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Lanthanide Chelates as Bilayer Alignment Tools in NMR Studies of Membrane-Associated Peptides

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The equimolar complex, consisting of the lipid-like, amphiphilic chelating agent 1,11-bis[distearylamino]-diethylenetriamine pentaacetic acid (DTPA-18) and Tm^{3+}, is shown by deuterium (^2H) NMR to be useful in aligning bicelle-like model membranes, consisting of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC). As shown previously (1996, R. S. Prosser *et al., J. Am. Chem. Soc.* 118, 269–270), in the absence of chelate, the lanthanide ions bind loosely with the lipid phosphate groups and confer the membrane with a sufficient positive magnetic anisotropy to result in parallel alignment (i.e., average bilayer normal along the field). Apparently, DTPA-18 sequesters the lanthanide ions and inserts into the phospholipid bilayer in such a manner that bilayer morphology is preserved over a wide temperature range (35–70°C). The inherent paramagnetic shifts and line broadening effects are illustrated by ^2H NMR spectra of the membrane binding peptide, Leu-enkephalin (Lenk-d_2, Tyr-(Gly-d_2)-Gly-Phe-Leu-OH), in the presence of varying concentrations of Tm^{3+}, and upon addition of DTPA-18. Two conclusions could be drawn from this study: (1) The addition of Tm^{3+} to the bicelle system is consistent with a conformational change in the surface associated peptide, and this effect is shown to be reversed by addition of the chelate, and (2) The paramagnetic shifts are shown to be significantly reduced by addition of chelate.

Key Words: **paramagnetic; chelate; membrane peptide; oriented model membrane; solid state NMR.**

INTRODUCTION

Bicelles, or bilayered micelles, have gained considerable popularity as a model system for structural and dynamical studies of membrane associated compounds, both in isotropic solution (*1*) and magnetically aligned phases (*2–6*). Bicelles are typically formed by mixing a long-chain phospholipid, such as dimyristoylphosphatidylcholine (DMPC), with a surfactant, preferably dihexanoylphosphatidylcholine (DHPC), which is chemically similar to DMPC. The phosphatidylcholines are believed to form disk-shaped aggregates in which the DMPC is organized in a planar bilayer and DHPC coats the hydrophobic edges of the bilayer. At sufficiently high (> 2–5% (w/w)) total lipid concentration and a molar ratio q = [DMPC]/[DHPC] between 2 and 6 (*7, 8*) bicellar solutions align spontaneously in the magnetic field. The resulting anisotropic phase is thought to be a nematic discotic with the average bilayer normal perpendicular to the field. The bicellar solutions are stable in terms of lipid concentration (2–40%), temperature (30–45°C), ionic strength, and pH, and enzyme activity is preserved for systems where detergents are known to fail (*4*).

NMR spectra of membrane associated peptides in aligned bicelles typically exhibit excellent resolution (*3*). Unfortunately, well-resolved spectra are not expected for larger membrane spanning proteins, if they do not undergo rapid reorientation inside the bilayer. Instead, the absence of rapid uniaxial averaging will lead to cylindrical powder patterns. It is consequently desirable to use a bilayered model system that spontaneously aligns with the bilayer normal parallel to the applied magnetic field. Parallel alignment of phospholipid bilayers has been shown to be feasible by altering the magnetic susceptibility anisotropy of the membrane through the addition of either large amounts of aromatic molecules (*9*) or small amounts of the trivalent lanthanide ions Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+} (*10, 11*). In particular, it has been recently shown that less than 1 mol% Tm^{3+} or Yb^{3+}, relative to DMPC, is needed to induce parallel alignment (*12*). The parallel aligned liquid-crystalline bilayered phases generated by addition of lanthanide ions are stable over a wide range of temperature, pH, and lanthanide concentration (*11*), and the bilayers were observed to be highly ordered with a well-defined repeat spacing (*13*). This makes these phases suitable for the determination of the disposition of membrane associated peptides or pharmacophores by low-angle diffraction techniques as well as spectroscopic techniques that may benefit from the presence of oriented transition dipole moments.

