THE JOURNAL OF PHYSICAL CHEMISTRY Letters

LETTER

pubs.acs.org/JPCL

Direct Measurement of the Membrane Dipole Field in Bicelles Using Vibrational Stark Effect Spectroscopy

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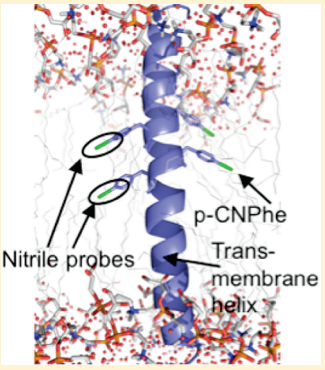
Supporting Information

ABSTRACT: Electrostatic fields in lipid bilayer membranes influence the structure and function of membrane-associated proteins. We present here the first direct measurement of the membrane dipole electrostatic field in lipid bicelles using vibrational Stark effect spectroscopy, in which a nitrile oscillator's vibrational frequency changes in response to its local electrostatic environment. We synthesized α-helical peptides containing the unnatural amino acid *p*-cyanophenylalanine (CN-Phe) at four locations along the helix. This peptide was intercalated into bicelles 5 and 15 nm in radius composed of mixtures of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). Changes in the vibrational absorption energy of the nitrile probe at each position along the helical axis were used to determine changes in the local electrostatic field of the probe. We measured the magnitude of the membrane dipole electrostatic field to be −6 MV/cm, changing rapidly near the membrane surface and more slowly in the low dielectric membrane interior.



SECTION: Biophysical Chemistry

Among the three distinct electrostatic potentials that are well known to be present in the lipid membrane bilayer (the transmembrane potential, surface potential, and dipole potential),[1,2] dipole potential is the least well characterized. Because it is located entirely within the membrane, it has been difficult to measure directly, and its influence on the structure, aggregation, and function of transmembrane and other membrane-associated proteins has not been studied as extensively as the transmembrane and surface electrostatic potentials. Despite this, a combination of indirect measurements such as ion transport rates,[3,4] nitroxide scanning,[5] fluorescence of voltage-sensitive dyes,[6,7] atomic force microscopy (AFM),[8] and other methods,[9−11] combined with computational approaches[12−14] has been used to estimate the magnitude of the dipole potential at a few hundred millivolts,[3,8] significantly larger than either the transmembrane (about 10−100 mV) or surface potentials (about 8−30 mV).[1] Because this dipole potential drops across a small distance of <4 nm within the low dielectric membrane interior, it generates a large electric field, typically 1−10 MV/cm compared with ∼0.25 MV/cm for the transmembrane field and ∼0.1 MV/cm for the surface field. This is believed to have a considerable influence on the structure and function of membrane-associated proteins.[2,3] Here we present a direct measurement of the membrane dipole field using vibrational Stark effect (VSE) spectroscopy in which changes in the absorption energy of a probe oscillator molecule are used as a local and directional probe of the electrostatic environment in which that oscillator is immersed.

The sensitivity of a vibrational oscillator to its local electrostatic environment is given by eq 1

$$\Delta E = hc\Delta\nu_{\mathrm{obsd}} = -\Delta\vec{\mu}\bullet\vec{F} \qquad (1)$$

where the transition energy, ΔE, of the absorption, measured as the vibrational frequency, $\Delta\nu_{\mathrm{obsd}}$, responds to the local electrostatic field, $\vec{F}$, through a sensitivity factor dependent on the difference dipole moment of the transition from ground to first excited vibrational state, $\Delta\vec{\mu}$, often called the Stark tuning rate. After the value of $\Delta\vec{\mu}$ has been calibrated independently in a known electrostatic field, the probe oscillator is inserted into the system of interest, in this case, the interior of lipid membrane bilayer, where measured changes in vibrational energy of the probe act as a local and sensitive reporter of its electrostatic environment. Several VSE probes have been demonstrated, including the nitrile group.[15] This probe is sensitive to its local field environment (large value of $\Delta\vec{\mu}$), possesses a vibrational transition in a clear region of the infrared spectrum, and is stable in aqueous and lipid environments.[16−19] Including this probe in the biological membrane will to some extent alter the electrostatic environment in the immediate vicinity of the nitrile, as has been previously observed in ion transport measurements across a membrane.[20−22] However, this effect, the extent of which is unknown, should be relatively consistent throughout the membrane

