Comparisons of Interfacial Phe, Tyr, and Trp Residues as Determinants of Orientation and Dynamics for GWALP Transmembrane Peptides

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*Supporting Information

ABSTRACT: Aromatic amino acids often flank the transmembrane alpha helices of integral membrane proteins. By favoring locations within the membrane–water interface of the lipid bilayer, aromatic residues Trp, Tyr, and sometimes Phe may serve as anchors to help stabilize a transmembrane orientation. In this work, we compare the influence of interfacial Trp, Tyr, or Phe residues upon the properties of tilted helical transmembrane peptides. For such comparisons, it has been critical to start with no more than one interfacial aromatic residue near each end of a transmembrane helix, for example, that of GWALP23 (acetyl-GGALW5(LA)6LW19LAGA-[ethanol]amide). To this end, we have employed 2H-labeled alanines and solid-state NMR spectroscopy to investigate the consequences of moving or replacing W5 or W19 in GWALP23 with selected Tyr, Phe, or Trp residues at the same or proximate locations. We find that GWALP23 peptides having F5, Y5, or W5 exhibit essentially the same average tilt and similar dynamics in bilayer membranes of 1,2-dilauroylphosphatidylcholine (DLPC) or 1,2-dioleoylphosphatidylcholine (DOPC). When double Tyr anchors are present, in Y4,5GWALP23 the NMR observables are markedly more subject to dynamic averaging and at the same time are less responsive to the bilayer thickness. Decreased dynamics are nevertheless observed when ring hydrogen bonding is removed, such that F4,5GWALP23 exhibits a similar extent of low dynamic averaging as GWALP23 itself. When F5 is the sole aromatic group in the N-interfacial region, the dynamic averaging is (only) slightly more extensive than with W5, Y5, or Y4 alone or with F4,5, yet it is much less than that observed for Y4,5GWALP23. Interestingly, moving Y5 to Y4 or W19 to W18, while retaining only one hydrogen-bond-capable aromatic ring at each interface, maintains the low level of dynamic averaging but alters the helix azimuthal rotation. The rotation change is about 40° for Y4 regardless of whether the host lipid bilayer is DLPC or DOPC. The rotational change (Δρ) is more dramatic and more complex when W19 is moved to W18, as Δρ is about +90° in DLPC but about −60° in DOPC. Possible reasons for this curious lipid-dependent helix rotation could include not only the separation distances between flanking aromatic or hydrophobic residues but also the absolute location of the W19 indole ring. For the more usual cases, when the helix azimuthal rotation shows little dependence on the host bilayer identity, excepting W18GWALP23, the transmembrane helices adapt to different lipids primarily by changing the magnitude of their tilt. We conclude that, in the absence of other functional groups, interfacial aromatic residues determine the preferred orientations and dynamics of membrane-spanning peptides. The results furthermore suggest possibilities for rotational and dynamic control of membrane protein function.

Model peptides have proven to be useful for studying protein–lipid interactions, which in turn are important for the regulation of biological function. Model systems offer particular advantages for establishing general principles because specific changes in peptide sequence and structure can be made, and the direct effects on interaction with a surrounding lipid membrane can be analyzed. Several membrane proteins, including gramicidin channels, reveal the preferred location of tryptophan residues at the lipid–water interface.1–3 For model peptide design, a core leucine–alanine sequence4 will enhance sensitivity of the peptide to membrane thickness because of hydrophobic mismatch. Then, by altering the identities or positions of aromatic anchors that flank the core sequence, the effects of these placements on the orientations and dynamics of transmembrane helices can be investigated and inferences for membrane proteins can be deduced.