Despite the advantages of parallel aligned phospholipid bilayers, lanthanide ions present certain disadvantages in the study of membrane proteins by NMR. First and foremost, since the binding affinity (~30 M^{-1}) of Ln^{3+} ions to the free carboxyl groups in aspartic and glutamic acid (*14*) is comparable

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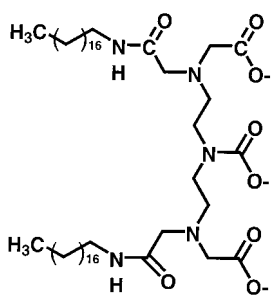
to their affinity for the phosphate groups in phospholipids (*15*), the lanthanide ions may introduce unacceptable conformational changes in a polypeptide. Second, the Ln^{3+} ions—well known as good shift reagents (*16, 17*)—perturb chemical shifts of peptide spins; this may make assignments and structural elucidation cumbersome. Finally, in some instances the presence of lanthanide ions may affect both longitudinal and transverse nuclear spin relaxation times, perhaps leading to unacceptable line broadening.

One way of overcoming such limitations is to incorporate an amphiphilic chelating agent which sequesters the lanthanides ions and is compatible with the phospholipid bilayer. The first priority for design of such an amphiphilic chelate is retention of positive magnetic susceptibility anisotropy. Second, the chelate should resemble a lipid molecule and should not induce phase separation when mixed with regular phospholipids. Finally, low concentrations of the lanthanide–chelate complex should be adequate for macroscopic alignment, so that the system represents a good membrane model. One such chelate, DMPE-DTPA, has been recently introduced and shown to have desirable properties (*5*). In this paper, we consider the use of another amphiphilic chelate, DTPA-18 (structure given in Scheme 1) as a sequestering agent for trivalent lanthanide ions in phospholipid bilayer solutions. In particular, we address the issue of lanthanide-induced conformational changes in a membrane associated peptide, the lanthanide–peptide paramagnetic interactions, and the efficacy of the chelate to reverse these effects, while preserving the orientation of the bilayer. For this purpose, we have employed deuterium NMR of the selectively labeled pentapeptide Leu-enkephalin, Tyr-(Gly-d_2)-Gly-Phe-Leu, henceforth referred to as Lenk-d_2. Portions of this work were presented previously (*18*).



SCHEME 1. DTPA-18.

MATERIALS AND METHODS

Synthesis of the Chelating Agent

1,11-Bis(octadecylamino)-1,11-dioxo-6-aza-3,6,9-tris(carboxymethyl)undecane (DTPA-18) was synthesized and purified by modifications of a previously reported procedure (*19, 20*). All materials were reagent grade. Octadecylamine (30.1 g; 111 mmol, Aldrich) was dissolved in 200 mL of anhydrous chloroform (Mallinckrodt). Diethylenetriamine pentaacetic acid dianhydride (20.0 g; 55.5 mmol, Aldrich) was added and the solution refluxed for 48 h. The white precipitate formed during the reaction was filtered off and air-dried. The resulting crude product was stirred for 3 h at 80°C in 1 L water, filtered hot, and dried in a vacuum desiccator. This product was then stirred in 1 L boiling anhydrous chloroform for 3 h, filtered hot, and dried in a vacuum desiccator. Yield: 25.0 g (49.9%). Elemental analysis of the product was performed by Galbraith Laboratories, Inc., Knoxville, Tennessee. Calcd. for $C_{50}H_{97}N_5O_8$: C, 66.99%; H, 10.90%; N, 7.81%. Found: C, 66.28%; H, 10.74%; N, 7.66%.

Leu-enkephalin-d_2 (Lenk, Tyr-(Gly-d_2)-Gly-Phe-Leu-OH) was prepared on an Applied Biosystems solid state peptide synthesizer at the UCSD Biopolymer Synthesis Laboratory (an NIH Research Resource Facility) using tBOC-chemistry. The crude product was found by mass spectrometry and high-resolution NMR spectroscopy to be 98% pure, eliminating the need for further purification. A 160 mM stock solution was prepared for subsequent addition to the bicellar NMR samples from 15.5 mg Lenk-d_2 and 180 μL of deuterium depleted water.

