Determination of Peptide Oligomerization in Lipid Bilayers Using 19F Spin Diffusion NMR

Jarrod J. Buffy,†‡ Alan J. Waring,§ and Mei Hong*†

Contribution from the Department of Chemistry, Iowa State University, Ames, Iowa 50011, and Department of Medicine, University of California at Los Angeles School of Medicine, Los Angeles, California 90095

Received October 20, 2004; E-mail: mhong@iastate.edu

Abstract: Aggregation or oligomerization is important for the function of many membrane peptides such as ion channels and antimicrobial peptides. However, direct proof of aggregation and the determination of the number of molecules in the aggregate have been difficult due to the lack of suitable high-resolution methods for membrane peptides. We propose a 19F spin diffusion magic-angle-spinning NMR technique to determine the oligomeric state of peptides bound to the lipid bilayer. Magnetization transfer between chemically equivalent but orientationally different 19F spins on different molecules reduces the 19F magnetization in an exchange experiment. At long mixing times, the equilibrium 19F magnetization is 1/M, where M is the number of orientationally different molecules in the aggregate. The use of the 19F spin increases the homonuclear dipolar coupling and thus the distance reach. We demonstrate this technique on crystalline model compounds with known numbers of molecules in the asymmetric unit cell, and show that 19F spin diffusion is more efficient than that of 13C by a factor of ~500. Application to a β-hairpin antimicrobial peptide, protegrin-1, shows that the peptide is almost completely dimerized in POPC bilayers at a concentration of 7.4 mol %. Decreasing the peptide concentration reduced the dimer fraction. Using a monomer–dimer equilibrium model, we estimate the ΔG for dimer formation to be ~10.2 ± 2.3 kJ/mol. This is in good agreement with the previously measured free energy reduction for partitioning and aggregating β-sheet peptides into phospholipid membranes. This 19F spin diffusion technique opens the possibility of determining the oligomeric structures of membrane peptides.

Introduction

The intermolecular association or aggregation of peptides and proteins is ubiquitous in nature, and can have beneficial or deleterious consequences. Amyloids, insoluble fibril-forming protein aggregates, are implicated in neurodegenerative diseases such as Alzheimer’s, Huntington’s, and bovine spongiform encephalopathy.1 A growing number of solid-state NMR investigations have been reported on the supramolecular assembly of amyloidogenic peptides.2,3 In biological membranes, many peptides and proteins require oligomerization of multiple subunits or self-association for function.4 Two well-studied cases of functionally important homooligomerization are the helical peptides glycoporphin A and the transmembrane domain of the M2 protein of influenza A.5–7 Despite the prevalence of membrane peptide oligomerization and the importance of understanding the thermodynamics of aggregation, no high-resolution methods are so far available to directly determine the oligomeric state of membrane peptides, let alone the detailed aggregate structure, due to the general difficulty of investigating peptide structure in lipid bilayers. Instead, analytical ultracentrifugation, equilibrium dialysis, and other biochemical approaches have been employed to probe the folding and self-association of membrane peptides.6–9

Solid-state NMR is becoming an increasingly important spectroscopic technique to study the three-dimensional structures of membrane peptides and proteins directly in the biologically relevant environment of the lipid bilayer. The orientation of membrane peptides relative to the bilayer normal,10 the high-resolution structure of protein–ligand interfaces,11 and peptide–lipid interactions12,13 can be determined with exquisite detail.
Reorientation during spin diffusion for static samples; thus, it suffers from inherently low sensitivity. Unfortunately, MQ NMR is most suitable among homonuclear dipolar-coupled spins to allow intermolecular multiple-quantum (MQ) NMR: excitation of MQ coherences enables the determination of the size of membrane peptide oligomers. The most promising approach is state NMR, which has not allowed for the determination of the size of compounds. However, the traditional arsenal of solid-state NMR is limited by the low sensitivity of MQ NMR.