Received: May 31, 2011
Accepted: July 13, 2011

Table 1. Amino Acid Sequences of the Four Polypeptides Described in This Work[a]

peptide	sequence
αLAX(25)	HHGGPGLALALALALALALALAXGPGGHH
αLAX(23)	HHGGPGLALALALALALALAXALGPGGHH
αLAX(21)	HHGGPGLALALALALALAXALALGPGGHH
αLAX(16)	HHGGPGLALALALXLALALALGPGGHH

[a] X = CN-Phe.

structure, and so changes in dipole potential as a function of probe location are still a physically meaningful quantity.[23]

Here we use VSE spectroscopy to measure local electrostatic fields in the interior of bicelle model membranes. Bicelles are discoidal bilayer structures composed of long-chain lipids in the planar region and short-chain lipids or detergents in the rim area.[24] They have been demonstrated to be useful model membrane systems through their ability to support and maintain the functions of reconstituted membrane-associated proteins and have emerged as a practical tool to study the structure and function of membrane-associated proteins by both solution and solid-state NMR.[25] The most common lipid composition for assembling bicelles is a combination of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) as the long-chain lipid and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) as the short-chain lipid. Here we describe VSE spectroscopic measurements of a nitrile probe moved systematically through the interior of bicelles composed of DMPC/DHPC. Bicelles of two sizes, q = 0.5 and 3 (q is the molar ratio of DMPC/DHPC), were studied. The sizes of these two bicelles were estimated to be 5 and 15 nm in radius, respectively, based on a previously published model.[26] The nitrile probe was incorporated into the membrane bilayer by intercalating a transmembrane helix containing *p*-cyanophenylalanine (CN-Phe) as the VSE probe. The transmembrane helix was composed of the polypeptide HHGGPGA$_m$L$_n$XGPGGHH where $m + n = 18$ and X = CN-Phe. We call these peptides αLAX(r), where r is the position number of CN-Phe in the peptide sequence. The sequences of these peptides are given in Table 1.

This peptide has been shown to be a stable transmembrane helix both in vivo and in molecular dynamics (MD) simulations, and to be suitable for introducing any amino acid into the membrane environment.[27,28] The chosen helix length (28.5 Å) approximately matched the hydrophobic thickness of the DMPC/DHPC bicelles (23 Å, based on the number of carbon atoms in the acyl chain)[29] and along with the helix-disrupting GGPG sequence ensured the helix intercalated perpendicular to the plane of the bilayer. Two histidine residues at the N- and C-termini were added to increase the solubility of the hydrophobic helix in the bicelle-forming buffer. The CN-Phe residue was moved systematically from the C- to N-terminus of the peptide to step the nitrile group from the bilayer surface to its interior. In doing so, the VSE probe was exposed to the local electrostatic environment at specific locations throughout the membrane bilayer. Changes in the absorption energy of the nitrile as a function of its location on the α-helix were then related to changes in electrostatic field due to the membrane dipole electrostatic field.

The structure of the bicelles both with and without intercalated peptides was characterized by ^{31}P NMR. These studies demonstrated that our preparation protocol does yield a bicellar

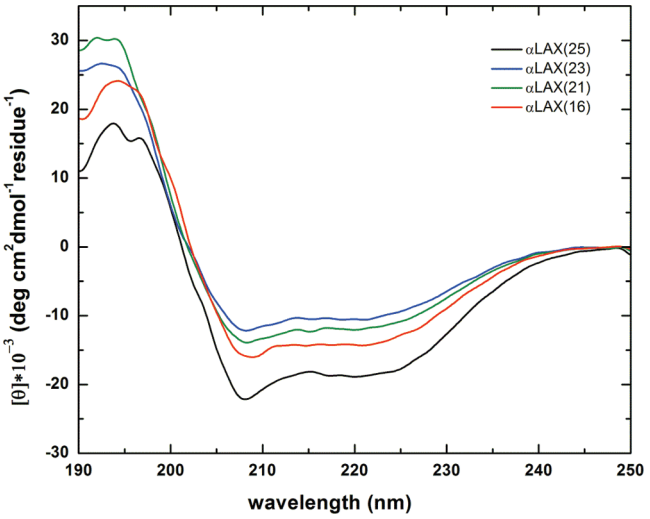


Figure 1. Representative circular dichroic spectra of peptides αLAX(25) (black), αLAX(23) (blue), αLAX(21) (green), and αLAX(16) (red) in q = 0.5 DMPC/DHPC bicelles, respectively. Spectral intensity is presented as mean molar ellipticity per residue.