The early model WALP peptides (acetyl-GWWA-(LA)ₙLWWA-[ethanol]amide), incorporating multiple Trp (W) anchors and the helical, hydrophobic repeating Leu–Ala core sequence, were important for helping to establish principles for the interfacial partitioning of Trp residues and the modulation of lipid phase behavior.4 Later, the four Trp residues were mutated to other aromatic or charged residues.
(Tyr, Phe, Lys, Arg, or His) to monitor the importance of the chemical and physical properties.\textsuperscript{5,6} The WALP family peptides adopt specific preferred tilted transmembrane orientations in lipid bilayer membranes,\textsuperscript{7,8} but, in addition to cone precession about the membrane normal,\textsuperscript{9} they also experience excessive dynamic averaging of solid-state NMR observables,\textsuperscript{10–13} caused by the presence of the four Trp residues.\textsuperscript{14} Importantly, the extent of dynamic averaging can be greatly reduced by decreasing the number of aromatic residues, as in GWALP23 (acyetyl-GGALW(LA)_5WLLAGA-[ethanol]amide),\textsuperscript{15} which possesses only two Trp residues and still maintains a preferred tilted orientation\textsuperscript{12,14,16} with low dynamic averaging. The tryptophans in GWALP23 thereby flank a hydrophobic Leu–Ala core of the same length as that of WALP19.\textsuperscript{4,7} With fewer aromatic residues, it becomes easier to assess the roles of each of them. The results to date suggest that four Trp anchors are so dominating, and possibly competing,\textsuperscript{17} that they induce significant peptide dynamics, mainly because of rotational “slippage” about the helix axis.\textsuperscript{14,16}

The favorable properties of GWALP23 have enabled this peptide to be employed as a highly suitable parent host framework for examining the influence of specific charged guest residues within the core hydrophobic sequence.\textsuperscript{18,19} It has furthermore been possible to examine the titration behavior of specific residues and the influence of ionization state upon helix orientation.\textsuperscript{20} Within the GWALP23 context, questions arose about the effects of changing the identity, number, and position of the aromatic residues. A peptide having a single Trp → Tyr replacement (Y’GWALP23) exhibits similar transmembrane orientation and dynamics as those of GWALP23,\textsuperscript{17} in three different lipids, such that the substitution of Tyr for Trp causes no fundamental change in the lipid–peptide interactions. However, when two Tyr residues are introduced to produce Y\textsuperscript{2}GWALP23, the extent of dynamic averaging increases dramatically,\textsuperscript{12} reminiscent of the earlier WALP peptides.

With the Trp- and Tyr-containing members of the GWALP23 family as a backdrop, we have examined the influence of phenylalanine by alternating the Tyr-containing models so as to have non-hydrogen-bonding Phe residues in F\textsuperscript{5}GWALP23 and F\textsuperscript{4,5}GWALP23 (acyetyl-GGAF\textsuperscript{5}(LA)_5WLLAGA-[ethanol]amide). We furthermore examined whether the dynamic behavior shown by Y\textsuperscript{5}GWALP23 could be caused by Y4 alone, as opposed to the Y4,5 combination. Placing a Tyr residue at position 4 also provided a way of determining the effects of moving a single aromatic residue one position (100°) around the α helix by comparison with Y\textsuperscript{5}GWALP23. A similar radial comparison between aromatic ring positions was made by moving Trp19 to position 18 to give GW\textsuperscript{5,18}ALP23.

### MATERIALS AND METHODS

Peptides F\textsuperscript{5}GWALP23, F\textsuperscript{4,5}GWALP23, Y\textsuperscript{5}GWALP23, and W\textsuperscript{5}GWALP23 (Table 1) were synthesized on a model 433A synthesizer from Applied Biosystems by Life Technologies (Foster City, CA) using solid-phase methods, as described previously.\textsuperscript{17} Typically, two deuterated alamines of differing isotope abundances were incorporated into each synthesized peptide. Peptides were purified as described\textsuperscript{18,21} using an octyl silica column (Zorbax Rs-C8, 9.4 × 250 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA) and a gradient of 97–100% methanol (with 0.1% trifluoroacetic acid) over 28 min. Final peptide purity (>97%) was confirmed by reversed-phase HPLC, and peptide identity, by mass spectrometry (Figure S1 of the Supporting Information).

Solid-state \textsuperscript{2}H NMR experiments, using mechanically aligned samples, were performed using methods that have been described previously.\textsuperscript{17} Mechanically aligned samples (1:60, peptide/lipid; 45% hydration, w/w) were prepared using DOPC, DMPC, or DLPC lipid from Avanti Polar Lipids (Alabaster, AL) and deuterium-depleted water from Cambridge Isotope Laboratories (Andover, MA). Bilayer alignment within each sample was confirmed using \textsuperscript{31}P NMR at 50 °C on a Bruker (Billerica, MA) Avance 300 spectrometer. Deuterium NMR spectra were recorded at 50 °C using both β = 0° (bilayer normal parallel to magnetic field) and β = 90° macroscopic sample orientations on a Bruker Avance 300 spectrometer utilizing a quadrupolar echo pulse sequence\textsuperscript{22} with 90 ms recycle delay, 3.2 μs pulse length, and 115 μs echo delay. Between 0.6 and 1.5 million scans were accumulated during each \textsuperscript{2}H NMR experiment. An exponential weighting function with 100 Hz line broadening was applied prior to Fourier transformation.