Here we propose and demonstrate a 19F spin diffusion technique, applicable under the magic-angle-spinning (MAS) condition to achieve high sensitivity, for determining the aggregation number of membrane peptides. We detect spin diffusion using the CODEX (centerband-only detection of exchange) pulse sequence, which was originally designed to probe slow molecular reorientations on the time scale of 1 ms or longer. The technique utilizes the recoupled chemical shift anisotropy (CSA) to sample the orientational change of molecules due to slow motion or spin diffusion between sites with different chemical shift tensor orientations. The CSA recoupling, achieved by rotor-synchronized 180° pulses, occurs in two stages that are separated by a mixing period, 90° pulses, respectively. DD dipolar decoupling, TPPM = two-pulse phase modulation, 19F-19F dipolar recoupling under MAS has been shown to yield intramolecular distances up to 12 Å in small organic compounds with several different fluorine sites.

Fluorinated amino acids such as [19F]-4-fluorophenylglycine have been incorporated into membrane peptides to determine peptide orientations with respect to the bilayer normal. Long-range distances between 19F and other spin-1/2 nuclei such as 13P and 1H have also been measured in a number of complex systems such as enzyme-substrate complexes.

In this work, we demonstrate the combined use of the 19F CODEX technique to probe 19F spin diffusion using crystalline amino acids, where the number of molecules in the asymmetric unit cell is known. We then apply the 19F CODEX technique to protegrin-1 (PG-1), a β-hairpin antimicrobial peptide known to destroy the cell membranes of a variety of microbial organisms.


(16) Schmitz-Rohr, K.; de13C/19F

(a) Pulse sequence for the CODEX experiment. Filled and open rectangles indicate 90° and 180° pulses, respectively. DD = dipolar decoupling. TPPM = two-pulse phase modulation to enhance the X nucleus spectral resolution. (b) Principle of CODEX for determining the number of molecules in an aggregate on the basis of orientational inequivalence. A molecule-fixed chemical shift tensor experiences different orientations in the aggregate. After complete exchange, the remaining magnetization on the initial molecule is 1/M. This is detected in the CODEX experiment as the S/S0 value at equilibrium.

(b) Principle of the CODEX experiment. Filled and open rectangles indicate 90° and 180° pulses, respectively. DD = dipolar decoupling. TPPM = two-pulse phase modulation to enhance the X nucleus spectral resolution.

Figure 1. (a) Pulse sequence for the CODEX experiment. Filled and open rectangles indicate 90° and 180° pulses, respectively. DD = dipolar decoupling. TPPM = two-pulse phase modulation to enhance the X nucleus spectral resolution. (b) Principle of CODEX for determining the number of molecules in an aggregate on the basis of orientational inequivalence. A molecule-fixed chemical shift tensor experiences different orientations in the aggregate. After complete exchange, the remaining magnetization on the initial molecule is 1/M. This is detected in the CODEX experiment as the S/S0 value at equilibrium.

(b) Principle of CODEX for determining the number of molecules in an aggregate on the basis of orientational inequivalence. A molecule-fixed chemical shift tensor experiences different orientations in the aggregate. After complete exchange, the remaining magnetization on the initial molecule is 1/M. This is detected in the CODEX experiment as the S/S0 value at equilibrium.

(b) Principle of CODEX for determining the number of molecules in an aggregate on the basis of orientational inequivalence. A molecule-fixed chemical shift tensor experiences different orientations in the aggregate. After complete exchange, the remaining magnetization on the initial molecule is 1/M. This is detected in the CODEX experiment as the S/S0 value at equilibrium.

(b) Principle of CODEX for determining the number of molecules in an aggregate on the basis of orientational inequivalence. A molecule-fixed chemical shift tensor experiences different orientations in the aggregate. After complete exchange, the remaining magnetization on the initial molecule is 1/M. This is detected in the CODEX experiment as the S/S0 value at equilibrium.
NMR studies showed that the PG-1 backbone is completely immobilized in POPC membranes while rotating uniaxially in thinner DLPC bilayers. The lack of motion in the POPC membrane suggests aggregation in the membrane. The present 19F CODEX experiments, conducted at 223 K when spin diffusion dominates the exchange process, indicate that PG-1 exists predominantly as dimers at a peptide concentration of 7.4%. Lowering the peptide concentration reduced the magnetization exchange, indicating decreased dimer formation. The concentration-dependent dimer fraction yields a free energy change of $-10.2 \pm 2.3$ kJ/mol for the monomer–dimer equilibrium. This is the first time membrane peptide oligomerization has been directly determined structurally in the lipid bilayer.

