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Influence of High pH and Cholesterol on Single Arginine-Containing Transmembrane Peptide Helices

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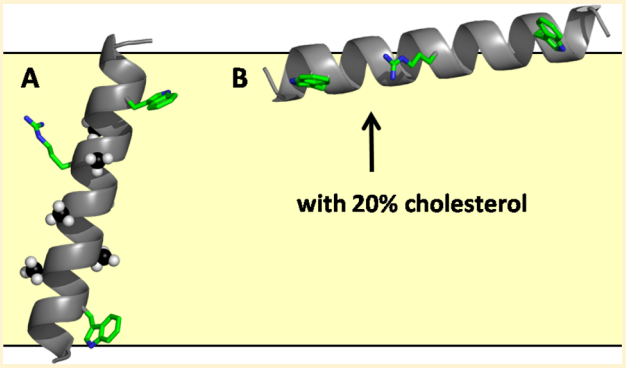
Supporting Information

ABSTRACT: An essential component of mammalian cells, cholesterol exerts significant influence on the physical properties of the cell membrane and in turn its constituents, including membrane proteins. Although sparse, polar amino acid residues are highly conserved in membrane proteins and play pivotal roles in determining specific structural and functional properties. To improve our understanding of particular polar residues in the membrane environment, we have examined two specific "guest" Arg residues within a well-defined and deuterium-labeled "host" framework provided by the transmembrane helical peptide GWALP23 (acetyl-GGALWLALALALALALALWLAGA-amide). Solid-state ^{2}H nuclear magnetic resonance (NMR) spectra from aligned bilayer membrane samples effectively report changes in the host helix properties because of the incorporation of the guest residues. The focus of this work is two-pronged. First, GWALP23-R14 was examined over a pH range of 2−13 in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) ester- or ether-linked bilayer membranes. Our findings indicate that the Arg guanidinium side chain remains charged over this entire range, in agreement with numerous molecular dynamics simulations. Second, GWALP23-R12 and GWALP23-R14 peptides were characterized in DOPC bilayers with varying cholesterol content. Our findings suggest that 10 or 20% cholesterol content has minimal impact on the orientation of the R14 peptide. Although the NMR signals are broader and weaker in the presence of 20% cholesterol, the deuterium quadrupolar splittings for [^{2}H]Ala residues in GWALP23-R14 change very little. Conversely, cholesterol appears to modulate the multistate behavior of GWALP23-R12 and to favor a major interfacial state for the helix, bound at the bilayer surface. These results indicate a conditional sensitivity of a complex multistate transmembrane Arg-containing peptide helix to the presence of cholesterol.



Membrane proteins perform a variety of functions critical to the survival of organisms, including serving as gateways that allow for communication between the interior of the cell and its external environment. Essential to many membrane proteins are polar amino acids, which often determine the structure and function of the protein itself. The particular sequence and length of a largely hydrophobic membrane protein determine its behavior and stability within the cell membrane. Interestingly, the more sparse polar amino acids may be critical for particular functional properties. The examination of the influence of particular individual polar amino acids on molecular properties, within the context of a lipid bilayer membrane environment, is possible through the use of synthetic, labeled model peptides such as those of the well-defined "GWALP" family.

The GWALP peptides were derived from an original "WALP" model peptide family[1] by reducing the number of tryptophan (W) residues.[2] WALP contained four aromatic W residues to serve as interfacial anchors for the membrane-spanning helix.[3] Although the WALP peptides have proven to be useful for studying peptide−lipid hydrophobic mismatch, their high dynamics renders them less than ideal for examining the effects of single-residue replacement within the helical core.[4−6]

GWALP23, developed initially as a control, was found to exhibit averaging that was less dynamic than that of WALP23.[6,7] Notably, two interfacial tryptophan residues (W), rather than four, led to better definition of the transmembrane helix orientation and lower dynamics;[2,8] GWALP23 [GGALW(LA)$_6$-LWLAGA] contains a single Trp residue flanking each end (positions 5 and 19) of a repeating leucine-alanine helical core sequence.[9,10] The (Leu-Ala)$_6$L leucine-alanine domain prefers the hydrophobic environment of the membrane interior, while the Trp residues prefer a location near the lipid−water interface.[11,12] GWALP23 and related model peptides therefore adopt defined tilted orientations that can be observed in bilayer membranes by means of solid-state nuclear magnetic resonance (NMR) spectroscopy. The solid-state NMR spectra allow for investigations of the influence imposed by a single "guest" residue, such as a strongly polar or charged amino acid within