Helix orientations were analyzed by means of a semistatic “GALA” method based on three adjustable parameters: the average tilt τ, of the helix axis, the average azimuthal rotation ρ, about the helix axis, and a principal order parameter S\textsubscript{222} as described.\textsuperscript{7,17} An additional three-parameter modified Gaussian method is available, based on τ,ρ, and a distribution width σ\textsubscript{ρ}, and a fixed σ\textsubscript{τ}.\textsuperscript{16} We also employed this modified Gaussian method, but σ\textsubscript{τ} was fixed at either 15° (DLPc) or 9° (DOPC; see Discussion) instead of the previously assumed value of 0° for σ\textsubscript{τ}. For the analysis of helix rotation, we analyzed some pairwise residue separation distances using a recently described procedure.\textsuperscript{23} Distances were compared to hydrophobic thicknesses of 20.9 Å for DLPC\textsuperscript{24} and 27.2 Å for DOPC,\textsuperscript{25} which are based on the location D\textsubscript{C} of the Gibbs dividing surface for the hydrocarbon region of the bilayer.

### RESULTS

The designed peptides (Table 1 and Figure 1) were successfully synthesized and purified, as confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Figure S1 of the Supporting Information). The repeating Leu–Ala sequence at the core of GWALP peptides favors folding into α-helical secondary structure within the hydrophobic region of the lipid bilayer. Indeed, the CD spectra for the new variants of GWALP23 (Figure 2) show a minimum near 208 nm and a broad shoulder near 222 nm, indicating that the secondary structure is indeed α-helical. The \textsuperscript{31}P NMR spectra for oriented samples of each peptide–lipid combination furthermore confirmed the presence of oriented lipid bilayers within samples that were aligned with

### Table 1. Sequences of GWALP23-Like Peptides with Aromatic Substitutions\textsuperscript{a}

| name | sequence |
|------|----------|
| GWALP23 | a-GWW\textsuperscript{5}LALALALALALALALALWWA-e |
| GWALP23 | a-GGALW\textsuperscript{5}LALALALALALALALWLAGA-e |
| Y\textsuperscript{5}GWALP23 | a-GGALY\textsuperscript{5}LALALALALALALWLAGA-amide |
| Y\textsuperscript{5}GWALP23 | a-GGAY\textsuperscript{5}LALALALALALALWLAGA-amide |
| F\textsuperscript{5}GWALP23 | a-GGALF\textsuperscript{5}LALALALALALALWLAGA-amide |
| F\textsuperscript{4,5}GWALP23 | a-GGAF\textsuperscript{4,5}LALALALALALALWLAGA-amide |
| Y\textsuperscript{5}GWALP23 | a-GGAY\textsuperscript{5}LALALALALALALWLAGA-amide |
| W\textsuperscript{5}GWALP23 | a-GGG\textsuperscript{5}LALALALALALALALWLAGA-amide |

\textsuperscript{a}Abbreviations: a, acetyl; e, ethanolamide.
the bilayer normal either parallel (β = 0°) or perpendicular (β = 90°) to the applied magnetic field. The spectra exhibit characteristic 31P resonances located close to −14.5 ppm for the β = 90° orientation and near +29 ppm when β = 0° (Figure S2 of the Supporting Information).

Solid-state 2H NMR spectra from oriented samples of GWALP23, Y4,5GWALP23, and Y5GWALP23 (blue), and Y4GWALP23 (red) in DLPC (1:60 peptide/lipid). The y-axis units for mean residue ellipticity (MRE) are deg cm² dmol⁻¹.