**Materials and Methods**

Crystalline l-Phe samples with 4-19F and 13C labels were recrystallized from concentrated HCl solutions. The amino acid structure was confirmed using single-crystal X-ray diffraction and found to be identical to the reported structure. Crystalline 13C-labeled Gly contained two polymorphs with distinct isotropic shifts: the major form is α-Gly, and the minor form is γ-Gly. Fluorinated PG-1 (NH2-RGGRLCYCRRFVCCVGR-CONH2) was synthesized on the 0.25 mmol scale using Fmoc solid-phase peptide synthesis protocols as described before. [19F]-fluoro-l-Phe was incorporated at Phe12. [13C]Val16, and [15N]Val16 were also incorporated in one sample to confirm the backbone rigidity of the peptide in POPC bilayers at room temperature. Herzfeld-Berger analysis of the CSA sidebands yielded the anisotropy parameter ($\hat{d}$) and the asymmetry parameter ($\gamma$). Fluorinated POPC-1 contained residual trifluoroacetic acid and the trifluoroacetate (TFA) countercations. These give rise to a 19F signal at $-75$ ppm, which partially overlaps with the edge of the rigid-limit 19F CSA sidebands of [19F]-4-fluoro-Phe. Thus, we removed residual TFA by repeated (3–4 times) washing and lyophilization of the peptide in 5 mM HCl solution, until negligible TFA signal was detected in the NMR spectrum. The purified fluorinated peptide was combined with POPC lipids (main phase transition temperature $-2^\circ$C) by codissolving them in methanol and chloroform solutions to achieve the desired P.L mole ratio. The combined solution was dried under a stream of nitrogen gas and the resulting membrane film redisolved in a small amount of cyclohexane and lyophilized. The samples were loaded into a 4 mm ZrO2 rotor equipped with borosilicate glass spacers and directly hydrated by repeated ($3^\circ$) hydrations. The combined solution was dried under a stream of nitrogen gas and the resulting membrane film redisolved in a small amount of cyclohexane and lyophilized. The samples were loaded into a 4 mm ZrO2 rotor equipped with borosilicate glass spacers and directly hydrated by repeated ($3^\circ$) hydrations. The combined solution was dried under a stream of nitrogen gas and the resulting membrane film redisolved in a small amount of cyclohexane and lyophilized.

NMR experiments were carried out on a Bruker DSX-400 spectrometer (Karlsruhe, Germany) operating at a resonance frequency of 400.49 MHz for $^1$H, 376.84 MHz for $^{19}$F, and 100.70 MHz for $^{13}$C. 19F experiments were conducted on a MAS probe with a 4 mm spinner module. A Bruker HFX unit containing a tuning splitter and a power combiner splits the $^1$H channel of the probe into two channels for simultaneous $^1$H and $^{19}$F radio frequency irradiation. Samples were spun at low temperature using air cooled through a Kinetics Thermal Systems XR air-jet sample cooler (Stone Ridge, NY). The temperature was maintained within $\pm 1$ K of the desired value, and the spinning speed was regulated to $\pm 3$ Hz. Typical 90° pulse lengths were 5 µs for 19F and 13C and 3.5–4.0 µs for 1H. A short $^1$H–$^{19}$F cross-polarization (CP) contact time of 0.3 ms was used, while $^1$H–$^{13}$C contact times were 0.5 ms. The typical recycle delays were 2 s, and acquisition times were $\sim 30$ ms for 13C and $\sim 3$ ms for 19F. 13C and 19F chemical shifts were referenced externally to the α-Gly 13C signal at 176.4 ppm on the TMS scale and the Teflon 19F signal at $-122$ ppm, respectively.