Received: September 5, 2016
Revised: October 19, 2016
Published: October 26, 2016

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DOI: 10.1021/acs.biochem.6b00896
Biochemistry 2016, 55, 6337−6343

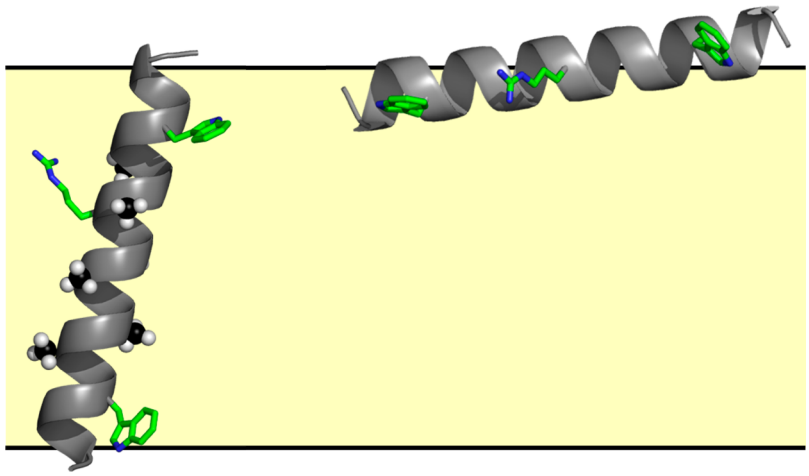

Figure 1. Models to illustrate experimental tilted orientations of charged peptides GWALP23-R14 (left) and GWALP23-R12 (right), with respect to a vertical bilayer normal of 20% cholesterol and 80% DOPC lipid bilayer membranes (drawn using PyMOL). The indole rings extending from both models are W^5 and W^{19} in the 23-residue sequence. The side-chain orientations are arbitrary. The six deuterated core alanine residues that allow for tilt analysis are shown on the GWALP23-R14 model as space filling.

the central core of the transmembrane helix. The constrained dynamics of GWALP23 make it more favorable than many other candidate "host" peptides for studies of specific guest residues.

Alanine residues within the GWALP23 core can be synthesized with deuterium labels to investigate the dynamics and orientation of the transmembrane peptide helix by means of solid-state deuterium (^{2}H) NMR spectroscopy. For the sake of convenience and experimental efficiency, alanine residues typically are labeled in pairs in core positions such as 7 and 9, 11 and 13, or 15 and 17. Within the labeled alanine pairs, one alanine may be labeled with 50% deuterium and the other with 100% deuterium. This allows for the respective quadrupolar splittings in the NMR spectra to be distinguished and assigned.

This work centers on GWALP23 peptides that contain a strongly polar, positively charged arginine (R) residue in place of a hydrophobic leucine (L) residue. Previous experiments have revealed that the Arg residue in GWALP23-R14 causes a 10° change in helix tilt, which is invariant over a pH range of 4−9, where R14 is charged.[13] The equivalent peptide with lysine (GWALP23-K14) exhibits an interesting pH-dependent tilt that indicates a pK_a value of 6.5 for the lysine side chain at 37 °C in membranes of DOPC.[14] On the basis of the established titration of the lysine, we have extended the NMR observations for GWALP23-R14 to pH 13 by using bilayers composed of the more stable ether-linked lipids (see Methods).

This work has also addressed the potential influence of membrane cholesterol on the transmembrane helices of GWALP23-R14 and GWALP23-R12 (Figure 1), which already display markedly different properties in the absence of cholesterol.[13] Some simulations have suggested that cholesterol may have a direct impact on the pK_a of buried arginine residues.[15] Experimental approaches have highlighted in some cases specific peptide/cholesterol recognition and binding interactions[16] and in other cases generalized membrane effects of cholesterol without specific binding.[17,18] Using a combination of DOPC phospholipids and 0−20% cholesterol, we conducted experiments with GWALP23-R14 and GWALP23-R12 in the lipid bilayers. The results reveal, in some cases, an influence of cholesterol on the preferred helix location but not on the arginine pK_a value.

