium sedimentation in 50% EtOH. Figure 4 shows the effect of 50% EtOH on the oligomeric state of GGN4 measured at the two different wavelengths (220 and 230 nm). Unlike HFIP, EtOH failed to cause the peptide to self-associate despite the increased helical content of GGN4 in its presence (Fig. 1A). Further increase of the percentage EtOH or MeOH to 70% had no effect (data not shown).

Fluorinated alcohols, such as 2,2,2-trifluoroethanol (TFE) and HFIP are known to induce the formation of α-helices in peptides and proteins. HFIP seems to be the strongest enhancer of α-helix formation (Kahn et al., 2000; Kumanran and Roy, 1999). In a melittin study, HFIP was found to be approximately 20 times more effective than ethanol in promoting helix formation (Hirota et al., 1997). A recent physicochemical analysis indicated that an HFIP-water binary solution formed micelle-like aggregates of maximum size at approximately 30% (v/v) HFIP or \( \chi_{\text{HFIP}} = 0.0671 \) (Hong et al., 1999; Yoshida et al., 2003). The tetrahedral-like water structures that predominate at 0% HFIP, began to be disrupted to form higher aggregates upon the addition of HFIP. Even though our experimental set-up of 15% (v/v) HFIP (\( \chi_{\text{HFIP}} = 0.0336 \)) is located at the transition of water to maximal micelle-like aggregates (Yoshida et al., 2003), it is possible that binding of water-HFIP co-solvents to GGN4 led us to overestimate GGN4 size (10-mer). However, the decameric GGN4 determined by equilibrium sedimentation reflects the maximum possible size of the oligomers in 15% HFIP if no co-solvation occurred. Until now, only HFIP-water systems have been
studied experimentally (Hong et al., 1999; Yoshida et al., 2003); and the effects of HFIP on real peptides have been examined only by molecular dynamic simulations applied to melittin (Roccatano et al., 2005).

Fluorescence properties of fluorescein-labeled GGN4 in HFIP To confirm the effects of HFIP on the structure of GGN4, we measured steady-state fluorescence emission spectra of fluorescein-labeled GGN4 (GGN4-F*). At higher HFIP concentrations, the emission intensity of GGN4-F* increased with a concomitant blue-shift in emission maxima (Fig. 5). The inset of Fig. 5 gives the emission maxima of GGN4-F* and those of free dye (FITC) at different HFIP concentrations. While the blue-shift of free dye was minimal ($\Delta \lambda = 3$ nm) and linear, the blue-shift of GGN4-F* was substantial ($\Delta \lambda = 9$ nm) and sigmoidal, which points to a cooperative conformational transition at higher HFIP concentrations.

Estimation of GGN4-induced pore sizes in planar lipid bilayers Recent electrochemical data suggest the presence of GGN4-induced cation-selective pores in planar lipid bilayers (Kim et al., 1999). To estimate the size of the GGN4 pores in artificial lipid bilayers, we used organic cations of different sizes. Figure 6 illustrates the current-voltage relation recorded under near-biionic conditions: 200 mM tetraethyl ammonium chloride (TEA-Cl) or N-methyl-D-glucamine (NMDG) and 200 mM KCl in the recording pipette. The respective permeability ratios of TEA$^+$ and NMDG$^+$ to K$^+$ ions ($P_{TEA}/P_K$ and $P_{NMDG}/P_K$), calculated from the GHK equation using the mean reversal potentials shown in Fig. 6, were 0.42 and 0.041. Although the channels formed by GGN4 displayed lower permeability to TEA$^+$ or NMDG$^+$ than to K$^+$, the generation of inward current by TEA$^+$ or NMDG$^+$ strongly suggests that the GGN4-induced pores were large enough for these large organic cations. Therefore, our results indicate that the diameter of the GGN4-induced pores must be at least 7.3 Å, since the geometrical mean diameters of TEA$^+$ and NMDG$^+$ are 6.6 and 7.3 Å, respectively (Villarroel et al., 1995).