The pulse sequence for the 19F and 13C CODEX experiments is shown in Figure 1a. After CP from $^1$H, the X spin magnetization evolves under the CSA interaction, which is recoupled by 180° pulses that are spaced at half a rotor period apart. The pair of 90° pulses immediately after CP allows the input of a trigger signal for active rotor synchronization of the two CSA recoupling pulse trains. The total CSA recoupling time, $\tau_{CSA}$, must be sufficiently long to detect small-angle orientational changes. For uniaxial chemical shift tensors, $\tau_{CSA}$ needs to satisfy the condition $\delta_{CSA} \sin \beta_R \geq 2$, where $\beta_R$ is the orientation change. Between the two CSA periods, the magnetization is stored along the z axis for a mixing time, $\tau_m$, without $^1$H decoupling, during which magnetization undergoes $^1$H-driven spin diffusion. This changes the CSA frequency, thus reducing the intensity of the stimulated echo. Spin diffusion among M differently oriented sites reduces the echo to a final intensity of 1/$M$. To correct for spin–lattice relaxation ($T_1$) effects during $\tau_m$, a $z$ filter ($\tau_x$) is added at the end of the second $\pi$-pulse train. Two experiments were conducted: a dephasing experiment (S) with the desired $\tau_m$ and a short $z$ filter ($<1$ ms) and a reference experiment (S0) with interchanged $\tau_m$ and $\tau_x$ values. Due to the short duration of the mixing between the two $\pi$-pulse trains, the S0 experiment does not have spin diffusion but has a total $z$ magnetization time identical to that of the S experiment. Thus, the intensity ratio $S/S_0$ represents the normalized exchange free of $T_1$ relaxation. Due to the strong coupling of the X spins to the surrounding protons, the empirically observed normalized exchange signal usually dephases exponentially with $\tau_m, S/S_0 = 1/M + \sum e^{-\alpha_{sd,i} \tau_m}$, where $\tau_{sd,i}$ is the spin diffusion time constant. A multexponential decay suggests the presence of multiple and significantly different X–X distances. However, the values of the decay constants cannot be quantitatively related to individual distances.

**Results and Discussion**

**Crystalline Amino Acids.** We demonstrate the principle of CODEX spin diffusion for determining the number of unique molecular orientations on two crystalline amino acids, Gly and Phe. α-Gly has four molecules per unit cell, two of which have unique orientations, while γ-Gly contains three magnetically inequivalent molecules per unit cell. 13C CODEX experiments on Gly were conducted using a $\delta_{CSA}$ of 7.2, which is sufficiently large to detect orientational differences as small as $\sim 15^\circ$. Figure 2A shows the 13C S/S0 values as a function of $\tau_m$ for both polymorphs of Gly. The CODEX curve decays to an equilibrium value of 0.49 for α-Gly and 0.32 for γ-Gly, consistent with the M values of 2 and 3, respectively. Both curves are well fit by single-exponential decays, with a decay constant of 270 ms for α-Gly and 120 ms for γ-Gly. The distances probed on this time scale are 4–5 Å: the nearest-neighbor C–C’ distances between unique orientations are 4.22 Å in α-Gly and 4.17 and 5.23 Å in γ-Gly.

The distance reaches of 13C and 19F spin diffusion in the CODEX experiment are compared using crystalline Phe, which has four different molecules per asymmetric unit cell. The p-19F site has a chemical shift anisotropy $\delta$ of 58 ppm and an asymmetric parameter $\eta$ of 0.67. The 19F CODEX experiment was conducted at a spinning speed of 9 kHz and with four rotor periods of CSA recoupling. This corresponds to $\delta_{CSA} = 9.7$, which is sufficiently large to detect $\sim 10^\circ$ orientational differences. The 19F data shown here were acquired at 253 K to

(32) Gurskaya, G. V. Kristalografiya 1964, 9, 839–845.
(35) Kozhin, V. M. Kristalografiya 1978, 23, 1211–1215.