structure that can be used as a mimic of the membrane bilayer structure for our purpose of measuring the dipole field in lipid membranes. (See the Supporting Information.) The secondary structure of the peptide in the bicelle was then investigated with circular dichroic (CD) spectroscopy to estimate the extent of α-helical character.

Figure 1 shows representative CD spectra of the four peptides αLAX(25), αLAX(23), αLAX(21), and αLAX(16) incorporated into bicelles of q = 0.5 composed of a DMPC/DHPC mixture. Peptides were insoluble in the buffer solution without the addition of the lipid mixture, indicating that the CD signal arose from the peptides incorporated into the formed bicelles. We observed two minima at 208 and 220 nm as well as a maximum at 195 nm, indicating a high fraction of α-helical character for all four of these peptides in the DMPC/DHPC bicelle samples.[30] The extent of helical character was estimated from the magnitude of the absorption at 222 nm[31] and was found to range from 32 to 56%. Given that the maximum possible helicity of these peptides is 60% (the helical alanine-leucine repeat sequence comprises 18 residues in each of these 30-mer peptides), the helical portion of these peptides remains intact when intercalated within the bicelle. We were unable to collect CD spectra on larger bicelles of q = 3 because the relatively high lipid concentrations required to form large bicelles caused substantial light scattering. Given the conclusion from NMR experiments that q = 0.5 and 3 bicelles have very similar discoidal shape and only differ in disk radius (Figure S1 of the Supporting Information), we assume that peptides intercalated in q = 3 bicelles should also be predominately helical.

CD spectroscopy has also been used to determine the extent of aggregation of α-helices in both buffer and biceller solutions based on the intensity ratio of the measured molar ellipticity at 220 and 208 nm.[30] To test the extent of any aggregation of α-helices incorporated into bicelles, we compared the CD spectra of two DMPC/DHPC bicelles in which the ratio of peptide/DMPC was increased by a factor of 2 from 1:100 to 1:50. Whereas both spectra demonstrated strong helical character,

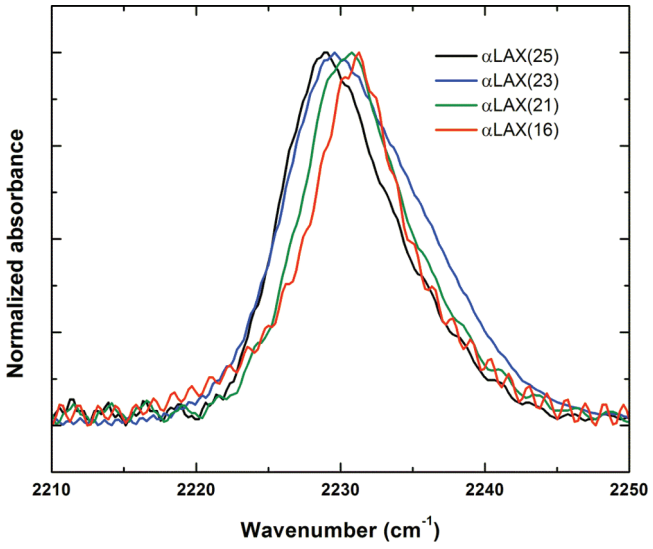


Figure 2. Representative normalized FTIR spectra for four peptides in q = 0.5 DMPC/DHPC bicelles collected at room temperature. The absorption spectra of each peptide are ordered from low to high energy as αLAX(25) (black), αLAX(23) (blue), αLAX(21) (green), and αLAX(16) (red), respectively.