Discussion

Although they differ widely in length and sequences, the amphipathic α-helices in various antibiotic peptides are important. The CD analyses in this and previous work (Park et al., 2000) demonstrated that GGN4 forms helical structures in membrane-mimicking environments. MeOH,
EtOH and HFIP each increased the helical content of GGN4, but to different degrees, in the order: 15% HFIP > 50% EtOH > 50% MeOH (Fig. 1A). Based on the CD data (Fig. 1B), GGN4 formed a helix from approximately 6% HFIP, which is close to the fluorescence emission transition point (Fig. 5). In addition, the fluorescence emission maxima of GGN4-F* blue-shifted in a sigmoidal manner, which points to a cooperative transition. An HFIP-induced cooperative transition of spin-labeled cer-cropin to a higher-order oligomer has also been reported. (Mchaourab et al., 1993).

Some antimicrobial peptides with the potential to form amphipathic α-helical structures aggregate into higher oligomeric structures in a lipid bilayer (He et al., 1996; Matsuzaki, 1995). In this study, we demonstrated that GGN4 underwent a coil to helix transition and formed oligomers of a maximum size corresponding to decamers (10-mers) in 15% HFIP (Fig. 3). Because HFIP is one of the most potent helix enhancers, decamers may be the largest aggregates that GGN4 can form. However, if HFIP forms micelles and the co-solvent binds to GGN4, the GGN4 aggregates may be smaller than this estimate. In order to understand the exact influence of HFIP on GGN4 aggregation, further equilibrium sedimentation analyses, which view the HFIP-GGN4 interactions as peptide-detergent complex systems (Fleming et al., 1997), are underway. Similar sizes of pore were observed with alamethicin, which forms 8 to 11-mer barrel-stave-type aggregates, depending on conditions (He et al., 1996), and magainin 2, which forms supramolecular structures of 4 to 7-mer peptide/lipid toroidal-type pores (Matsuzaki et al., 1995; 1996). The relationship between peptide aggregation state and antibiotic activity is not yet understood.

However, recent studies indicate that oligomerization of peptides can have dramatic effects on antibacterial action (Feder et al., 2000) and membrane leakage properties (Mazzuca et al., 2005).

An estimate of the GGN4 pore size in lipids was also obtained from the electrochemical data (Fig. 6). From the patch-clamp analysis, the internal diameter of the pores in the lipid bilayer was estimated to be larger than 7.3 Å in order for the NMDG⁺ ion to pass through the channel. From this we were able, with the following equation (Matsuzaki et al., 1996), to estimate the lower limit of the oligomeric state of the GGN4 pore:

$$D = 2^\ast r\ast[(1/\sin(\pi/n_p)) - 1]$$

(Rinaldi, 2002)

where, D is the internal diameter of the pore, r the radius of the peptide helix which was approximated at 5 Å, and n_p the number of peptide molecules. From this equation we estimated the internal diameters of the tetrameric (n_p = 4) and pentameric (n_p = 5) pores to be 5.4 Å and 8.7 Å, respectively. Therefore, in order to allow the NMDG⁺ ion (7.3 Å) to pass through the channel, the pore has to be generated by at least a pentamer (5-mer), assuming a barrel-stave type GGN4 pore. However, considering that lipid membranes, as well as peptide-lipid interactions, are dynamic (Matsuzaki et al., 1996), the appearance of GGN4 pores may be transient and too unstable to comprise a unique form of homogeneous oligomer. This possibility is also reflected in a recent study (Kim et al., 1999a; 2004) of the voltage-dependence and cation selectivity of GGN4 channels, which showed that these channels are highly heterogeneous in their conductance and
gating. It is likely that the GGN4 pores are also heterogeneous in size and transient in their nature in a real membrane environment due to the fluidity of the lipid bilayer and the complex cellular environment.

In summary, we have demonstrated that GGN4 is a helical peptide that can form oligomeric structures in lipid membranes as well as in membrane-mimicking environments. We estimated the minimal and maximal sizes of the pores as pentamers (5-mers) and decamers (10-mer), using analytical ultracentrifugation and patch-clamp techniques, respectively. The CD and fluorescence data demonstrated that the oligomeric transition was cooperative and accompanied by a large secondary structure change from a near random coil to a helix.

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