2H NMR Studies of a Myristoylated Peptide in Neutral and Acidic Phospholipid Bicelles†

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ABSTRACT: Deuterium NMR spectra of Myr-d27-GNAAAAKGSEQES (Cat14), the N-terminal 14-residue peptide from the catalytic subunit of cAMP-dependent protein kinase A (PKA), illustrate how magnetically aligned neutral and acidic phospholipid bicelles can be used to characterize the ordering and mode of binding of peptides to membranes. Since Cat14 is electrically neutral, the major interaction responsible for binding is the insertion of the myristoyl group into the hydrophobic core of the bilayer. The inclusion of 25% phosphatidylserine or phosphatidylglycerol into phosphatidylcholine bicelles results in a moderate increase in the ordering of the peptide relative to the bicelle normal, presumably because of favorable electrostatic interactions between the phospholipid headgroups and the two lysines in positions 7 and 8. Successful preparation of acidic bicelles was achieved by careful adjustment of lipid composition, pH and ionic strength.

Since the first identification in 1982 (1) of an N-terminal myristic acid/glycine amide linkage in the catalytic subunit (C) of cAMP-dependent protein kinase A (PKA), this blocking group has been found in numerous eukaryotic and viral proteins (2). In most cases, such proteins are targeted toward cellular membranes, and the myristoyl chain has been recognized as the entity that serves as a transient anchor of a protein to either the membrane itself or, perhaps, to a hydrophobic pocket or patch on a membrane-bound protein. The association of myristoylated peptides with phospholipid bilayers has been documented frequently during the past decade (3, 4), and the binding energy has been found to agree with Tanford’s estimate (5) of 3.5 kJ/mol per alkyl chain carbon (6). Still, a precise description of the conformation and the orientation of the peptide myristoyl group is missing, and such information is needed for assessment of the configurational entropy of the myristoyl group. The 2H NMR data presented here for a magnetically aligned bicellar solution of the N-terminal 14-residue peptide Myr-d27-GNAAAAKGSEQES from C demonstrate how such information can be obtained, and simultaneously illustrate the utility of neutral and acidic phospholipid bicelles in studies of protein–membrane interactions.

N-Terminal myristoylation is found in a variety of eukaryotic proteins whose function in the signal transduction cascade relies on the simultaneous localization of several proteins near the inner surface of the cell membrane. Examples include the myristoylated alanine-rich C kinase substrate (MARCKS) (7) and the α-subunit of G-proteins (8), both of which are ubiquitous in eukaryotes. Other eukaryotic signal-processing proteins that make use of a myristoyl group as a temporary hydrophobic anchor are recovering in retinal rod cells (9) and several homologous neuronal calcium sensors, in which Ca2+-ion binding triggers the translocation of the protein to the membrane. N-terminal myristoylation is also used by membrane-binding viral proteins, such as the transforming protein of Rous sarcoma virus, and the Gag proteins of retroviruses, as well as in several products of the nef gene in human immunodeficiency virus type 1 (10, 11).

A common property of myristoylated signal-transduction proteins is that they cycle between the cytosol and the membrane. During the cytosolic phase, the myristoyl group rests in a hydrophobic pocket on the protein surface (9, 12), but given the appropriate signal, the myristoyl group is released and made available for binding to an external hydrophobic entity. Its task completed, the protein is released from the membrane, perhaps via phosphorylation of a strategically placed serine residue. As discussed, in particular, by Resh, McLaughlin, and their co-workers (2, 4, 6, 13, 14), the myristoyl group is not by itself sufficient to firmly anchor a protein to the membrane. Instead, three structural motifs may combine to ensure the binding of
proteins to phospholipid bilayers: (1) an N-terminal fatty acid chain, (2) two or more lysines or arginines, and (3) an amphiphilic helical segment. Interference with any one of these binding motifs weakens the interaction and may cause the release of the protein from the membrane.