![Figure 1](image)

![Figure 2](image)

Table 2. 2H NMR Quadrupolar Splittings (Δνq in kHz) for Labeled Alanine CD₃ Groups in F₅GWALP23, F₄,5GWALP23, and Y₅GWALP23

| Ala-d₅ | DLPC       | DMPC       | DOPC       |
|--------|------------|------------|------------|
| 7      | 17.8       | 23.7       | 15.8       |
| 9      | 20.4       | 23.5       | 9.2        |
| 11     | 21.9       | 25.7       | 18.8       |
| 13     | 13.8       | 19.6       | 9.2        |
| 15     | 18.3       | 23.4       | 18.8       |
| 17     | 1.0        | 1.8        | 1.3        |

Note: "Sample orientation is β = 0°. Each value (in kHz) is the average of the magnitude observed at β = 0° and twice the magnitude observed for a β = 90° sample orientation. Values that are absent (--) were not recorded. The labeled alanines are identified, and the positions of the N-flanking aromatic amino acids are indicated as F₅, F₄,5, and Y₅. The C-flanking W₁⁹ is present in all samples except for W³⁴."
the range of $\Delta \nu_q$ for alanines in F4,5GWALP23 is about 24 kHz, from 1.8 to 25.7 kHz. In DOPC, the values cover a range of about 17 kHz, from 1.9 to 18.6 kHz (Table 2). These differences suggest that F4,5GWALP23 may be tilted to greater extent or may undergo less dynamic averaging than the single-anchored X4GWALP23 peptides. It is striking that the results for F4,5GWALP23 differ greatly from the previous characterization of Y4,5GWALP23. The larger quadrupolar splittings that are observed in $^2$H NMR spectra for the F4,5 peptide are greatly reduced in the spectra for Y4,5 (Figure 3). The ranges of the quadrupolar splitting magnitudes from alanines in Y4,5GWALP23 therefore span only 11 and 9 kHz in DLPC and DOPC (Figure 4). In DLPC, the wave amplitude actually is larger for F4,5GWALP23 than for F4,5GWALP23 (Figure 4A), suggesting somewhat lower dynamic averaging for F4,5GWALP23, and the wave amplitudes for both F4,5GWALP23 and F4,5GWALP23 are notably very much larger than that for Y4,5GWALP23 in DLPC. In DOPC, these three peptides have rather similar small tilt angles (Figure 4B and Table 3), yet the dynamic averaging remains much larger for Y4,5GWALP23 (see Discussion).

For the case of moving the tyrosine residue from position 5 to position 4 in the sequence, the helix tilt and dynamics remain unchanged, yet a phase change is seen in the quadrupolar wave plot (Figure 5), indicating a change in the helix azimuthal rotation ($\rho_z$) or direction of the tilt. Semistatic GALA analysis of Y4,5GWALP23 indicates that the rotation of the peptide changes by about 31° in DLPC and 46° in DOPC (Table 3) when Y5 is moved radially by 100° to position 4. A top view or helical wheel illustrates these differences in the direction of tilt for Y4,5GWALP23 and Y5GWALP23 (Figure 5B). Importantly, the extensive dynamics observed for double-Tyr derivative Y4,5GWALP23 are not caused by either Y4 or Y5 alone, but rather by the presence of the two tyrosines together.
Because of the change in azimuthal rotation that accompanies the moving of Y5 to Y4, we decided to investigate the consequence of moving W19 to position 18 in GWALP23. The results (Figure 6) indicate a larger rotational shift of almost 100° for the peptide helix in DLPC when W19 in GWALP23 is moved by 100° to W18. This large shift suggests a dominant role for W19 in determining the azimuthal rotation preference of GWALP23 in lipid bilayer membranes. Yet, a similar comparison between GWALP23 and W18GWALP23 in DOPC brings a surprise (Table 3). Namely, the preferred azimuthal rotation ρo about the helix axis does not vary appreciably when each particular helix is moved from DLPC to DMPC or DOPC. For cases of excess dynamics, namely, W2,22GWALP23 and Y4,5GWALP23, which also incorporate extra aromatic residues, σρ is large and ρo is not well defined from lipid to lipid. In this respect, W18GWALP23 stands alone with low dynamic averaging and yet has a value of ρo that changes by about 135° from DLPC to DOPC (Table 3). Even if one considers a Gaussian analysis (see Discussion), the value of ρo still varies widely from DLPC to DOPC. We therefore sought to consider possible reasons for this lipid-dependent variation in helix azimuthal rotation.