METHODS

Peptides GWALP23-R12 and GWALP23-R14 (Figure 1) were synthesized using solid-phase Fmoc methods on an Applied Biosystems 433A synthesizer from Life Technologies (Foster City, CA), as described previously.[9] Protected amino acids were purchased from NovaBiochem (San Diego, CA). Two deuterated alanine residues were incorporated into the core of each peptide with 50 or 100% relative abundance to distinguish the quadrupolar splittings by solid-state deuterium NMR spectroscopy. Following completion of synthesis, peptides were cleaved from resin using a trifluoroacetic acid (TFA) cocktail composed of an 85:5:5:5 TFA:triisopropylsilane:H_2O:phenol ratio (v/v, w/v for phenol). After filtration of the cleavage mixture, the crude peptide was precipitated using a 50:50 mixture of hexane and methyl-*tert*-butyl ether and then lyophilized from a 50:50 mixture of acetonitrile and water.

Peptides were purified by reversed-phase HPLC on an octyl-silica column (Zorbax Rx-C8, 9.4 mm × 250 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA) using a gradient of 92 to 96% methanol, with 0.1% TFA (v/v), over 24 min. The peptide molecular mass and deuteration pattern were confirmed by MALDI-TOF mass spectrometry, as previously illustrated.[19]

Mechanically aligned samples (1:60, peptide:lipid) were prepared for solid-state ^{2}H NMR experiments using ester- or ether-linked DOPC lipids (Avanti Polar Lipids, Alabaster, AL). Peptide/lipid films were deposited on thin glass slides from 95% methanol, dried under vacuum (10^{-4} Torr for 48 h), and hydrated (45% w/w) with either deuterium-depleted water (Cambridge Isotope Laboratories, Andover, MA) or 10 mM glycine, citrate, acetate, 4-(cyclohexylamino)-1-butanesulfonate, or phosphate buffer in deuterium-depleted water at specific pH values between 2 and 13. Ether-linked lipids have been found to be more suitable for experiments at high pH because they are resistant to base-catalyzed degradation (unlike ester-linked lipids). Small but measurable changes in ^{2}H NMR spectra (0−2 kHz) are observed when DOPC bilayers are formed with ether lipids instead of ester lipids (Figure 2), but the minor differences are inconsequential for the results presented here.

Samples with cholesterol were prepared by combining cholesterol (Sigma-Aldrich, St. Louis, MO) and DOPC in

DOI: 10.1021/acs.biochem.6b00896
Biochemistry 2016, 55, 6337−6343

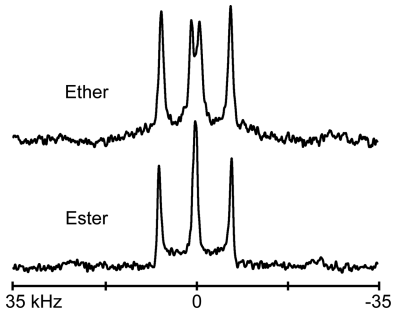

Figure 2. Comparison of ^{2}H NMR spectra of labeled GWALP23-R14 with ether-linked or ester-linked DOPC phospholipids at neutral pH. Alanines 15 and 17 are ^{2}H-labeled. The spectra are similar, with only small changes (0−2 kHz) in the magnitudes of the ^{2}H quadrupolar splittings when the ester lipids are replaced with ether lipids.

chloroform with cholesterol as 0−20% of the total lipid content, with the remainder as ester-linked DOPC. Peptide/lipid samples (1:60, peptide:total lipid) were deposited on glass slides from a chloroform/methanol solvent, dried as described above, and hydrated [45% (w/w)] with deuterium-depleted water. The hydrated slides were stacked in 8 mm cuvettes and sealed with epoxy.

Bilayer formation and alignment were confirmed for each sample by means of ^{31}P NMR spectra recorded at 50 °C using a Bruker (Billerica, MA) Avance 300 spectrometer (Figure 3).