J. AM. CHEM. SOC. ■ VOL. 127, NO. 12, 2005 4479
eliminate possible side chain motion. However, the CODEX results are identical between 253 and 292 K, indicating that the 19 F spin and the resulting stronger homonuclear dipolar coupling reduce the time needed to reach complete exchange by 2 orders of magnitude. This is crucial for studying membrane molecules in each unit cell. Note the 2 orders of magnitude decrease in the spin diffusion equilibration time for 19 F CODEX compared to 13 C CODEX in (B) due to the stronger 19 F– 19 F dipolar coupling.

Lipid-Free [19 F]-4-fluoro-PG-1. While 19 F CODEX can unequivocally determine orientational inequivalences in amino acids, its ability to measure the number of molecules in peptide aggregates, where the internuclear distances are expected to be much larger, is still untested. Thus, we conducted 19 F CODEX experiments on lyophilized [19 F]-4-fluoro-PG-1 prior to membrane binding. In the absence of water or lipids, the peptide should be closely packed and have no particular structural order. The intermolecular F– F distances represent a lower limit that the CODEX technique must be able to detect to be useful for membrane-diluted peptides. Figure 3A shows a representative pair of 19 F CODEX spectra, for a mixing time of 300 ms, where a significant difference intensity, ∆S = S0 − S, is observed. The S/S0 value decays to 0.26 after 1 s, and is best fit to a single-exponential function with τex = 200 ms. This demonstrates that 19 F spin diffusion is able to detect at least four PG-1 molecules sufficiently aggregated. The decay constant is 1–2 orders of magnitude larger than those of Phe, suggesting that the minimum F– F distance between PG-1 molecules is indeed much larger than in crystalline Phe. The decay of the S0 signal indicates a 19 F T1 relaxation time of 1.7 s, which is sufficiently long for measuring spin diffusion at long mixing times.

[19 F]-4-fluoro-PG-1 in POPC Membranes. To determine whether PG-1 is aggregated in the lipid bilayer, we prepared PG-1/POPC liposomes with a P:L molar ratio of 1:12.5, corresponding to a peptide concentration of 7.4 mol %. This was chosen to exceed the membrane disruption and aggregation thresholds based on our previous NMR experiments.31 P spectra of oriented membranes showed that PG-1 disrupts the POPC membrane order above a concentration of 3.3%,13 while 13 C MAS experiments indicated that the peptide backbone is immobilized in POPC bilayers at a concentration of 4%.31 Since lipid molecules are dynamic solvents that can induce significant peptide motion, we conducted the 19 F CODEX experiments at 223 K. The 19 F CSA sideband pattern (not shown) at this temperature gives a δ of 57 ppm and an η of 0.69, comparable to the rigid-limit values measured for lipid-free PG-1. The experiment was conducted at a spinning speed of 8 kHz with two rotor periods of CSA recoupling; thus, δτCSA = 5. Figure 3B shows that the peptide 19 F signal decays to a final value of 0.56 ± 0.06 in 1 s with a single-exponential time constant of 225 ms. This equilibrium value indicates that PG-1 mainly associates as dimers in POPC membranes. The fact that the equilibrium value is slightly higher than 0.50 suggests that a small fraction of the peptide remains as monomers. Then the measured S/S0 is the weighted average of the values of the monomer (S/S0 = 1) and dimer (S/S0 = 0.5). The fraction of the dimer, fD, can be obtained from 0.5f0 + (1 − f0) = 0.56, resulting in fD = 88%. The single-exponential fit indicates that
there is only a single resolvable $^{19}$F CODEX dephasing curve. Figure 3A shows a $\Delta S/S_0$ decay to $\sim 0.25$, indicating that at least four PG-1 molecules are aggregated in close proximity. The $^{19}$F T$_1$, measured from the $S_0$ decay, is 1.7 s. (B) PG-1 bound to POPC lipids at 223 K. A representative set of $S_0$ and $\Delta S$ spectra are shown. Key: filled squares and solid line, 7.4% PG-1 concentration; open circles, 2.8% PG-1, open tilted squares between 0 and 200 ms, CODEX with $^1$H decoupling during the mixing time for the 7.4% PG-1/POPC sample. The reduction of the $S_0/S_0$ values with $^1$H decoupling indicates spin diffusion to be the main exchange mechanism.