Sample Preparation

All samples were prepared with deuterium depleted water from Cambridge Isotopes Laboratories, Cambridge, Massachusetts. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), chain-perdeuterated DMPC (DMPC-d_{54}), and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Long- and short-chain lipids were combined in a molar ratio of [DMPC]/[DHPC] = 3.2 in a dry-bag and dissolved in water. All samples investigated here contained 20% (w/w) phospholipid corresponding to a DMPC concentration of 156 mM, and for NMR samples without peptide a small fraction of the DMPC was of the chain-deuterated variety, DMPC-d_{54}. Homogeneous transparent bicellar solutions were typically obtained in less than an hour by stirring or vortexing, alternatingly heating to 40°C and cooling to 10°C for 10–20 min at a time. Since 20% DMPC/DHPC bicellar solutions are very viscous above 25°C, they were chilled to near 0°C, where the viscosity is close to 1 cP (= 1 mPa s) (*8*) before transfer to the NMR sample tube. The chelating agent was added to the bicelle sample in a ratio of 1 mol of DTPA-18 to 40 mol of DMPC. Following complete dissolution of the DTPA-18, enough thulium chloride hexahydrate (99.999%, Aldrich, Milwaukee, Wisconsin) was added to the samples to ensure a slight excess (10%) of DTPA-18 relative to Tm^{3+}.

For ^{2}H NMR studies of bilayer associated peptide, Lenk-d_2, DTPA-18, and thulium chloride were added to a 400 μL 20% (w/w) q = 3.2 DMPC/DHPC bicelle solution in the following order: (1) 12 L of the 160 mM peptide stock solution (1.9 mol Lenk-d_2), (2) 5 μL of a 520 mM thulium chloride solution

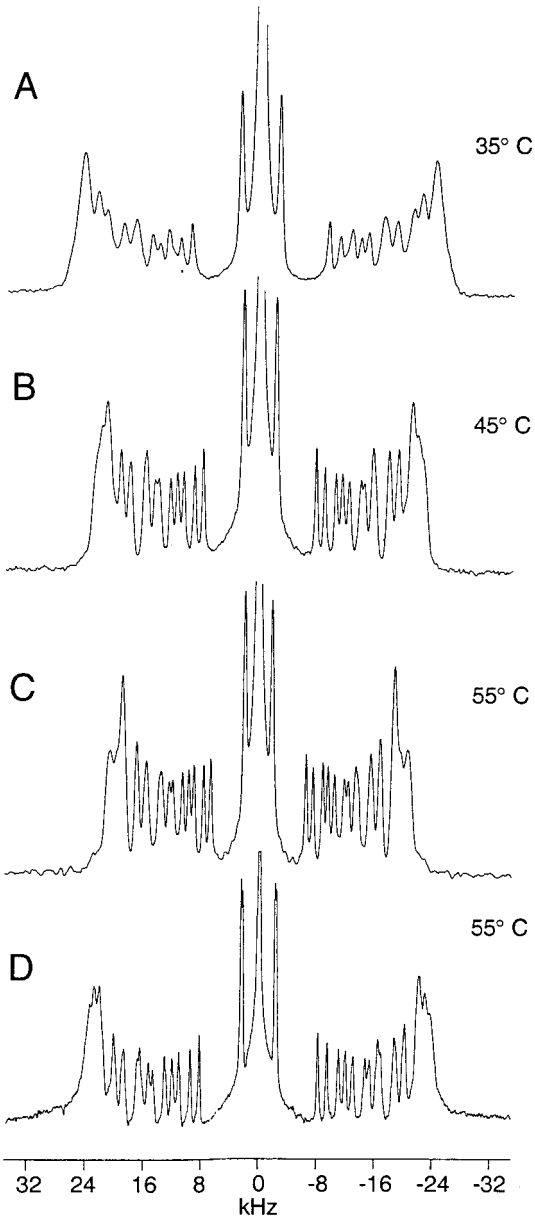


FIG. 1. A 55.3 MHz deuterium quadrupole echo spectra of DMPC-d_{54} in magnetically aligned aqueous DMPC/DHPC solutions containing 20% (w/w) DMPC + DHPC in a ratio of $q = 3.2$ corresponding to 156 mM DMPC and 49 mM DHPC. Spectra A, B, and C were obtained from a sample to which 3.9 mM Tm^{3+} and 4.3 mM DTPA-18 were added. The bottom spectrum, D (from Ref. *11*, Fig. 5), was recorded at 38.4 MHz. This sample contained slightly less Tm^{3+} (3.1 mM) and no chelating agent and was recorded at 55°C.

