Solution and Membrane Bound Structure of a Peptide Derived from the Protein Kinase C Substrate Domain of Neuromodulin†

Stacey L. Wertz, Yoko Savino, and David S. Cafiso*

Department of Chemistry and Biophysics Program, University of Virginia, Charlottesville, Virginia 22901

Received May 27, 1996; Revised Manuscript Received July 1, 1996⊗

ABSTRACT: The solution, micelle, and membrane bound structure of a peptide based on the protein kinase C and calmodulin binding domain of neuromodulin was studied using a combination of NMR, EPR, and circular dichroism. NMR spectroscopy on this peptide indicates that there is little secondary structure in aqueous solution or detergent micelles, but that the peptide is helical in methanol. This finding is in agreement with EPR experiments utilizing double spin-labeled derivatives of the peptide as well as circular dichroism. The membrane bound structure of this peptide was investigated with EPR by synthesizing a series of spin-labeled peptides based on the protein kinase C and calmodulin binding domain of neuromodulin. These peptides exhibit no binding to neutral membranes containing phosphatidylcholine, but associate strongly with membranes containing negatively charged lipids such as phosphatidylserine. The depth of penetration of the spin label was estimated using continuous wave power-saturation EPR and demonstrates that labels at the ends of the peptide are localized slightly outside the membrane interface, but that spin labels in the central portion of the sequence are near or within the membrane interface. In addition, the peptide is in an extended structure when bound to membranes containing acidic lipid with its more hydrophobic side chains interacting with the membrane interior. The results demonstrate that the binding of these peptides to membranes is not driven by purely electrostatic interactions, but includes the interaction of hydrophobic side chains with the membrane interior.

Many critical protein−protein interactions in cell signaling take place at the cytoplasmic membrane interface. The reduction in dimensionality that occurs at the membrane interface facilitates protein−protein interactions (McLaughlin & Aderem, 1995), and attachment to the membrane is an essential step for the activation of some enzymes. Proteins that are water soluble can become associated with the membrane interface through at least two different mechanisms. Acylation of proteins, for example, myristoylation or palmitoylation, can attach proteins to membranes and is driven by the hydrophobic effect (Towler et al., 1988). In addition, electrostatic interactions between basic residues on proteins and membranes containing negatively charged lipids will also serve to anchor proteins to the interface (McLaughlin & Aderem, 1995).

Several protein kinase C (PKC)[1] substrates, such as neuromodulin and the myristoylated alanine rich C kinase substrate (MARCKS), associate with membranes through a combination of acylation and electrostatic interactions. Neuromodulin (also known as B-50, GAP-43, g5, P-57, GAP-48, pp46, and F1) is a membrane associated, 24.7 kDa, neurospecific calmodulin (CaM) binding protein that is a substrate of PKC (Zwiers et al., 1978; Skene & Willard, 1981; Rodnight, 1982; Andreasen et al., 1983; Benowitz & Lewis, 1983; Katz et al., 1985; Chan et al., 1986). Neuromodulin is conserved in all vertebrates, and it has been implicated as a key player in phosphoinositide metabolism (van Dongen et al., 1985), neurotransmitter release (van Hoof et al., 1988), and long term potentiation (Nelson et al., 1989). Neuromodulin contains a basic domain which can associate electrostatically with membranes containing acidic lipids and bind to CaM. This basic domain is phosphorylated by PKC at serine 41 and dephosphorylated by the CaM-dependent phosphatase calcineurin (Liu & Storm, 1989). Unlike MARCKS, which is myristoylated and dissociates from the membrane when phosphorylated, neuromodulin is palmitoylated at cysteines 3 and 4, and remains permanently attached to the membrane (Peitzsch & McLaughlin, 1993). A synthetic peptide derived from the basic domain of neuromodulin corresponding to residues 37−53 with the sequence KIQASFRGHITRKKLKG binds to membranes containing acidic lipids, and is phosphorylated by PKC (Apel et al., 1990). Membrane binding of this peptide is blocked in the presence of CaM and is reversed by PKC phosphorylation (Houbre et al., 1991). Thus the CaM, membrane, and PKC interactions of this protein can be mediated by a peptide from its basic domain. Although the exact molecular function of neuromodulin has yet to elucidated, it has been proposed to sequester CaM and release it in response to PKC activation (Liu & Storm, 1989).

† This work was supported by a grant from the National Science Foundation (MCB-9418318).

* All correspondence should be addressed to this author at the Department of Chemistry.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1996.