It is important to verify that the observed exchange at 223 K is due to spin diffusion rather than slow peptide motion. Two types of motion could occur in the Phe side chain: phenylene ring flip and rotation around the Cα–Cβ bond. The former does not change the $p$-$^{19}$F position or the F–F dipolar coupling. The latter is unlikely at 223 K on the basis of the similarity of its CODEX curve to temperature changes. To verify the lack of slow motion in PG-1 at 223 K, we carried out a CODEX experiment with $^1$H decoupling active during $t_m$. For sites with identical isotropic chemical shifts but different anisotropic shifts, $^1$H decoupling during $t_m$ speeds up spin diffusion by increasing the overlap integral $f_{ij}(0)$ but does not affect motional exchange. Thus, if spin diffusion is the main mechanism of exchange, lower $S/S_0$ values are expected with $^1$H decoupling than without. We applied moderate $^1$H decoupling field strengths of 50 and 30 kHz for 30 and 100 ms, respectively, due to the shared use of the $^1$H channel for both $^1$H and $^{19}$F irradiation. Figure 3B shows that the two $S/S_0$ values acquired using $^1$H decoupling (tilted squares) are indeed lower than the values without $^1$H decoupling, thus confirming that spin diffusion is the dominant exchange mechanism at this temperature.

Interestingly, at 292 K the Phe side chain of PG-1 exhibits fast motion: the $^{19}$F CSA is averaged to $\delta = -28$ ppm and $\eta = 1.0$. This suggests that the liquid-crystalline bilayer indeed promotes significant side chain conformational motion, a phenomenon that has been observed previously. The corresponding CODEX curve (Supporting Information Figure S1) shows a $\sim 25\%$ component of fast decay $(t_{\text{rel,1}} \approx 10$ ms), which we attribute to $\chi_1$ torsion dynamics on a time scale near the CODEX detection limit. The remaining nonmotional exchange can be fit excellently, although not uniquely, by equal weights $(\sim 37\%)$ of a constant offset and a slow decaying component with a $t_{\text{rel,2}}$ of $\sim 1.5$ s. We attribute this to $^{19}$F spin diffusion, where the equal weights are consistent with dimer formation of the peptide concluded at low temperature. The fact that the slow diffusion time constant is much larger at 292 K is understandable since the $^{19}$F–$^{19}$F dipolar coupling is attenuated by motion, decreasing the coupling parameter, $K_{ij}$.

Our previous $^{31}$P spectra of oriented POPC membranes containing PG-1 indicate that the bilayer structure is severely disrupted above a peptide concentration of 3.3%. To determine if PG-1 aggregation depends on the concentration and potentially correlates with bilayer disruption, we conducted the CODEX experiment at a lower P:L of 1:34.5, corresponding to a peptide concentration of 2.8%. Due to the lower sensitivity, only two mixing times, 300 ms and 1 s, were measured. Figure 3B shows that the diluted PG-1 sample gives an $S/S_0$ of 0.75 at 300 ms and 0.70 at 1 s (circles). The latter value is most likely the equilibrium value, since the difference between the two data points is small and the 7.4% sample has equilibrated at 1 s. The error bar indicates that the difference of this $S/S_0$ value from that of the concentrated sample is statistically significant. The higher equilibrium value indicates an increased fraction of dimers. Since the higher concentration data support monomer–dimer equilibrium, the more dilute sample cannot have higher order aggregates. Thus, the equilibrium value of 0.70 corresponds to a dimer fraction of 60% and a monomer fraction of 40%. Moreover, the best fit for the 2.8% CODEX data has the same decay constant as that for the 7.4% sample within experimental uncertainty. This indicates that the dimers that do form at lower concentration have a structure similar to that of the high-concentration sample.