(2.6 μmol); and (3) 2.6 mg (2.9 mol) DTPA-18. ^{2}H NMR spectra were recorded at 92.1 MHz following the addition of each component.

Apparatus and NMR Measurements

Deuterium quadrupole echo NMR spectra of bicellar solutions containing DMPC-d_{54} were acquired at 55.3 MHz, using a GN500 spectrometer equipped with an 8.5 T Oxford Instruments wide-bore magnet, an ENI LPI-10 RF amplifier, and a home-built probe delivering 2.5 s /2-pulses to the sample via a 12-turn solenoid coil. The normal $\pi/2$–τ–$\pi/2$–acq quadrupole echo pulse sequence (*21*) with a pulse spacing $\tau = 50$ μs was used, and 5000 transients were acquired with a repetition time of 1 s. In studies involving Lenk-d_2, a Bruker DRX600 spectrometer was used. Here a simple 1-pulse experiment with a 20 μs $\pi/2$-pulse was found to be adequate. For the peptide solutions 90,000 transients were accumulated with a repetition time of 1 s. The probe temperature was maintained to $\pm$ 0.1 K with a LakeShore temperature controller.

RESULTS AND DISCUSSION

The 55.3 MHz deuterium quadrupole echo spectra of DMPC-d_{54} presented in Fig. 1 illustrate the effects of adding thulium chloride and chelating agent to bicellar solutions. All four spectra were obtained from solutions containing 20% (w/w) DMPC + DHPC with $q = 3.2$. The top three spectra (A, B, and C) were obtained from samples containing thulium chloride at a concentration of $[Tm^{3+}]/[DMPC] = 0.023$ as well as DTPA-18 in the amount of 1.1 DTPA-18 for each Tm^{3+} ion, i.e., [DTPA-18]/[DMPC] = 0.025 The bottom spectrum (D) was recorded earlier (*11*) at 55°C from a similar sample without chelating agent and a ratio of $[Tm^{3+}]/DMPC = 0.02$. The relatively large quadrupolar splittings, 40–50 kHz, show that the samples contain parallel aligned phospholipid bilayers. The similarity of B and C to D indicates, as described by Prosser *et al.* (*11*), that the majority of the DMPC exists in smectic layers separated by isotropic or very weakly ordered aqueous layers containing, probably, nonoriented DHPC micelles with some DMPC enclosed. The two bottom spectra C and D, which were both recorded at 55°C are seen to be nearly identical. The strong similarity of these two spectra suggests that the Tm^{3+} ions coordinated directly to the phospholipid headgroups and the Tm^{3+} chelate are equally effective in inducing parallel alignment for the phospholipid bilayers and that the myristoyl chain conformations are similar in the two samples. Higher concentrations (0.050 and 0.10 relative to DMPC) of Tm^{3+}–DTPA-18 gave rise to spectra with marginally larger order parameters, but otherwise very similar to those presented here. It should be noted, of course, that the spectra presented in Fig. 1 do not by themselves prove that the Tm^{3+} ions are ligated only to DTPA-18, but the peptide spectra discussed below demonstrate the isolation of the Tm^{3+} ions by the chelating agent.

To explore the potential of Tm^{3+}-doped phospholipid bilayers in structural studies of membrane associated peptides and simultaneously investigate the efficacy of the ligand in sequestering lanthanide ions, deuterium NMR spectra were obtained for a $q = 3.2$ DMPC/DHPC bicellar solution of Leu-enkephalin (YGGFL), selectively deuterated at the α-position of Gly-2. Figure 2A shows the 92.1 MHz ^{2}H spectrum of Lenk-d_2 in a typical negatively aligned bicellar solution containing 20% (w/w) DMPC + DHPC. The two doublets, which arise from