Recently, a method was presented for correlating helix rotational preference with flanking residue positions on a tilted transmembrane helix. We have applied this method to W18GWALP23 (Figure 7) as well as to the parent GWALP23

Figure 5. (A) Quadrupolar wave plots for Y4GWALP23 (red, circles) and Y5GWALP23 (blue, triangles) in DOPC. (B) Helical wheel diagram to illustrate the relative azimuthal rotation ρ for Y4GWALP23 (red circle) and Y5GWALP23 (blue circle) in DOPC, offset by ~50°. The labels Y4 and Y5 represent the respective radial locations of the tyrosines, which differ by 100° on the helical wheel.

Figure 6. (A) Quadrupolar wave plots for W18GWALP23 (red) and GWALP23 itself (blue) in DLPC. (B) Helical wheel diagram to illustrate the relative azimuthal rotation ρ for W18GWALP23 (red circle) in relation to (W19)GWALP23 (blue circle) in DLPC, offset by ~100°. The labels W18 and W19 represent the respective radial locations of the tryptophans, which differ by 100° on the helical wheel.

Figure 7. Aromatic (W18−W5) and hydrophobic (L20−L4) residue Cβ separation distances in angstroms along the bilayer normal as functions of rotation of W18GWALP23 about its tilted helix axis (A) when τo = 18° in DLPC or (B) when τo = 17° in DOPC. The preferred ρo values are shown by the arrows. The respective bilayer thicknesses are indicated by the dashed segments.

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Recent reports have compared the influence of Tyr versus Trp in the N-flanking position $S^{-1}$ and the C-flanking position 19 $26$ in membrane-spanning GWALP23 peptides. The main findings were that either Tyr or Trp at each location will support a similar tilted transmembrane helix orientation, with only about a 10 relative azimuthal rotation about the helix axis when Tyr is substituted for Trp. Furthermore, the extent of dynamic averaging of solid-state NMR observables remains low as long as only one interfacial aromatic residue is present at each end of the membrane-spanning GWALP23 helix (with no extra aromatic residues potentially competing for lipid headgroup interactions).

In the present work, we compare the results for Trp/Tyr with the consequences of Phe substitutions at positions 4 and 5 in GWALP23. Additionally, we examine the consequences of moving a single aromatic side chain, either Y5 or W19, 100° around the helix axis by interchanging either Y5 and L4 or W19 and L18. Key findings are (a) an unexpectedly low extent of dynamic averaging for F4,GWALP23 and (b) a preference of azimuthal rotation of W4,GWALP23 that depends upon the identity (thickness) of the host lipid bilayer membrane. We will discuss first the results with the tyrosine-to-phenylalanine substitutions. Because of the differences in dynamics, we will also present the outcomes for a modified Gaussian treatment of the helix rotational dynamic averaging. Then, we will discuss the changes in rotational preference when aromatic and leucine residue locations are switched on the N-flanking side or the C-flanking side of the core helix.

**Changes Involving Y5 → F5 or Y4,Y5 → F4,F5.** A significant question concerns the hydrogen-bonding property of the OH group on the Y5 ring, which could interact favorably, albeit transiently (under conditions of dynamic hydrogen-bond exchange), with lipid heads groups and interfacial water molecules. The potential for hydrogen bonding is nevertheless absent when the F5 phenyl ring is substituted for Y5. With F5,GWALP23, we find that hydrogen bonding by the N-flanking aromatic ring is not necessarily needed to define a preferred, stable transmembrane orientation, with limited dynamic averaging, for the core (LA)$_L$ transmembrane helix. In this context, the indole ring of W19 seems to have special importance for defining the orientation and low dynamics (see below) and should not be overlooked or underestimated. Notably, F5,GWALP23 exhibits a similar transmembrane orientation as that of Y4,GWALP23 and W4,GWALP23 (Figure 4 and Table 3), with low dynamic averaging. Furthermore, F5,GWALP23, as well as its Y5 and W5 cousins, adapts to changes in the lipid bilayer thickness mainly by changing its tilt (Figure 4 and Table 3), with little change of the helix azimuthal rotation. The slightly smaller amplitude of the quadrupolar wave for F5,GWALP23 in DLPC (Figure 4) nevertheless suggests increased dynamic averaging, which indeed is borne out by the lower estimate of 0.58 for $S_\alpha$ for F5,GWALP23 in DLPC (Table 3). This order parameter is notably low compared to those observed for gramicidin A $29$ and Y4,GWALP23 or GWALP23 itself (Table 3). Moreover, we note the uniformly lower estimates for $S_\alpha$ from the semistatic GALA analysis of each peptide helix in DLPC compared to DOPC (Table 3).