[1] Abbreviations: CaM, calmodulin; COSY, 2D correlated spectroscopy; CD, circular dichroism; DCM, dichloromethane; DQF-COSY, double quantum filtered COSY; DMF, dimethylformamide; EDT, ethanedithiol; MARCKS, myristoylated alanine rich C kinase substrate; MBHA, *p*-methylbenzhydrylamine; NMM, *N*-methylmorpholine; MTSSL, *S*-[(1-oxy-2,2,5,5-tetramethylpyrrolin-3-ylmethyl] methanethiolsulfonate spin label; NiEDDA, nickel ethylenediaminediacetic acid; NOE, nuclear Overhauser effect; NOESY, 2D nuclear Overhauser effect spectroscopy; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine, PKC, protein kinase C; PyBOP, [(benzotriazolyl)oxy]-tripyrrolidinophosphonium hexafluorophosphate; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

S0006-2960(96)01248-2 CCC: $12.00

The structures of highly basic peptides such as those derived from MARCKS and neuromodulin are important to characterize because they will facilitate an understanding of PKC substrate requirements. At the present time, however, the membrane bound structures of these basic peptides are not well characterized, primarily because determination of membrane bound structures presents a number of unique difficulties. For example, the slow rotational correlation time of membrane associated macromolecules makes conventional high-resolution NMR impossible. Circular dichroism will provide an indication of overall secondary structural trends, but can be difficult to reliably use for highly charged peptides. At lipid concentrations low enough to avoid light scattering problems, extremely high peptide:lipid ratios must be employed. Recent advances in microwave resonator technology have improved both the sensitivity and versatility of EPR. As a result, continuous wave power-saturation measurements on nitroxides can be easily carried out and used to determine the position of nitroxide labels along the bilayer normal (Altenbach et al., 1994); in addition, experiments on double-labeled peptides have also been performed and establish EPR as a tool for making secondary structure determinations (Millhauser, 1992).

Recently, the membrane bound structure of a peptide derived from MARCKS was determined using EPR and site-directed spin-labeling (Qin & Cafiso, 1996). In this study, a 25 amino acid peptide derived from the PKC and CaM binding domain of MARCKS was shown to be an extended conformation when bound to membrane interfaces containing PS. The peptide backbone was at the interface, with the more hydrophobic residues being buried in the interface. The data suggest that several phenylalanine residues may function to stabilize the highly charged domain of MARCKS at the membrane−solution interface. In the present work, we describe the synthesis and nitroxide labeling of 22 spin-labeled derivatives of a peptide based on neuromodulin. This peptide has the sequence acetyl-KIQASFRGHITRKKLKG-amide. EPR spectroscopy is used in combination with NMR and CD to determine the solution and membrane bound structure of this peptide. Although the peptide has the capacity to adopt a helical conformation under some conditions, it is extended on the membrane interface. The central domain of the peptide appears to be strongly associated with the membrane interface with each end positioned on the aqueous side of the membrane−solution interface. Peptides such as those derived from MARCKS and neuromodulin do not bind to membranes containing zwitterionic phosphatidylcholine; as a result, the binding of these peptides is thought to be dominated by electrostatic interactions with the membrane interface. The work described here demonstrates that this peptide binding is driven by both electrostatic and hydrophobic interactions with the membrane interface.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain phosphatidylserine (PS) and dimyristoylphosphatidylglycerol (PG) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Egg phosphatidylcholine (PC) was purified from egg yolks as described previously (Singleton et al., 1965) and stored in chloroform under argon at −20 °C until needed. The methanethiosulfonate spin label, *S*-[(1-oxy-2,2,5,5-tetramethylpyrrolin-3-ylmethyl] (MTSSL) was obtained from Reanal (Budapest, Hungary). Nickel ethylenediaminediacetic acid (NiEDDA) was the generous gift of Dr. Christian Altenbach. [(Benzotriazolyl)oxy]tripyrrolidinophosphonium hexafluorophosphate (PyBOP), and all N^{α}-Fmoc L amino acids were obtained from Novabiochem (La Jolla, CA). *N*-Methylmorpholine (NMM) and piperidine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Peptide synthesis grade dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) was obtained from Applied Biosystems (Foster City, CA), and acetonitrile was purchased from Mallickrodt Chemicals (Chesterfield, MO). Perdeuterated sodium dodecyl sulfate (SDS) was obtained from Cambridge Isotopes (Woburn, MA).