they displayed different intensities in molar ellipticity (θ) at 220 and 208 nm; the ratio of $\theta_{220}/\theta_{208}$ was ∼0.85 for 1:100 samples and 1 for 1:50 samples. Previous reports have concluded that a ratio of 0.85 suggests that most of the peptide helix is monomeric, whereas ratio of 1 suggests that helix association or aggregation has occurred. Based on the stoichiometry of the peptide-to-lipid ratio in the preparation solution, we expect that in bicelles of q = 0.5 there is on average 0.8 peptide per bicelle for 1:100 samples. Using a Poisson distribution, this means that ∼45% of the bicelle population contains no peptides, 36% contains one peptide, and only ∼14% of the population contains two or more peptides. The measured vibrational energy is therefore dominated by bicelles containing only one peptide, and there is minimal risk that the measured vibrational frequency is a convolution of electrostatic and aggregation effects. For bicelles prepared from a peptide/DMPC ratio of 1:50 (i.e., twice as concentrated in peptide), self-assembly stoichiometry indicates that there are 1.6 peptides per bicelle, assuming a homogeneous distribution of peptides throughout all available bicelles.[32] However, Poisson statistics indicate that there is a wider distribution of possible peptide/bicelle ratios in this scenario, with the probability that bicelles contain zero, one, two, or more than two peptides all greater than 20%. All spectra reported here are from solutions formed from a ratio of 1:100 peptide/DMPC and thus predominately on bicelles containing a single peptide to avoid convoluting factors in the vibrational absorption of the nitrile caused by aggregation of multiple peptides in the same bicelle. However, controlled aggregation of helices in the bicelle will be a powerful experimental tool for future studies investigating the function of electrostatic fields in causing the association and aggregation of membrane-associated peptides and proteins.

Representative vibrational absorption spectra of the nitrile probe on the four α-helices studied here intercalated in bicelles composed of DMPC/DHPC lipids of size q = 0.5 at room temperature are shown in Figure 2. Complete frequency data for peptides intercalated in both q = 0.5 and 3 bicelles over a range of temperatures are reported in Table S2 of the Supporting Information. As the nitrile probe was moved from αLAX(25) to αLAX(16) and thus immersed deeper into the bilayer, the absorption energy of the nitrile probe increased by 2.3 cm^{-1}. For q = 3 DMPC/DHPC bicelles, we measured slightly different vibrational energies of nitrile probe (e.g., the nitrile on αLAX-(23) intercalated in q = 3 bicelles absorbed at a frequency 0.7 cm^{-1} lower in energy than in the q = 0.5 bicelles), but the pattern of increasing absorption energy as the probe was inserted deeper into the membrane was identical. Furthermore, we observed a systematic decrease in the full width at half-maximum (fwhm) of the absorption peaks as the nitrile probe was moved toward the center of the membrane. The fwhm of the nitrile absorption peaks of peptides intercalated in DMPC/DHPC bicelles of q = 0.5 were 10.7 ± 0.5, 11.4 ± 0.2, 9.2 ± 0.2, and 6.9 ± 0.7 cm^{-1} for αLAX(25), αLAX(23), αLAX(21), and αLAX(16), respectively. The relative magnitude of the fwhm of the absorption peak has been proposed as a measurement of the homogeneity of the local chemical environment in the immediate vicinity of the probe.[17] When the peptide is immersed in the hydrophobic lipid bilayer, only the probes on αLAX(25) and αLAX(23), which are closest to the membrane surface, are possibly interacting with the lipid headgroup or water molecules immersed in the membrane. The probes on the other two peptides, αLAX(21) and αLAX(16), are located beyond the point of possible penetration by water molecules and thus are only exposed to the aliphatic lipid tail, seen as the systematic reduction of the fwhm.[33] The effect of complicating factors on the vibrational absorption energy of the nitrile bond, such as hydrogen bonding or interactions with water, is therefore negligible, and the observed spectral shifts are primarily due to a Stark effect based on electrostatic fields within the membrane bilayer.