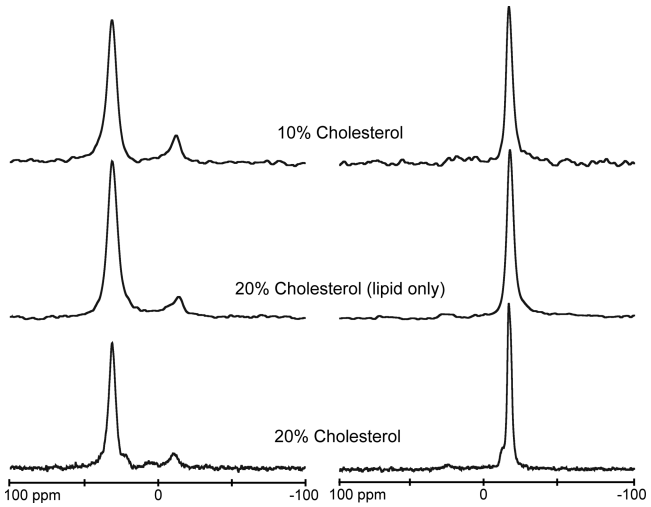

Figure 3. ^{31}P NMR spectra of DOPC bilayers aligned on glass plates and containing 10 or 20% cholesterol with or without the peptide GWALP23-R14 (1:60, peptide:total lipid). Major peaks in $\beta = 0°$ (left) and $\beta = 90°$ (right) spectra indicate that the phospholipid bilayer phase is present and well-aligned. The minor peaks at $\beta = 0°$ represent unaligned material, yet notably, the amount of unaligned phospholipids is not influenced by the cholesterol.

Deuterium NMR spectra were recorded at 50 °C, at $\beta = 0°$ (bilayer normal parallel to the magnetic field) and $\beta = 90°$ macroscopic sample orientations using a Bruker Avance 300 spectrometer, using a quadrupolar echo pulse sequence[20] with full phase cycling, a 90 ms recycle delay, a 3.2 μs pulse length, and a 115 μs echo delay. During each ^{2}H NMR experiment, between 0.7 and 1 million scans were recorded. Prior to Fourier transformation, an exponential weighting function with 100 Hz line broadening was applied.

Peptide helix orientations were analyzed using the semistatic "geometric analysis of labeled alanines" ("GALA") method, which is based on three adjustable parameters. These include the average tilt τ of the helix axis, the average azimuthal rotation ρ about the helix axis, and the principal order parameter S_{zz}, as described previously.[21,22] A modified Gaussian approach based on τ, ρ, a distribution width $\sigma\rho$, and a fixed $\sigma\tau$ was employed as previously described.[23]

RESULTS

Lipid bilayer formation was observed for all of the macroscopically aligned lipid/peptide samples, including those with ether- and ester-linked DOPC lipids and those containing up to 20% of the lipid as cholesterol. Importantly, a level of 20 mol % cholesterol preserves the bilayer phase and avoids any onset of lipid phase change or phase separation. The ^{31}P NMR spectra (Figure 3) illustrate that all of our cholesterol-containing DOPC/peptide samples retain the bilayer phase and the bilayer orientation as, notably, the extent of misalignment of ^{31}P headgroups does not change between 0 and 20% cholesterol (Figure 3). Importantly, we stay below 30% cholesterol, to avoid the possibility of a nonbilayer phase that one may observe with closely related peptides (Figure S1). The bilayer preservation is an important consideration in light of the finding that some of the peptide helices change orientation in the presence of cholesterol (see below).

For the Arg residue in GWALP23-R14, it has been known that the guanidinium group remains positively charged between pH 4 and 9.[13,14] By using ether-linked DOPC lipids, we were able to examine the range between pH 9 and 13. The results at high pH reveal no significant changes in the ^{2}H quadrupolar splitting magnitudes for Ala methyl groups on the core helix over the extended pH range between pH 2 and 13 (Figure 4).

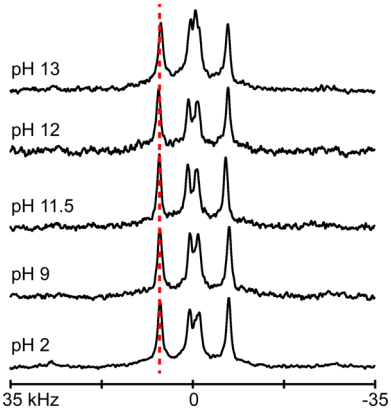

Figure 4. ^{2}H NMR spectra of labeled A15 (50% deuteration) and A17 (100% deuteration) of GWALP23-R14 in oriented DOPC ester- or ether-linked bilayers, hydrated with 10 mM buffer at the indicated pH values. Samples above pH 8.5 require ether-linked lipids. $\beta = 90°$ sample orientation; 1:60 (peptide:lipid); $T = 50$ °C.