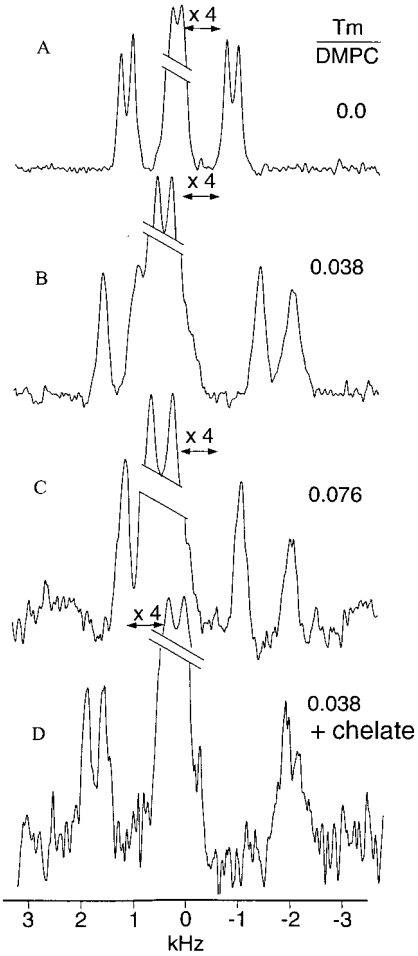


FIG. 2. A 92.1 MHz deuterium NMR spectra recorded at 38°C and pH 5.5 of Lenk-d_2 incorporated into a magnetically aligned $q = 3.2$ DMPC/DHPC solution containing 20% (w/ w) lipid. The top spectrum (A) was recorded prior to the addition of either thulium chloride or chelating agent. The spectra in B and C were recorded after the addition of 5.9 and 11.8 mM thulium chloride, respectively. The bottom spectrum (C) was obtained following the addition of DTPA-18 to a concentration of 6.5 mM. The central, off-scale doublet is due to weakly ordered HOD, and for clarity the top of the doublet has been expanded horizontally by a factor of 4. The HOD quadrupolar splittings were (from top to bottom) 42, 68, 99, and 76 Hz.

the two distinct glycine α-deuterons, have splittings of 1.82 and 2.04 kHz. This corresponds to orientational order parameters $S_{\bar{n}p} \equiv \frac{1}{2}\langle 3\cos^2\beta_{\bar{n}p} - 1\rangle$ of 0.014 and 0.016 for principal components (p) of the two quadrupole coupling tensors relative to the director, i.e., the average bilayer normal, $\bar{n}$.

This low ordering of the peptide may be due to relatively weak association with the bilayer (*22*), to large amplitude internal motion in the peptide, to an orientation fortuitously close to the magic angle, or to a combination of these factors.

The central resonance, which is off-scale in Fig. 2, is due to HOD and has a splitting of 42 ± 4 Hz in the undoped sample (A). The average center of the two glycine doublets is observed to be 55 Hz = 0.6 ppm upfield from the HOD resonance, which agrees well with typical glycine α-proton shifts relative to water (0.5–1.0 ppm upfield) observed in high-resolution proton NMR spectroscopy (*22*). Although the chemical shift anisotropies for HOD and CD_2 are quite different, the effects are clearly too small to be observable.

When sufficient thulium is added to a bicellar solution to realign the bilayer normal along the magnetic field, all shifts and quadrupolar splittings are expected to double in magnitude (*11, 12*), because the order parameter $S_{l\bar{n}}$ for the average bilayer normal relative to the laboratory frame (l) changes from $-\frac{1}{2}$ to $+1$. If the lanthanide ion, which is known to coordinate with phosphate groups, is in the vicinity of the Gly-2 deuterons, then one also expects to observe a (dipolar) paramagnetic shift of the peptide resonances. Depending on the average relative orientation of the ^2H–Tm^{3+} interatomic vector, this shift may be positive or negative (because the solution is anisotropic) and its magnitude will be proportional to the ensemble average $\langle(3\cos^{-2}\beta_{id} - 1)r^{-3}\rangle$, where r is the distance between the Tm^{3+} ion and the deuteron and β_{id} is the angle between the interatomic vector and the direction of the external magnetic field. If, in addition, the lanthanide ion coordinates directly to particular peptide sites, conformational changes may affect the peptide spectrum. The sequestering power of a chelating agent can therefore be tested simply by comparing the spectra of Tm^{3+}-doped samples with and without chelating agent, as shown in Figs. 2B and 2D.