As a first step in an NMR study of the interaction between myristoylated polypeptides and membranes, we have investigated the behavior of Myr-d_{17}-GNAAAAKGSEQES (Cat14), the first 14 residues at the N-terminus of the catalytic subunit of PKA. The dormant form of PKA is a tetrameric complex that consists of two regulatory (inhibitory) subunits and two catalytic subunits. Activation is initiated by two molecules of cAMP binding to each of the regulatory subunits, which unleashes the active C subunit. In the X-ray structure of the mammalian C-subunit (12), the myristoyl group is tucked into a hydrophobic pocket next to the amphiphilic A helix (15–32), and the comparison of this structure with that of a recombinant enzyme missing the myristoyl group (13) suggests that the myristoyl group serves to stabilize the overall fold of the C subunit. The myristoyl group may also participate in the signal transduction process, by being released and interacting with either the inner leaflet of the plasma membrane or with a specific membrane receptor. If so, the A helix may be involved in the membrane binding, and the two lysines in positions 7 and 8 may contribute to the stabilization of the complex as well.

As an essential part of testing such hypotheses, we have developed a reliable protocol for the preparation of acidic phospholipid bicelles that contain phosphatidylserine or phosphatidylglycerol in addition to the phosphatidylcholines normally used for bicellar samples (16). The new bicelles are negatively charged, and they are typically stable over a narrower range of temperature, pH, and ionic strength than neutral bicelles. As shown here, however, well-aligned samples for use in NMR spectroscopy can be prepared, and a full description of the preparation protocol is forthcoming. With these new membrane mimetics in hand, we were able to show conclusively that Cat14 binds to the phospholipid bilayer by insertion of the myristoyl group and that the binding is enhanced by the addition of acidic phospholipids to the bilayered section of the bicelles.

**MATERIALS AND METHODS**

**Peptide.** The 14-residue peptide, Cat14, was synthesized using FMOC chemistry on a PerSeptive Biosystems solid-state peptide synthesizer following a procedure described elsewhere (17). The free carboxyl group of myristic-d_{27} acid (CDN) was coupled to the amino terminus by a standard peptide synthetic scheme using HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as the activator in the coupling reaction. The peptide was purified by HPLC (Waters) on a C18 column with a 0.1% TFA–acetoniitrile gradient, and its identity and purity were confirmed with electrospray mass spectrometry.

**Sample Preparation.** All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. A pH 5.5 20% (w/w) stock solution of the extremely hygroscopic DHPC was prepared first in a drybox from deuterium-depleted water (Cambridge Isotopes, Cambridge, MA) containing 50 mM MES buffer. Weighed amounts of the long-chain lipids, peptide, and water were mixed and vortexed to obtain a homogeneous slurry. Then, the DHPC solution was added, and a good mixing was obtained by vortexing the solution at below 20 °C, where the viscosity of the solutions is similar to that of pure water. The total peptide concentration was 6 mM, with 180 mM long-chain phospholipids. Sufficient 4 M KCl (buffered with MES to pH = 5.5 and stored over Chelex Resin (Biorad)) was then added to reach a final salt concentration of 0.1 M. NMR samples were prepared from 200 µL of these (transparent) solutions in short 5 mm sample tubes and sealed tightly. Following insertion in the magnetic field and before any data acquisition, the samples were kept at 38 °C for 1 h to allow equilibrium alignment to be established. When not in use, the samples were stored at −20 °C.

**NMR Measurements.** Deuterium quadrupole echo NMR spectra were recorded at 55.3 MHz and 38 °C on a Chemagnetics CMX-250/360 NMR spectrometer equipped with an ENI LPI-10 rf amplifier and an 8.5 T Oxford Instruments wide-bore magnet with a homogeneity of 50–60 Hz. The standard π/2 − τ − π/2 − τ − acq quadrupole echo sequence with τ = 50 µs, 2.1 µs π/2 pulses, and a repetition time of 1 s was used. 4 K data points were acquired from the quadrupole echoes, fractionally left-shifted to start the record at the top of the echo, baseline corrected, zero-filled to 16 K points, and multiplied with an exponential apodization function of 50 Hz prior to Fourier transformation. The probe temperature was maintained by a LakeShore 91C controller, and the temperature stability and gradient across the sample were both better than 0.1 °C. Because only a small fraction of the DMPC was chain-perdeuterated, 2000 and 64 000 scans were required for the peptide-free and peptide-containing samples, respectively, to ensure an adequate signal-to-noise ratio.