For interpretation of how these changes in absorption energy are related to the electrostatic field created by the membrane dipole potential, it is necessary to know both the distance between the nitrile positions at each of the four locations that CN-Phe was placed on the α-helix and the orientation of the nitrile with respect to the membrane normal. To this end, models of each of the four peptides studied here were made with Avogadro 1.00, an open-source molecular builder and visualization tool.[34] After energy minimization, the atomic coordinates of the peptides were input into Mathematica 7 for measurements. The axis of the peptide was determined by numerically fitting a line through the center of the peptide backbone. The distances between adjacent nitrile probes were determined from the distance between the nitrogen atoms of the nitriles projected onto the peptide axis. The axis-projected distance between the two nitrile probes on αLAX(25) to αLAX(23) was 1.8 Å, from αLAX(23) to αLAX(21) was 3.1 Å, and from αLAX(21) to αLAX(16) was 8.3 Å for a total distance from the nitrile probe on αLAX(25) to αLAX(16) of 13.2 Å. The angle of the nitrile with respect to the helical axis (and thus presumably the membrane normal) was determined by the angle between the peptide axis (defined above) and the line defined by the carbon and nitrogen atom pair of the nitrile bond on CN-Phe. The angle between these two lines was found to be 57 ± 4°; the error is the standard deviation of the nitriles on the four helices measured here and shows little dependence on the location of CN-Phe along the helix, which agrees well with a previous study.[15] With the total frequency change caused by moving the nitrile probe from αLAX(25) to αLAX(16) of 2.3 cm^{-1}, using these distance, angle, and vibrational energy measurements and a value of Stark

tuning rate of 0.67 cm^{-1}/(MV/cm) in eq 1,[35] we estimate an electrostatic field perpendicular to the plane of the membrane and extending from the intermediate region near the lipid headgroup to the hydrophobic center of the bilayer to be approximately −6 MV/cm.

The magnitude of this field is well within the range of the previous experimental estimates of dipole electrostatic fields within lipid membranes, measured by other diverse experimental methods in lipid bilayers and vesicles.[5,6] The incremental frequency changes as the position of the nitrile probe was moved through the biceller structure were measured to be 0.7, 1.1, and 0.5 cm^{-1} as the chromophore moved from αLAX(25) to αLAX(23), from αLAX(23) to αLAX(21), and finally from αLAX(21) to αLAX(16). Using eq 1 for these incremental frequency steps, the electrostatic field from αLAX(25) to αLAX-(23) is −1.91 MV/cm, from αLAX(23) to αLAX(21) is −3.01 MV/cm, and from αLAX(21) to αLAX(16) is −1.38 MV/cm. Because the distance the nitrile moves between αLAX(25) and αLAX(23) (1.8 Å) is much shorter than the distance from αLAX(21) to αLAX(16) (8.3 Å), this demonstrates that the electrostatic field falls off rapidly near the membrane surface and more slowly in the low dielectric alkane interior. Although there is a limited number of lipids that have demonstrated stable bicellar structures beyond the one studied here, we are extending this investigation to lipids that may indeed demonstrate large difference in membrane dipole field than those measured here. We are also investigating the role that small molecules that intercalate in the bilayer play in modulating the magnitude of the dipole field. Preliminary data on the effct of the incorporation of the phloretin molecule into DMPC/DHPC bicelles is described in the Supporting Information; a more extensive investigation will be reported in further publications.

In these experiments, we have assumed that the peptide is positioned parallel to the membrane normal. The measured value of the dipole field could be affected by hydrophobic mismatch between the hydrophobic thickness of the lipid bilayer and the hydrophobic stretch of the transmembrane helical peptide, which could cause the helical axis to tilt with respect to the bilayer normal.[29] Given the hydrophobic thickness of DMPC/DHPC bicelle of 23 Å and helical stretch of 28.5 Å, a tilt angle as large as 35° is possible. This would cause an error of ∼2.5 MV/cm in the estimated dipole field. Thus, even this error would result in a dipole field that is in the range of 1−10 MV/cm that has been previously estimated from indirect measurements. As a further test, we prepared and measured bicelles of $q = 0.5$ prepared with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as the long-chain lipid and intercalated with the 4 CN-Phe-containing peptides. This slightly longer lipid has a hydrophobic thickness of 26.5 Å, but the measured vibrational frequencies of the nitrile probes in these bicelles were essentially identical to those measured in bicelles composed of DMPC. We therefore conclude that possible hydrophobic mismatch in this system is not substantially affecting our results. MD simulations are currently underway to test this issue further and will be reported in a later publication.