Inspection of Fig. 2B shows that the addition of thulium chloride causes the two glycine doublets to shift upfield by 374 and 396 Hz, using the absolute resonance frequency as a reference. Simultaneously, the water doublet shifts downfield by 600 Hz (6.5 ppm). This trend is observed to continue with still higher concentrations of lanthanide (Fig. 2C). As the $[Tm^{3+}]$ is doubled, the water resonance shifts further downfield, thereby masking the downfield resonance of the inner glycine doublet, while the outer glycine doublet shifts upfield by an additional 233 Hz. The direction of the paramagnetic shift of the glycine deuterons is reasonable, since both Tm^{3+} ions and glycine deuterons are located on or near the bilayer surface. The glycine deuterons are consequently found in the transverse plane, or shielded region, of the dipolar field generated by the Tm^{3+} ions. Note that the quadrupolar splittings of the parallel aligned system in Fig. 2B are 2.37 and 3.67 kHz, while the HOD splitting is 68 Hz. Since we know from the ^2H NMR spectra in Fig. 1 of a similar sample containing chain-perdeuterated DMPC that the bilayer is parallel aligned, a factor of 2 was expected, barring any significant change in ordering of the local bilayer director with the average director. The observed increases in the glycine splittings by factors of 1.67 and 1.31 (both ±0.05), respectively, are probably due to a conformational change in the peptide, induced by direct, if weak (*15*), coordination of the lanthanide ion directly to the C-terminal carboxyl group. A change in the mode of binding of the peptide to the bilayer may also be involved.

The effects of adding the chelating agent DTPA-18 to the sample used for Fig. 2B is illustrated by the spectrum in Fig. 2D. The spectrum is clearly more symmetric than those in Figs.

2B or 2C, reflecting the relative decrease in the difference between the paramagnetic upfield glycine shifts and the downfield water shifts. Moreover, the glycine quadrupolar splittings (3.53 and 3.89 kHz) are now much closer to the expected factor of two times their respective splittings observed in the undoped sample (namely, 1.94 and 1.91 times those in Fig. 2A). Thus, whatever caused the differential, "nonideal" effects on the two glycine resonances, the chelating agent significantly reduces this effect. Unfortunately, the quality of this spectrum is poor, due to the formation of a precipitate during the long acquisition time. Recently, a careful optimization of lanthanide-doped, parallel aligned model systems revealed that Yb^{3+} is preferable to Tm^{3+} for minimizing paramagnetic effects, and minimal lanthanide concentrations could be lowered in systems consisting of 25% amphiphile and $q = 2.8$ (*12*). Furthermore, a similar study of a lanthanide chelated model membrane system revealed that lanthanide-mediated interbilayer association (the likely result of the precipitation observed in our experiments) could be avoided by the addition of a small amount of negatively charged lipid (*5*). Therefore, the instabilities observed in the peptide sample consisting of DTPA-18 could probably be easily controlled by altering the amphiphile concentration ($q = 2.8$, 25% (w/w)) and using slightly less Tm^{3+}:DTPA-18, in combination with a small amount of negative lipid, such as dimyristoyl-*sn*-glycero-phosphatidylglycerol (DMPG), such that [DMPC]/[DMPG] ~20.

CONCLUSION

The results presented here demonstrate that the addition to a bicellar solution of an amphiphilic lipid-like lanthanide chelate, which can be incorporated into a DMPC bilayer, generates a phase in which the bilayer normal is parallel to the magnetic field. In the absence of chelate, the lanthanide ions, which are necessary to establish a large positive magnetic susceptibility anisotropy and parallel alignment, may bind specifically to individual sites on a membrane-associated polypeptide and may alter the mode of association between polypeptide and membrane. In either case, the conformational changes associated with lanthanide ion coordination may be unacceptable in structural studies. The addition of chelating agent appears to minimize the direct paramagnetic effects on the peptide spectrum and reverse conformational changes by sequestering the lanthanide ions. The use of a lanthanide chelate should consequently facilitate resonance assignment and accumulation of structural information for membrane bound polypeptides.

In closing we note that the paramagnetic shifts induced by the addition of nonchelated lanthanide ions may of course be a useful means for obtaining information about the mode of binding, orientation, and structure of a membrane-associated peptide. In particular, paramagnetic shifts and, perhaps, increased relaxation rates will be observed only for those parts of the peptide exposed to the aqueous phase. In general, however, when rates of reorientational and translational molecular motion are reduced enough to demand the use of parallel aligned bilayers, the use of a lanthanide chelate is recommended over that of "free" lanthanide ions.

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