As the positive charge on R14 has already been established at low pH, on the basis of the substantial change in helix orientation when R14 is introduced into the transmembrane helix of GWALP23,[13,14] the spectra in Figure 4 confirm that the R14 guanidinium group remains charged up to at least pH 13. Again, the helix orientation, reported by the ^{2}H NMR spectra, is extremely sensitive to the side-chain charge of residue 14, as

DOI: 10.1021/acs.biochem.6b00896
Biochemistry 2016, 55, 6337−6343

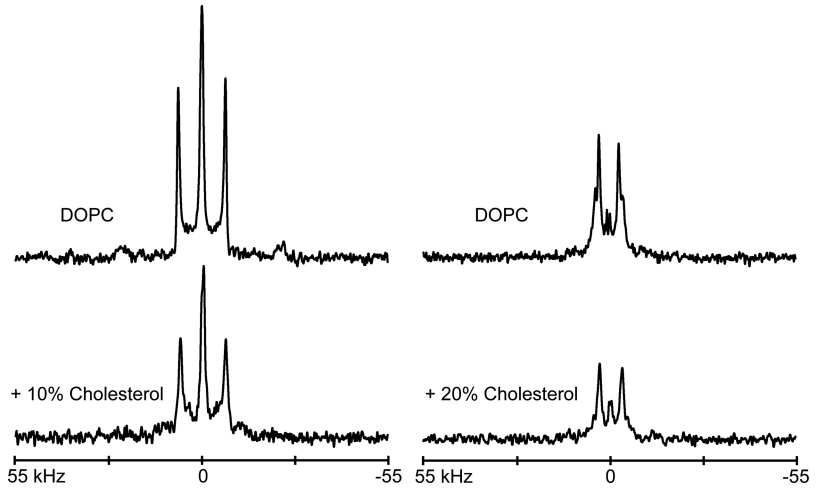

Figure 5. ^{2}H NMR spectra for labeled alanines in selected R14 peptides in aligned, hydrated, unbuffered DOPC bilayers with varying amounts of cholesterol. Spectra in the left column display A15, deuterated 50%, and A17, deuterated 100%. Spectra in the right column display A11, deuterated 50%, and A13, deuterated 100%. β = 90° sample orientation; 1:60 (peptide:lipid); T = 50 °C.

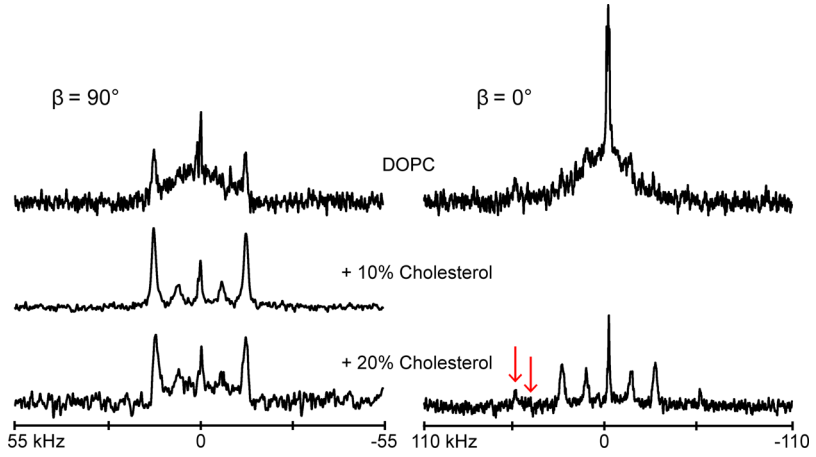

Figure 6. ^{2}H NMR spectra for labeled A7 (50% deuteration) and A9 (100% deuteration) in GWALP23-R12 in aligned, hydrated, unbuffered DOPC bilayers with varying amounts of cholesterol. Spectra are shown for β = 90° (left) and β = 0° (right) sample orientations; 1:60 (peptide:lipid); T = 50 °C.

seen by the large changes when K14 is titrated,[14] effectively serving as a control for the experiments presented here.