**Aggregation Model of PG-1 in POPC Membranes.** Anti-microbial peptides, part of the innate immune system of many...
organisms against bacterial, fungal, and viral attacks,\textsuperscript{41} are thought to kill invading microbes by destroying their cell membranes via aggregation or oligomerization. Three leading models have emerged for describing the mode of action of these small defensive peptides. The barrel-stave model postulates that transmembrane peptides form helical bundles\textsuperscript{42,43} that deplete the membrane potential. The carpet model hypothesizes that peptides aggregate on the surface of the membrane at low concentrations, but at high concentrations they micellize the bilayer, thus destroying the membrane.\textsuperscript{44} The toroidal-pore model suggests that aggregated peptides form pores where the lipid orientations change, connecting the upper and lower leaflets of the bilayer.\textsuperscript{45} Although oligomerization is central to all these models, there is so far little direct evidence of peptide aggregation\textsuperscript{46} due to the lack of suitable techniques. The spin diffusion NMR technique presented here is thus relevant for determining the aggregation of these membrane-active peptides in the lipid membrane.

Evidence of PG-1 aggregation in DPC micelles was reported based on $^1$H NOEs between residues far apart in the $\beta$-hairpin structure, and based on the slowness of $^1$H/$^2$H exchange for amide hydrogens not involved in intramolecular hydrogen bonds.\textsuperscript{47} These data indicated an antiparallel dimer, with the C-terminal strands adjacent to each other, in an NCCN fashion. Although further association of the dimer was hypothesized, evidence for it was lacking, since no intermolecular NOEs for such higher order aggregates were observed.\textsuperscript{47} The current $^{19}$F CODEX data indicate that, in the lipid bilayer at a peptide concentration of 7.4\%, PG-1 mainly (88\%) exists as dimers. Although the CODEX experiment presented here in principle cannot simultaneously determine both the fraction and the size of the aggregate in a heterogeneous mixture, the scenario of having higher order oligomers rather than dimers in PG-1 is unlikely for several reasons. First, the CODEX decay curve is single exponential (Figure 3B); thus, only a single oligomeric species (other than a monomer) is present, and a complex equilibrium such as monomer–dimer–tetramer can be ruled out. Second, if the sample consisted of monomers and tetramers, then the fraction of the monomer would be very high, 40\%, to give the observed equilibrium value of 0.56. For such different molecular weight species to both be present at significant percentages, there should be observable chemical shift and line width distribution in the $^{13}$C, $^{15}$N, and $^{19}$F spectra of the peptide. No such spectral heterogeneity was observed (Supporting Information Figure S2). Finally, other membrane peptides whose oligomerization has been characterized are completely oligomerized at concentrations above 1\%.\textsuperscript{6}

Modeling of the PG-1 structure (PDB accession code 1PG1)\textsuperscript{48} suggests that the $^{19}$F–$^{19}$F distances in both parallel and antiparallel NCCN dimers are 11–14 Å, a distance range that is accessible by $^{19}$F CODEX (Figure 4A). More detailed distance measurements are required to further delineate the structure of the dimer interface.

A PG-1 dimer appears too small to be completely immobilized in the POPC membrane at room temperature.\textsuperscript{13,31} On the basis of the size and viscosity dependence of rotational diffusion rates in two-dimensional lipid membranes,\textsuperscript{49} one would expect the PG-1 dimer to undergo whole-body uniaxial rotation. We hypothesize that the reason for the lack of this overall mobility is the hydrophobic mismatch between the lipid bilayer and the PG-1 dimer. This hydrophobic mismatch may induce highly curved membrane defects that trap the PG-1 dimers (Figure 4B, right). Evidence of such membrane defects is seen from the distorted $^{31}$P line shape of the POPC membrane in the presence of PG-1\textsuperscript{13} and the emergence of an isotropic $^{31}$P peak when anionic POPG lipids are added to the membrane.\textsuperscript{50} In addition, it is possible that loose aggregates of dimers may exist to line the defects in the lipid membrane. Such larger aggregates of dimers may not be detectable on the $^{19}$F spin diffusion length scale.