**RESULTS AND DISCUSSION**

The deuterium quadrupole echo spectra of chain-perdeuterated DMPC-d_{54} in magnetically ordered neutral and acidic bicelles were obtained at 38 °C and pH 5.5 from samples containing 20% (w/w) total lipid and 0.1 M potassium chloride. The bicelles were prepared with long-chain and short-chain phospholipid molecules in a molar ratio of q = [long]/[short] = 3.5 from DHPC with DMPC (Figure 1, C) or from DHPC with a 3:1 mixture of either DMPC and DMPS (Figure 1, C + S) or DMPC and DMPG (Figure 1, C + G). The spectra shown in Figure 1 are typical of chain-perdeuterated oriented phospholipid bilayers (18) and quite similar to those published previously for DMPC/DHPC bicelles (16). Analysis of the DMPC-d_{54} spectra also shows that the quadrupolar splittings of essentially all CD_{2} and CD_{3} groups increase slightly in the order C < C + S < C + G. These trends reflect slight changes in chain order induced by the different sizes and electrostatic properties of the phospholipid headgroups. NMR spectra (not shown) were also obtained for bicellar solutions without salt, and the effect of adding KCl to the bicellar solutions is to reduce the largest quadrupolar splittings by 0.5–1.0 kHz, a small but reproducible amount. These observations will be discussed in more detail in a future publication.

In the absence of special paramagnetic agents (19), phospholipid bicelles orient with their normals perpendicular to the magnetic field. At 38 °C, the morphology of the
samples is discotic nematic, and the phase and molecular symmetry is effectively uniaxial by virtue of rapid molecular and bicellar reorientation. Under these conditions, the observed quadrupolar splitting for a methylene deuteron in an alkyl chain may be expressed (20) as

$$\Delta = \Delta_p S_{ln} S_{nm} S_{mp}$$  (1)

where $\Delta_p = \frac{1}{2} Q \alpha^2 Q$ represents an orientational order parameter linking the coordinate axis systems $i$ and $j$ to one another. Thus we need (1) the average orientation of the bicellar normal with respect to the magnetic field in the lab frame, $\beta_{ln}$; (2) the angle, $\beta_{nm}$, between the average bilayer normal, $\hat{n}$, and the instantaneous, or local, bilayer normal, $\hat{n}$; (3) the orientation $\beta_{nm}$ of a molecular axis $m$ with respect to $n$; and (4) the local angle $\beta_{mp}$, between the molecular axis and the principal axis $p$ of the quadrupolar tensor along the CD bond. If we choose the molecular axis as the normal to the plane of the CD$_2$ group, $\beta_{mp} = 90^\circ$.

Using a (vibrationally averaged) quadrupole coupling constant $Q = 168$ kHz, we then find that the observed splitting (in kHz) $\Delta = 63 S_{ln} S_{nm}$, where $S_{ln} = S_{nm} S_{ln}$ defines an internal order parameter. Observed values of $\Delta$ for the “plateau region” (P) of the lipid chains (L), i.e., the similarly ordered methylene groups closest to the phospholipid head-group, are listed as $\Delta_{LP}$ in the third column in Table 1. The internal order parameter, $S_{ln}$, is seen to be approximately 0.32 for all three bicellar samples, which is slightly less than the internal order parameter $S_{nm} = 0.39$ observed for multilamellar hydrated phospholipid bilayers without surfactant. Conversely, if we assume that the extents of phospholipid motion in bicelles and multilamellar bilayers are the same, we find that the bicellar order, expressed by $S_{ln}$, is reduced by 15--20% relative to that in pure DMPC bilayers. We ascribe this decrease to bicellar “wobble”, defined more precisely as “diffusion-in-a-cone” (21, 22) of the bicelle normal. Using the cone model, for which $S_{ln} = \frac{1}{2} \cos \beta_0 (1 + \cos \beta_0)$, the reduction in order corresponds to a cone half-angle $\beta_0 = 28$--32° for the $q = 3.5$ bicelles with diameter $= 500 \text{ Å}$ (23) used in this study. The bicellar wobble is presumably cooperative in nature, given the spatial constraints imposed by neighboring bicelles (24), and the wobble amplitude calculated from the cone model is safely below the “critical” angle of 45° where the magnetic torque is at its maximum.