The structured α-helix carries a helix-long dipole moment from the negatively charged C-terminus to the positively charged N-terminus of approximately 3.5 D per amino acid residue and oriented parallel to the helical axis.[36−38] The experiments reported here are not able to distinguish the effects of the electrostatic field created by this helix dipole moment from the surrounding lipid medium. The magnitude of the electrostatic field created by the helix is proportional to the length of the helix up to ∼7 residues, after which additional residues have only a marginal effect on increasing the field.[36] Because of this, we placed our VSE probe near the C-terminal end of the peptide, no closer than nine amino acids from the N-terminal end of the α-helix. In a peptide of this size, the magnitude of the field at the positions of the probe molecules is estimated to be ∼4 MV/cm.[37] Therefore, any electrostatic field due directly to the helical dipole should be constant for each of the four probe locations discussed here, and any change in field experienced by moving the probe should be due entirely to the dipole potential supported by the membrane bilayer. Future experiments will explore the interaction between the N-terminal helical field and the membrane dipole field through VSE probes that are near the N-terminus of the peptide.

In conclusion, the membrane dipole field is often cited as being important in membrane organization and the function of membrane-associated proteins. A variety of experimental methods have identified the magnitude of the membrane dipole field between 1 and 10 MV/cm, which we have verified with VSE spectroscopy in bicelles composed of DMPC and DHPC. This direct measurement uses a diatomic spectroscopic probe that can be placed synthetically on a variety of transmembrane components, including the lipids or peptide elements interacting with the membrane interior, and that is significantly smaller than molecular probes used in alternative techniques such as nitroxide scanning or incorporation of voltage-sensitive fluorescent dyes. This therefore raises the possibility of measuring electrostatic fields in the membrane interior in the presence of transmembrane elements with an appropriately placed small VSE probe. In combination with alternative techniques to estimate the membrane dipole field discussed above, this technique offers the opportunity for a new suite of experimental investigations for determining the molecular origin of the dipole field and the mechanism by which it may influence the lateral organization of biological molecules in membranes of increasingly complex composition.

EXPERIMENTAL METHODS

The lipids were purchased as dry powders from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. D_2O was purchased from Cambridge Isotope Laboratories (Andover, MA). $PrCl_3 \cdot 6H_2O$ (99.9%), THF, and NaN_3 were purchased from Sigma Aldrich (Milwaukee, WI). Fmoc-*p*-cyanophenylalanine was obtained from PepTech (Burlington, MA). HPLC-grade water was used to prepare all aqueous solutions. Four peptides, αLAX(25), αLAX(23), αLAX(21), and αLAX-(16) (Table 1) were synthesized using standard Fmoc solid-state peptide synthesis. Bicelle samples were prepared following previous procedures.[39] ^{31}P NMR experiments were carried out to characterize the bicellar structure at 202.349 MHz on a Varian INOVA 500 spectrometer. CD spectra were measured on JASCO J-815 CD spectrometer in a 1 mm cell under instrument conditions of 0.2 nm resolution, 50 nm/min scanning rate, and 1 s response time to verify the helical conformation of peptides. Infrared spectra were recorded on a Bruker Vertex 70 Fourier transform infrared spectrometer with a liquid N_2-cooled indium antinomide (InSb) detector. The spectra were averaged from 250 scans collected at 0.5 cm^{-1} resolution, and the baseline of the background-subtracted spectra was corrected using an interactive polynomial option in the OPUS software package from Bruker.

■ ASSOCIATED CONTENT

Supporting Information. ^{31}P NMR characterization of the bicelles, temperature dependence of FTIR spectra, further results of the effect of peptide aggregation on FTIR spectra, and the effect of intercalation of phloretin in bicelles. This material is available free of charge via the Internet at the http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This work was supported by the Burroughs Wellcome Fund (1007207.01). L.J.W. holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund. We thank Ms. Michelle Gadush at the Institute for Cell and Molecular Biology at The University of Texas at Austin for peptide synthesis and Dr. Ignacio Gallardo for assistance in constructing and measuring model peptides.

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