The change from Y4,GWALP23 to F4,GWALP23 brings big changes to the properties of the transmembrane helix, mainly to reduce the very extensive dynamic averaging that occurs when

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**DISCUSSION**

maximum L20-to-L4 distance in DOPC (Figure 7B). Notably, the tilt angle for W19,GWALP23 appears to be essentially the same in DOPC as in DLPC (Table 3, Figure 7), with the main adaption seeming to involve the helix rotation. We will revisit these issues using a modified Gaussian analysis (see Discussion).

However, the rotation of GWALP23 (Figure 8) seems not to correlate with either the W19-to-W5 distance or the L20-to-L4 distance. Indeed, we surmise that W19 itself may play a special role. Being near the C-terminal, the indole side chain of W19 must adopt non-standard torsion angles $27,28$ in order to aim its aromatic residue locations are switched on the N- and C-flanking position 26 suggesting a special role for the W19 indole. Within this context, the tilted GWALP23 helix in DLPC is rotated so as to provide an (perhaps fortuitous) exact match between the W19 $C_{N}$ distance from the bilayer center and half of the DLPC bilayer’s hydrophobic thickness (Figure 8A). In the thicker DOPC, the GWALP23 helix is less tilted such that the W19 $C_{N}$ position, with respect to the bilayer center, varies less steeply with rotation (Figure 8B). There is a detectable, yet minor, change in $\rho_{N}$ so as to move the W19 side chain outward, but only slightly, with the main adaption being the smaller helix tilt in the thicker lipid bilayer. In contrast, W15,GWALP23 adapts to the changing lipid environment more by changing its helix rotation than by changing its tilt (Figure 7).

![Figure 8.](https://example.com/figure8.png)
Y4 and Y5 are present together. It has been noted that Y4,5-GWALP23 exhibits dramatically more dynamic averaging than is the case when only one tyrosine is present in Y4-GWALP23. What happens when the hydrogen-bonding ability is removed from the Y4 and Y5 aromatic rings? Remarkably, the dynamic averaging is seen to diminish when F4 and F5 are present (compare Figure 4 to Figure 6B in ref 17). We surmise that the phenyl rings of F4 and F5 are favorably placed if they are just “in the neighborhood” of the interfacial region, without need of any specific interactions with the lipid head groups. Phenol rings Y4 and Y5, on the other hand, may compete, perhaps alternately, for direct hydrogen-bonding interactions with lipid head groups. Such competition, if not able to optimize simultaneously a Y4 interaction and a Y5 interaction, could lead to increased helix rotational dynamics of the type suggested previously.11,13 Importantly, neither Y4 nor Y5 alone causes the extensive dynamic averaging (see below); rather, it is the pair of tyrosines together that leads to the high level of dynamics, as has been noted also for pairs of tryptophans.12

**Modified Gaussian Treatment of the Dynamics.** We sought to compare a semistatic treatment with a modified Gaussian treatment of the dynamic properties of each of the transmembrane peptides. The semistatic GALA treatment7,8 employs three adjustable parameters: tilt, $\tau_\theta$, and azimuthal rotation, $\rho_\phi$, to describe helix orientation, together with a principal order parameter, $S_{zz}$, to provide a highly abbreviated estimate of overall dynamics. A Gaussian treatment offers advantages of describing estimates for helix “wobble” $\sigma_\rho$, and rotational “slippage” $\sigma_\rho$, but it does so at the expense of requiring four adjustable parameters instead of three.13 As an alternative, a modified Gaussian treatment has been suggested,16 in which $\sigma_\rho$, is held constant and dynamic differences are embodied in an adjustable $\sigma_\rho$. We have employed this modified procedure16 but differing in that we set $\sigma_\rho$ to a small finite value instead of to zero while maintaining $S_{xx}$ of 0.88 as an estimate of internal motion of the peptide.14