Synthesis and Purification of Neuromodulin Analogs. A 17 amino acid peptide that encompassed residues 37−53 of neuromodulin and a peptide having the cysteine substitution Ala-4-Cys in this sequence were obtained from Multiple Peptide Systems (San Diego, CA). Both peptides were acetylated at the N-terminus and amidated at the C-terminus. The native peptide was further purified by HPLC on a C18 reverse phase column (POROS R2/H 4.6 × 100mm, Perseptive Biosystems, Cambridge, MA) using a gradient of 15−27% acetonitrile in water run at 3 mL/min over a 10 min period. Twenty-one additional peptides were synthesized on an Gilson Automated Multiple Peptide System (AMS 422) using a rink amide *p*-methylbenzhydrylamine (MBHA) resin. To make a complete set of single spin-labeled peptides, every position in the peptide was replaced by cysteine. In addition, five double cysteine substituted peptides were also produced with cysteines at positions 4−6, 4−7, 4−8, 10−14, and 11−14. For this synthesis the protecting groups used were: pentamethylchroman-6-sulfonyl for Arg; S-trityl for Cys, Gln, and His; Boc for Lys; and *tert*-butyl for Ser and Thr. Single coupling was carried out on each amino acid, and the peptides were acetylated using acetic anhydride before cleavage. A 5 h cleavage was performed in a mixture of 89% TFA, 3% EDT, 3% thioanisole, 2% anisole, and 3% water, and the peptides were precipitated in cold ether redissolved in a mixture of 5% acetic acid in water, dried down, and redissolved in buffer (1 mM MOPS, 100 mM KCl, pH = 7). Peptides were then spin-labeled using an excess of the MTSSL which was dissolved in a small amount of acetonitrile and added to the peptide in the same buffer solution. After 30 min in the dark, the mixture was purified by HPLC using the C18 reverse phase column described above. The peptide was purified on a gradient of acetonitrile−water at a flow rate of 3 mL/min. Typically, the gradients ran from 10% to 40% acetonitrile and the labeled peptides eluted in approximately 5 min. The conditions for HPLC purification of the double labeled neuromodulin peptides varied, with a continuous gradient of two solvents, water and acetonitrile, which were narrowed from 5% to 60% acetonitrile over 10 min. Fourier transform mass spectrometry was used to verify the molecular weights of the peptides both before and after spin-labeling. The purity of these peptides was estimated from HPLC to be at least 98−99%.

Vesicle Preparation. For EPR spectroscopy, lipid mixtures containing PC and 25 mol % PS were obtained by weighing lipids from stock solutions in chloroform followed by vacuum desiccation overnight. An aqueous solution of 100 mM KCl/1 mM MOPS, pH 7, was added to the dried lipid,

and the suspension was vortexed and freeze-thawed five times in liquid nitrogen. This solution was then extruded ten times through 500 Å polycarbonate filters using a commercially available unit (Avestin, Inc., Ottawa, Canada). The extruded vesicles were then centrifuged at 10 000 rpm using a Sorvall SS-34 rotor for 15 min to remove nonvesicular lipid. The total phospholipid content was determined using a modified Fiske and Subarrow assay (Bartlett, 1959). For CD measurements, lipid mixtures of 25 mol % PG in PC were formed and hydrated as described above and were then sonicated to form small 300 Å diameter vesicles using a procedure described previously (Castle & Hubbell, 1976).

NMR Spectroscopy. High-resolution NMR spectra were obtained using a General Electric Omega 500 spectrometer operating at a frequency of 500.13 MHz. All spectra were recorded at 25°C, and chemical shifts were referenced to internal H_2O at 4.75 ppm. The sweep width was 5 kHz, and the water resonance was suppressed by a 1.5 s CW irradiation at the start of all experiments. The data were analyzed using the program FELIX version 2.1 (Biosym Technologies, San Diego, CA) running on a Silicon Graphics Indigo R4000 XZ. Spectra were run in methanol, 500 mM perdeuterated SDS micelles, and aqueous solution. For these experiments the peptide was at a concentration of 5 mM and a pH of 4.3.

DQF-COSY spectra were obtained as described previously (Piantini et al., 1982; Rance et al., 1983) and contained 1024 and 2048 complex points in the t_1 and t_2 dimensions, respectively. A sine squared function shifted by 30° was used to apodize both time domains. The t_1 dimension was zero filled to 2048 points. The TOCSY spectra (Braunschweiler & Ernst, 1983; Bax & Davis, 1985) were obtained with a 100 ms clean (Griesinger et al., 1988) MLEV-17 spin lock, where Z filters preceded and followed the spin lock (Rance, 1987). NOESY spectra (Jeener et al., 1979; Macura & Ernst, 1980) were obtained with a mixing time of 100 ms. The number of complex points collected for TOCSY and NOESY spectra was 512 and 2048 in the t_1 and t_2 dimensions, respectively; the spectra were apodized with a 3 Hz exponential in t_1 and a 90°-shifted sine bell in t_2; the t_1 dimension was zero filled to 1024 points.