As an additional control, we also examined GWALP23-R12 at high pH. With its centrally located arginine, GWALP23-R12 occupies multiple states in DOPC bilayers at neutral pH.[13] When we examine samples of GWALP23-R12 in ether-linked DOPC bilayers at high pH, we find that the multistate behavior persists up to pH 13 (Figure S2). Because, once again, the system properties do not change, we conclude that R12 as well as R14 remains charged from neutral pH to at least pH 13.

Having established the retention of charge by R14 and R12, we sought to examine the possible influence of membrane cholesterol on the bilayer-incorporated GWALP23-R14 and GWALP23-R12 helices. The results observed for the two Arg-containing helices are strikingly different. With GWALP23-R14 in DOPC, one sees relatively minor spectral changes when the cholesterol content is changed from 0 to 20 mol % of total lipids (Figure 5), other than a reduction in the signal-to-noise ratio for the ^{2}H resonances. Importantly, the ^{2}H quadrupolar splittings that define the helix orientation do not change for the GWALP23 transmembrane helix when the cholesterol content is increased from 0 to 20%.

A very different picture emerges for the response of GWALP23-R12 to cholesterol. In the absence of cholesterol, it has been known[13] that GWALP23-R12 occupies multiple states in DOPC. At least three populated states are observed by ^{2}H NMR spectroscopy and coarse-grained molecular dynamics simulations. The simulations suggest that the major states consist of the Arg residue extending ∼30% of the time toward the N-terminus and ∼30% of the time toward the C-terminus or exiting the bilayer while pulling the entire helix to a surface orientation ∼40% of the time.

Remarkably, in the presence of 20% cholesterol in DOPC, the ^{2}H NMR spectra for labeled alanines in GWALP23-R12 become simplified, as multiple minor states merge into one predominant molecular orientation (Figure 6). The broad spectral feature and a number of minor ^{2}H peaks disappear, in favor of increasingly intense resonances corresponding to the major state as the cholesterol content is increased (Figure 6). Notably, some resonances from backbone Cα-D groups also become visible when 20% cholesterol is included in the bilayers (Figure 6).

What is the major membrane-bound orientation for the GWALP23-R12 helix in the presence of 20% cholesterol in

DOI: 10.1021/acs.biochem.6b00896
Biochemistry 2016, 55, 6337−6343

Table 1. Observed ^{2}H Quadrupolar Splitting Magnitudes (kilohertz) for Labeled Alanines in Aligned Samples of GWALP23 and Arg-Containing Derivatives in DOPC Bilayers[a]

				$\lvert\Delta\nu_q\rvert$ (kHz) for the indicated labeled Ala residue in sequence					
peptide	label type[b]	pH	cholesterol content[c]	A7	A9	A11	A13	A15	A17
GWALP23	CD_3	–	0 or 20%	16.6	1.7	16.7	1.5	15.4	2.6
GWALP23-R14	CD_3	2–13	0 or 20%	26.5	5.5	16	13.1	1.3	28.1
GWALP23-R12	CD_3	5.5	20%	27	56	–	–	21.5	29.3
GWALP23-R12	CαD	5.5	20%	95	111	–	–	–	–

[a]Oriented bilayer samples were prepared on glass plates with 10 mM buffer between pH 2 and 13 (see Methods). The data for GWALP23 (unbuffered) in DOPC are from ref 2. [b]Side-chain CD_3 or backbone CαD label on the indicated Ala residue, A7–A17. [c]Amount of cholesterol (mole percent of total lipid) in the DOPC bilayer. The results for GWALP23 and GWALP23-R14 do not depend upon the cholesterol content. GWALP23-R12 occupies multiple states in the absence of cholesterol.[13]