Notably, the results of such a modified Gaussian analysis (Table 4) agree substantially with the conclusions from a semistatic GALA analysis (Table 3). Both methods indicate that each peptide (except possibly W18GWALP23; see below) adapts from DOPC to DLPC by increasing its tilt. The methods in all cases give the same preferred values of $\rho_\phi$ in each lipid and are in agreement concerning the small variations when the identity of aromatic residue 5 is changed. Additionally, the same trends for in $\rho_\phi$ are seen with either method when a peptide helix is moved from DOPC to DLPC. Generally, the change of lipid involves only a small $\Delta \rho_\phi$, excepting the case of W18GWALP23 (see below) and the highly dynamic Y4,5-GWALP23, noted previously.17

The respective outcomes for fitting the dynamics also are similar. The semistatic analysis (Table 3) leads to estimates for $\sigma_\rho$ that are higher in DOPC than in DLPC, seemingly because the relatively smaller tilt angles in DOPC do not demand a downgrade of $S_{xx}$ during the fitting process. For the modified Gaussian analysis, we set $\sigma_\rho$ to 15° in DLPC (where $\tau_\theta$ is larger) and to 9° in DOPC (where $\tau_\theta$ is smaller). (When presented with a binary choice of either 9 or 15°, the analytical software selected 15° for each peptide in DLPC but 9° for each peptide in DOPC; the values subsequently were fixed.) With these constraints, the resulting fitted values for $\sigma_\rho$ are similar between DLPC and DOPC (Table 4), with somewhat larger values of $\sigma_\rho$ emerging for the fits in DOPC (perhaps reflecting the somewhat smaller choice for $\sigma_\rho$). For the highly dynamic Y4,5-GWALP23, $\sigma_\rho$ is seen to be very large in both DLPC and DOPC, thereby confirming earlier conclusions based on semistatic analysis.17 With a caveat that the helix behavior should be examined in more than one lipid, similar conclusions about the extent of dynamic averaging (whether limited, moderate, or high) for each transmembrane helix emerge from the semistatic GALA and modified Gaussian analyses. It is useful and productive to apply and compare both methods.

**Changes in Rotational Preference when Y5 Is Moved or W18 Is Moved.** We observed sizable changes in the preference for helix azimuthal rotation when a key aromatic ring, the side chain of either residue 5 or residue 19, has been moved by 100° around the helix axis (Tables 3 and 4). In this regard, the Trp residue, W19, seems once again to have a special importance, as the changes to the rotational preference are larger and lipid-dependent when this tryptophan is moved. When Y4 is moved to Y5, the preferred $\rho_\phi$ changes by about 30–35° in DLPC and by about 40–45° in DOPC. Conspicuously, the deduced values of $\Delta \rho_\phi$ are similar between the semistatic and modified Gaussian methods of analysis (Tables 3 and 4). The rotational change furthermore is slightly less than half of the 100° radial shift that accompanies the move of Tyr from position 5 to position 4. The results suggest that a compromise may be imposed by the interplay of W19 (fixed) and either Y4 or Y5 (variable), with the influence of W19 on the rotational preference nevertheless dominating to a small extent over that of Y4 or Y5 (because $\Delta \rho_\phi < 50°$ in both lipids). We recall also that the identity of residue 5 exerts a smaller influence on the azimuthal rotation.17 Interestingly, $\rho_\phi$ decreases ∼10° when W5 is changed to Y5, but it increases ∼10° when W5 is changed to F5; the molecular reasons that may underlie these small but opposite values of $\Delta \rho_\phi$ are elusive at this time.