Figure 4B summarizes our current model of PG-1 dimerization and its relation to its membrane disruptive function. We postulate that dimerization is required to maximize peptide insertion into the membrane. The oligomeric peptides create structural defects in the membrane, as manifested in the $^{31}$P NMR line shape.\textsuperscript{13} These defects in turn constrain the peptide and prevent it from undergoing whole-body rotation. At low concentrations, more peptides are monomeric and may remain on the membrane surface, as indicated by X-ray lamellar diffraction and oriented circular dichroism data.\textsuperscript{51,52}
Energetics of PG-1 Dimer Formation in POPC Membranes. We can estimate the free energy of PG-1 dimer formation, $\Delta G$, using a simple monomer (M)−dimer (D) equilibrium model, $2M \rightleftharpoons D$. The equilibrium association constant, $K_a$, is $[D]/[M]^2$, where $[D]$ and $[M]$ depend on the peptide concentration, $c$, and dimer fraction $f_D$ as $[D] = c f_D/2$ and $[M] = c(1 - f_D)$. $\Delta G$ is determined from $K_a$ according to $\Delta G = -RT \ln K_a$. On the basis of the experimental data, we found a $\Delta G$ of $-10.2 \pm 2.3$ kJ/mol in favor of dimer formation.

What is the driving force for PG-1 dimerization? We postulate that it is primarily the formation of intermolecular hydrogen bonds, which reduces polar interactions in the membrane. Reduced exposure of polar side chains to the membrane may also contribute. On the basis of equilibrium dialysis experiments on model peptides transferred from aqueous solution to membrane environments, White and co-workers estimated the free energy reduction for $\beta$-sheet aggregation in the membrane to be $\sim 0.5$ kcal/mol (2.09 kJ/mol) per residue, which is also the value per hydrogen bond. Since three residues on the C-terminus strand of PG-1 can form intermolecular hydrogen bonds, a total of six intermolecular hydrogen bonds may be gained per molecule due to aggregation. Thus, the total free energy reduction is $\sim 12.6$ kJ/mol, in good agreement with the NMR-deduced energy change.

The free energy estimation further argues against a monomer−tetramer equilibrium for PG-1, since the equilibrium mole fractions in that model would correspond to a small free energy change of $-5.6$ kJ/mol, which is inconsistent with the number of intermolecular hydrogen bonds that would be formed in a tetramer.

Conclusion

We have shown the utility of $^{19}$F CODEX for determining the aggregation of peptides bound to lipid membranes. Demonstration on crystalline amino acids proves unambiguously that the equilibrium exchange value, $S(S_0)$, is indicative of the number of unique orientations in the unit cell. The use of the $^{19}$F spin instead of $^{13}$C significantly reduces the time required to reach complete exchange, thus increasing the distance reach of $^1$H-driven spin diffusion. Application of the $^{19}$F CODEX technique to the membrane-bound antimicrobial peptide PG-1 at low temperature where peptide motions are frozen indicates that, at a concentration of 7.4%, PG-1 exists predominantly as dimers in POPC membranes. This is consistent with the observation of PG-1 in DPC micelles in solution. Lowering the peptide concentration reduced the dimer fraction. On the basis of the equilibrium constant, we estimate the free energy reduction for dimer formation to be $\sim 10.2$ kJ/mol. This is in good agreement with the previously measured free energy change of $\beta$-sheet aggregation, and supports the view that the main driving force for PG-1 aggregation is the formation of intermolecular hydrogen bonds.

We anticipate this $^{19}$F spin diffusion approach to be generally applicable to the study of the association of peptides in complex environments, such as membrane peptides that form channels in lipid membranes, coiled coil proteins, and amyloid peptides. For peptides that exist in more complex equilibria with multiple oligomerization states, the basic CODEX pulse sequence may be concatenated into a four-time CODEX experiment to detect the spin diffusion of only those molecules that are part of an aggregate. By removing the monomer signals and selectively detecting the aggregate signals, one may be able to obtain additional constraints to determine both the fractions and the sizes of the aggregates in such mixtures.

Acknowledgment. This work is supported by NIH Grants GM-066976 to M.H. and AI-22839 and AI-37945 to A.J.W. and R. I. Lehrer. We thank R. Mani for help in the Phe experiments.

Supporting Information Available: $^{19}$F CODEX data of PG-1 at room temperature and $^{13}$C and $^{15}$N MAS spectra of the 7.4% PG-1 sample (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA043621R