DOPC? We could address this question by analyzing the pattern of side-chain methyl and backbone Cα ^{2}H quadrupolar splitting magnitudes from labeled alanines in the core helix of GWALP23-R12 (Table 1). Indeed, the backbone Cα ^{2}H quadrupolar splitting magnitudes are essential for the analysis, as several possible helix orientations may appear to be feasible if only four side-chain CD_3 groups are considered. A unique answer emerges nevertheless from a combined analysis[6] that addresses not only the CD_3 but also the CαD quadrupolar splitting magnitudes. This answer reveals a surface-bound helix, oriented ("tilted") ~82° from the bilayer normal and described by the blue quadrupolar wave in Figure 7. Two methods for analyzing the helix dynamics yield similar results (Table S1). A semistatic "GALA" analysis[21] fits the quadrupolar wave with values of τ = 84°, ρ = 304°, and a principal order parameter S_{zz} of 0.9. A modified Gaussian analysis[23] fits the quadrupolar wave with values of τ = 81°, ρ = 301°, and $\sigma\rho$ = 0°, when $\sigma\tau$ is fixed at 5° so that there are only three adjustable parameters. The fitted value of ρ is referenced to an origin defined by van der Wel,[21] and the helix orientation defined by the fits of τ and ρ is illustrated in Figure 1B. The surface orientation for the helix (Figure 1B) matches the orientation observed for GWALP23-H12 at low pH.[24]

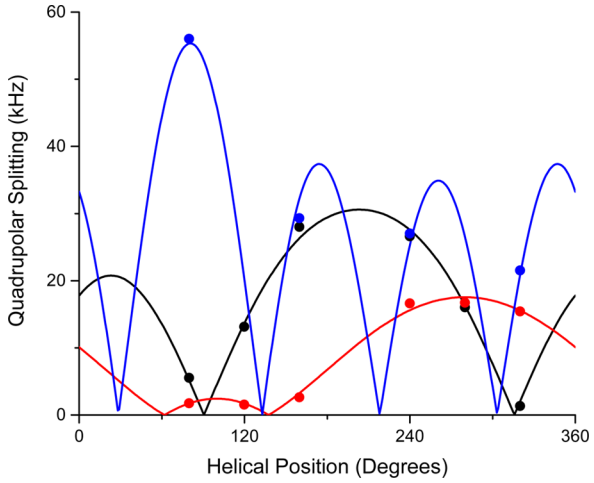

Figure 7. GALA quadrupolar wave results for tilted transmembrane peptides in lipid bilayers. Charged GWALP23-R14 (black; tilt τ = 15°, and rotation ρ = 247°) maintains the same orientation in DOPC bilayers regardless of cholesterol content (0–20%). Charged GWALP23-R12 (blue; tilt τ = 84°, rotation ρ = 304°, pH 5.5) is oriented at the surface of the DOPC bilayer when 20% cholesterol is present. This is a 78° change in helix tilt compared to GWALP23 (red; tilt τ = 6°, and rotation ρ = 323°).

DISCUSSION

Extensive theoretical and experimental investigations have converged to general consensus agreement that the side-chain guanidinium group of arginine remains positively charged in lipid bilayer membranes[13,25−29] and in the hydrophobic interior regions of soluble proteins.[30] For the membrane environment, lipid bilayer deformation can play a major role to help stabilize the positive charge.[13,27,31,32]

For the membrane systems, the pH range for experimental investigations usually has been limited to pH ≤9 because of the lability of the phospholipid esters under alkaline conditions.[13,14] In the work presented here, we employ ether-linked lipids and extend the range up to pH 13, at which still the Arg residues in the transmembrane helix of GWALP23-R14 and the multistate helix of GWALP23-12 remain charged. In this regard, it is notable furthermore that the aqueous pK_a for the Arg side chain has been revised upward to a value of 13.8.[33] Computational treatments, e.g., ref 27, have predicted only a small pK_a shift limited to ≤4.5 units for Arg in a lipid bilayer, yet here we observe that the shift is very much less than 4.5 units. Moreover, we affirm that the pK_a shift for Arg in a bilayer membrane is much smaller than the shift of ~4 units already observed for Lys at the same location on a transmembrane helix.[14] The combined experimental and computational insights reveal that the lysine side chain is influenced more by the lipid membrane environment than is the arginine side chain. For membrane protein function, moreover, it is clear that the Arg side chain will remain charged under all conditions whereas the Lys side chain is able to vary its protonation state within a relevant physiological range of, e.g., pH 6.5–8.0.