Inspection of the three spectra shown in Figure 1 reveals subtle differences in spectral resolution among the bicellar samples, although the degree of order is very similar. We believe that this variation in spectral quality mainly reflects the lack of consistency or reproducibility in bicelle preparation. Any variation in the concentration of trace impurities, perhaps associated with minor amounts of phospholipid hydrolysis at high or low pH, shows up in the quality of the NMR spectra. Also, the extent of sample handling, variations in temperature during mixing, and the duration of annealing affect the spectroscopic properties of the sample. The addition of peptidic solutes as described below certainly affects the approach to equilibrium alignment, if not the final degree of order. It should be pointed out that acidic bicelles, in particular, require careful handling, because they appear to be more sensitive to chemical degradation, perhaps hydrolysis. Thus, storage at $-20^\circ \text{C}$ is mandatory when measurements are not in progress. Basically, we found the DMPS- and DMPG-containing bicelles to be stable for extended periods (weeks) in the pH range 5.5--7.0, but below pH 5.5 the sample alignment deteriorated irreversibly in a few days. We did not explore conditions above pH 7.

Three bicellar samples containing Cat14 with a perdeuterated myristoyl anchor were prepared with a 0.033 molar ratio of peptide to long-chain phospholipid. The three samples were, except for the added Cat14, formally identical to those described above, and their deuterium quadrupole echo spectra are presented in Figure 2. The spectra drawn with thin lines represent samples in which only the peptide myristoyl group was deuterated. After these spectra were recorded, a small amount of $q = 3.5$ bicellar solution containing perdeuterated DMPC was added to the samples, and the resulting spectra are plotted with thick lines. The quadrupolar splittings $\Delta_{pm}$ for the terminal methyl group and
\[ \Delta_{PP} \text{ for the plateau CD}_{2} \text{ groups of the peptide myristoyl chain are listed in columns 6 and 7 and are compared with the corresponding splittings } \Delta_{LM} \text{ and } \Delta_{LP} \text{ of the DMPC myristoyl chain (columns 8 and 9) in Table 1.} \]

The following major features characterize the \(^2\text{H}\) spectra of Cat14 containing bicelles: (1) The peptide myristoyl chain is macroscopically ordered in bicellar solution, but the plateau splittings from the CD\(_2\) groups closest to the glycine are smaller than the phospholipid plateau splittings by factors of 0.64, 0.69 and 0.75 for the peptide in neutral, DMPS- containing, and DMPG-containing samples, respectively. (2) The \(^2\text{H}\) quadrupolar splittings \(\Delta_{PM}\) associated with the terminal Cat14 methyl groups are, however, reduced by only 3\% from the DMPC methyl splittings, \(\Delta_{LM}\), in the acidic bicelles and by only 10\% from the DMPC methyl splittings in neutral bicelles. (3) Both methyl and methylene group splittings of Cat14 are larger in the bicelles containing 25\% acidic phospholipid than in the neutral bicelles. (4) The plateau splittings observed for the DMPC (thick lines) are not reduced significantly by the addition of peptide to the DMPG- and DMPS-containing samples and by only \(\approx 10\%\) for the neutral bicelles, but the overall quality of the spectra is lower than that of the spectra of the peptide-free bicellar solutions in Figure 1. (5) An isotropic peak (actually an unresolved doublet), which arises from a small amount of HOD from exchangeable peptide protons, appears near the center of all three spectra.

The simplest and most obvious explanation for the different reduction factors for deuterons along the myristic acid chain of the peptide is that the chain, on average, is bent or kinked in the bilayer. The terminal CD\(_3\) and CD\(_2\) splittings are as well ordered as those observed for the phospholipid, suggesting that the average orientation of this part of the chain is parallel to that of the phospholipid molecules. Meanwhile, the plateau region of the myristoyl chain is either kinked at an angle of 24\(\text{°}\) to 29\(\text{°}\) relative to the rest of the chain or undergoing librational motion over the relatively large cone angle of 35\(\text{°}\) to 42\(\text{°}\) relative to the average orientation of the phospholipid molecules. At present, we cannot distinguish between these two possibilities.

**CONCLUSIONS**

The \(^2\text{H}\) NMR spectra presented here illustrate the practical utility of neutral DMPC/DHPC bicelles and their acidic analogues in studies of peptide association with bilayers. DMPC/DHPC bicelles containing up to 25\% DMPS or DMPG align well in the magnetic field at 20\% (w/w) total lipid concentration, and the bicellar solutions are stable for extended periods of time at 35\(\text{°}\) to 40\(\text{°}\) C and pH 5.5–7.0, provided the samples are stored at \(-20\text{°}\) C when not actually in use.