The movement of W19 to position 18 has major influence on the helix azimuthal rotation. Interestingly, an interchange of W19 and L18 leads to a change in $\rho_\phi$ of ∼90° in DLPC but −60° in DOPC in spite of the fact that $\sigma_\rho$ remains low in DLPC and moderate in DOPC (Tables 3 and 4). These results

### Table 4. Modified Gaussian Analysis of Orientations and Dynamics for Peptides of the GWALP23 Family$^{a,b}$

| Peptide        | DLPC            | DOPC            |
|----------------|-----------------|-----------------|
|                | $\tau_\theta$  | $\rho_\phi$    | $\sigma_\rho$ | RMSD (kHz) | $\tau_\theta$  | $\rho_\phi$    | $\sigma_\rho$ | RMSD (kHz) |
| W5             | 23              | 304            | 33            | 0.7        | 9             | 321            | 48            | 0.7        |
| Y4             | 23              | 295            | 32            | 0.0        | 6             | 313            | 34            | 1.0        |
| Y5             | 17              | 314            | 21            | 1.7        | 9             | 329            | 40            | 0.6        |
| Y5,5GWALP23    | 14              | 259            | >90$^d$       | 1.7        | 6             | 344            | 72            | 0.9$^d$    |
| F5             | 18              | 314            | 0$^d$         | 0.7        | 10            | 329            | 54            | 1.6        |
| F5,5GWALP23    | 11              | 328            | 22            | 0.9        | 11            | 353            | 56            | 0.4        |
| W5,W18         | 18              | 35             | 8$^d$         | 1.4        | 17            | 264            | 50            | 1.2        |

$^a$The N-flanking aromatic residues are indicated by the abbreviation for each peptide. C-flanking W5$^d$ is also present in all samples except when the aromatic residues are W5 and W5.$^d$ Analysis followed Strandberg et al.$^{16}$ but $\sigma_\rho$ was assigned a finite value instead of 0 (see Methods). Except as noted, $\sigma_\rho$ was set to 15° in DLPC or 9° in DOPC. $^b$The value of $\sigma_\rho$ was 20° because no satisfactory solution was found when $\sigma_\rho = 15°$. $^b$The value of $\sigma_\rho$ remains >90° even if $\sigma_\rho$ is set to 20°. $^{15}$The value of $\sigma_\rho$ was 13° because no satisfactory solution was found when $\sigma_\rho = 9°$. $^d$The value of $\sigma_\rho$ is perhaps artificially low because of the choice of $\sigma_\rho$. Because of the limited data set, further solutions were not explored.
once again highlight the major importance of W19, or more generally of the C-flanking aromatic residue, for the helix azimuthal rotation. Moreover, the semistatic and modified Gaussian methods agree on not only the absolute magnitudes but also the extent of change in both lipids (Tables 3 and 4). Why does the preferred rotation of W19GWALP23, but not of the other peptides that experience low dynamic averaging, depend upon the host lipid bilayer? While the possible answers to this question remain incomplete at this time, some clues may emerge from consideration of Figures 7 and 8. With W18 and W5 separated by 140° on the helical wheel (Figures 1 and 6), the transmembrane helix azimuthal rotation seems not to correlate with the W5-to-W18 separation distance along the bilayer normal (Figure 7) but rather with the L4-to-L20 distance, which could better describe the longitudinal hydrophobic length of the helix. Indeed, W19GWALP23 seemingly rotates so as to minimize the L4-to-L20 transmembrane distance in DLPC and to maximize the L4-to-L20 transmembrane distance in DOPC (Figure 7). While more examples would be needed to establish a causation relationship instead of a mere correlation, this principle could help to explain the lipid-dependent helix rotation for the special case of the W19GWALP23 helix.

The particular importance of W19 for the transmembrane orientation of GWALP23 is evident in Figure 8. Notably, in GWALP23, the two Trp residues are on the same side of a helical wheel, with a radial displacement of only 40° (Figures 1 and 5). Rather than any dependence on the W5-to-W19 longitudinal separation, GWALP23 rotates in DLPC so that the distance from Cβ of W19 to the bilayer center matches the hydrophobic thickness of a DLPC monolayer (Figure 8A). From DLPC to DOPC, the main adjustment is that GWALP23 is less tilted in DOPC. The rather minimal rotational adjustment serves to move W19 slightly farther away from the DOPC bilayer center (Figure 8B).

## CONCLUDING REMARKS

For the model membrane-spanning helix of acetyl-GGALW-(LA)6LWLAGA-amide (GWALP23), a well-defined transmembrane orientation with only limited dynamic averaging, other than long-axis precession about the bilayer normal, is retained following the single aromatic residue substitution of F5 or Y5 in place of W5. By contrast, all known model transmembrane peptide helices having more than two aromatic and charged residues.

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