Cholesterol Effects. It has been predicted that the Arg pK_a value may depend on the presence of cholesterol in a lipid bilayer membrane.[15] Our results, nevertheless, indicate no change in the arginine pK_a for either GWALP23-R14 or GWALP23-R12 in DOPC with 20% cholesterol, but ejection of the more buried Arg residue from the DOPC bilayer membrane. Importantly, a level of 20 mol % cholesterol in DOPC, without other lipid components, is below the condition for any lipid phase separation,[34−36] and the bilayer phase is maintained (Figure 3). Indeed, the tilted transmembrane helix of GWALP-R14 shows no response when the membrane cholesterol content is changed from 0 to 20% (Figure 5). By contrast, GWALP23-R12 shows a major response to cholesterol.

In DOPC bilayer membranes, it has been known that GWALP23-R12, with its central Arg residue, populates multiple states.[13] The solid-state ^{2}H NMR spectra suggest at least three different states with approximately equal populations. Coarse-grained molecular dynamics simulations likewise describe three

DOI: 10.1021/acs.biochem.6b00896
Biochemistry 2016, 55, 6337−6343

major populated states. The simulations predict that the R12 residue either snorkels up, snorkels down, or exits the bilayer, with the respective probabilities being ~30, 30, and 40%.[13] How does the presence of 20% cholesterol influence the situation? The answer is that with 20% cholesterol a single major state predominates (80%), and this population consists of a surface-bound helix (Figure 1) in which the R12 residue indeed has exited the bilayer. The NMR spectra have been clarified from a multistate population in which individual states could not be assigned experimentally, to now a major surface-bound state that can be assigned in the presence of 20% cholesterol (Figure 7). The coarse-grained predictions[13] showed general agreement that one state from the multistate population is essentially the surface-bound state for the helix that is observed with 20% cholesterol in DOPC. How much cholesterol is required to favor the surface-bound state of GWALP23-R12? Indeed, Figure 6 suggests that the transition of the helix to the surface orientation may be essentially complete with as little as 10 mol % cholesterol in the DOPC bilayers. Further experiments could narrow more precisely the present range of 0−10% for the cholesterol dependence of the helix transfer.

It is of further interest that the azimuthal rotation that fits the surface orientation of GWALP23-R^+12 in DOPC with 20% cholesterol matches the orientation observed for GWALP23-H^+12 in DOPC at low pH without cholesterol.[24] In each case, the cationic H^+12 or R^+12 side chain and the aromatic W5 and W19 side chains, on the same face of the helix, align approximately parallel to the membrane surface, such that these side chains point neither into nor out of the membrane. As noted,[24] the rotational preference for GWALP23-R^+12 again appears to be a compromise among the aromatic Trp and charged Arg side chains atop a cholesterol-containing bilayer. The situation contrasts with the rotational preference observed for some amphipathic antimicrobial peptides, such as PGLa at a bilayer surface, with four lysine residues pointing away from the bilayer.[37]

In summary, the two major findings of this endeavor are that (1) Arg in a bilayer membrane exposed to lipids retains its positive charge up to pH 13 and (2) 20% cholesterol in DOPC drives a helix-attached Arg residue out of the bilayer to the membrane surface. Numerous previous simulations have suggested that membrane deformation serves to aid the hydration and stabilization of the positively charged arginine. It is tempting therefore to speculate that cholesterol may interfere with the balance of forces favoring exit of a helix from the DOPC bilayer as opposed to membrane deformation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00896.

NMR spectra and analysis of helix orientation (PDF)

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Funding

This work was supported in part by National Science Foundation MCB Grant 1327611 and by the Arkansas Biosciences Institute. The peptide, NMR, and mass spectrometry facilities were supported in part by National Institutes of Health Grants GM103429 and GM103450. J.K.T. was supported by grants from the Arkansas Department of Higher Education and the University of Arkansas Honors College.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Venkatesan Rajagopalan for helpful discussions and Bethany Doss for Figure S1.

■ ABBREVIATIONS

DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPC ether, 1,2-di-*O*-[9(*Z*)-octadecenyl]-*sn*-glycero-3-phosphocholine; Fmoc, fluorenylmethoxycarbonyl; GALA, geometric analysis of labeled alanines; GWALP23, acetyl-GGALW$(LA)_6$-LWLAGA-amide; TFA, trifluoroacetic acid

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DOI: 10.1021/acs.biochem.6b00896
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