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**Table 1: Myristoyl Chain \(^2\text{H}\) Quadrupolar Splittings of Cat14-\(d_{27}\) and DMPC-\(d_{54}\) in 0.1 M KCl Bicellar Solutions at 38 °C and pH 5.5**

<table>
<thead>
<tr>
<th>sample</th>
<th>methyl (\Delta_{LM}) (kHz)</th>
<th>plateau (\Delta_{LP}) (kHz)</th>
<th>methyl (\Delta_{PM}) (kHz)</th>
<th>plateau (\Delta_{PP}) (kHz)</th>
<th>methyl (\Delta_{PM}/\Delta_{LM})</th>
<th>plateau (\Delta_{PP}/\Delta_{LP})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C + G</td>
<td>2.66</td>
<td>20.1</td>
<td>2.81</td>
<td>20.2</td>
<td>2.72</td>
<td>15.2</td>
</tr>
<tr>
<td>C + G</td>
<td>2.53</td>
<td>19.1</td>
<td>2.62</td>
<td>19.4</td>
<td>2.53</td>
<td>13.4</td>
</tr>
<tr>
<td>C</td>
<td>2.51</td>
<td>18.7</td>
<td>2.44</td>
<td>17.7</td>
<td>2.20</td>
<td>11.4</td>
</tr>
</tbody>
</table>

*The quadrupolar splittings were obtained from the NMR spectra in Figures 1 and 2. “Plateau splitting” refers to the separation between the major, outermost doublet that belongs to the CD\(_2\) groups closest to the carboxyl group.*

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**FIGURE 2:** Deuterium quadrupole echo NMR spectra of the N-terminal 14-residue peptide Myr-\(d_{27}\)-GNAAAAKKGSEQES (Cat14) of the catalytic subunit of cAMP-dependent protein kinase A incorporated into the bilayer of \(q = 3.5\) phospholipid bicelles in a 20\% (w/w) solution. The peptide concentration is 6 mM, and the long-chain phospholipid concentration is approximately 180 mM. Spectra of bicellar samples with only the peptide myristoyl chain deuterated are presented as thin lines, whereas the thicker line spectra were obtained following the addition of a small amount of bicellar solution prepared with chain-perdeuterated DMPC-\(d_{54}\). The “extra” resonance near the center of the spectra arises from HOD generated by protiated amide and ammonium groups in Cat14.
The NMR data obtained for Cat14, the N-terminal myristoylated 14-mer from the catalytic subunit of protein kinase A, confirms the notion that such peptides bind via insertion of the myristoyl group into the hydrophobic core of the bilayer. On the basis of the magnitude of observed quadrupolar splittings, we conclude that the chain termini are highly ordered, i.e., as well ordered as the termini of the phospholipid myristoyl chains. However, the plateau region of the peptide myristoyl group, i.e., the chain segment closest to the myristic acid group is wobbling in a cone of half-angle of 35°–42° relative to the membrane surface. This result that may lend support for the wobble model.

Whether this segment of the chain is tilted at a (more or less) fixed angle of 24°–29°. Results from two-dimensional proton NMR spectra (Hauer et al., to be published) of Cat14 in bicellar solution show the peptide backbone to be quite flexible and free to move relative to the membrane surface, a result that may lend support for the wobble model.

To obtain quantitative information about the binding of Cat14 to the bicelles, ultracentrifugation measurements are underway in our laboratory. Also in progress are NMR experiments on the myristoylated N-terminal peptides from the src family of tyrosine kinases (14). Several of these peptides contain an excess of basic residues, which have been found to stabilize the interaction between them and phospholipid membranes (13), and a comparison of the NMR spectra of the src peptides with those of Cat14 should provide valuable information about differences in the modes of interaction.

Finally, it is important to emphasize that it is still unclear whether myristoylated proteins such as the C-subunit of PKA bind directly to the membrane in vivo. Their catalytic action may well be facilitated by temporary membrane localization, but they may also function as a “switch” by binding to a membrane bound receptor. In either case, deuterium quadrupole echo spectroscopy appears to offer a valuable tool for studying their mode of binding.

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