Proton chemical shift assignments were made following a general procedure described previously (Wüthrich, 1986). In the first step of this procedure, the spin systems were identified using DQF-COSY and TOCSY experiments followed by the use of NOESY spectra to establish sequential assignments from NH−NH cross peaks.

EPR Spectroscopy of the Neuromodulin Analogs. EPR spectra were measured on a Varian E-line Century series spectrometer using an X-band loop gap resonator with a standard two loop one gap configuration. A microwave power of 2 mW was used with a peak-to-peak modulation amplitude of 1.6 G. Quartz capillary tubes, i.d. = 0.6 mm, o.d. = 0.84 mm (Vitro Dynamics, Rockaway, NJ), were used to hold the nitroxide samples and were filled with 7 μL of sample. For power saturation measurements, samples were placed in gas permeable TPX capillary tubes (Medical Advances, Milwaukee, WI).

Power saturation measurements were carried out on spin-labeled neuromodulin peptides that were totally membrane bound to PC/PS lipid vesicles containing 25 mol % PS. Peptides were added to a final concentration of about 50 μM to a lipid mixture at a concentration of 50 mM. The microwave power was increased from 0.1 to 190.0 mW, and the peak-to-peak amplitude of the $m_I = 0$ peak, $A_{m_I=0}$, was measured at regular power intervals. These power saturation data were fit to the function:

$$A_{m_I=0} = IP^{1/2}\left[1 + (2^{1/\epsilon} - 1)\frac{P}{P_{1/2}}\right]^{-\epsilon} \quad (1)$$

as described previously (Altenbach et al., 1994), where P represents the microwave power, I is a scaling factor, $P_{1/2}$ is the power required to reduce the resonance amplitude, A, to half its unsaturated value, and ϵ is a measure of the homogeneity of saturation of the resonance. In this fit, $P_{1/2}$, ϵ, and I were allowed to be adjustable parameters. Power saturation data were obtained for each sample under three different sets of conditions: equilibrated with a nitrogen atmosphere, equilibrated with air, and equilibrated with nitrogen in the presence of 20 mM NiEDDA in the aqueous phase. Values for $\Delta P_{1/2}$ were then obtained from the difference in the $P_{1/2}$ values in the presence and absence of the relaxation reagents (O_2 and Ni(II) in this case). The parameter Φ was calculated using the following equation from the experimentally measured values of $\Delta P_{1/2}$ obtained in the presence of air and NiEDDA:

$$\Phi = \ln\left(\frac{\Delta P_{1/2}(O_2)}{\Delta P_{1/2}(\text{NiEDDA})}\right) \quad (2)$$

As described previously, this parameter is directly related to the difference in the standard state chemical potentials of O_2 and NiEDDA and varies as a function of depth in the bilayer (Altenbach et al., 1994). Thus, Φ provides an estimate of the nitroxide depth in the bilayer. For the bilayers used in the present study, which contain 25 mol % PS in PC, a calibration curve obtained previously for the parameter Φ as a function of depth within the bilayer was used (Qin & Cafiso, 1996).

Measuring Partition Coefficients Using EPR. The binding of the spin-labeled neuromodulin derived peptides to lipid bilayers was measured using EPR as described previously (Archer et al., 1991). To obtain an estimate of the partition coefficient, β, 0.1−0.2 μL of a 160 mM lipid solution containing 25 mol % PS was added to a solution of approximately 50 μM spin-labeled peptide. The amplitude of the $m_I = -1$ resonance was recorded as a function of the lipid concentration and was then used to calculate the population of membrane bound to free aqueous peptide, λ. The binding of the peptide is expected to fit the equation shown below:

$$\frac{1}{C_L} = \beta A_L \frac{1}{\lambda} + V_L \quad (3)$$

where C_L is the lipid concentration, A_L is the area per lipid, and V_L is the volume per mole of lipid. β has units of length, and values for A_L and V_L of 66 Å^2 and 1255 Å^3 were used here, respectively (Flewelling & Hubbell, 1986).

CD Measurements on Neuromodulin Based Peptides. CD measurements were made on a Jasco J-720 spectropolarimeter using a 1 mm quartz cell at room temperature. Unless otherwise indicated, samples were in Tris buffer at both high and low ionic strengths with a peptide concentration of 25 μM. Each spectrum was an average of 10 scans taken at 50

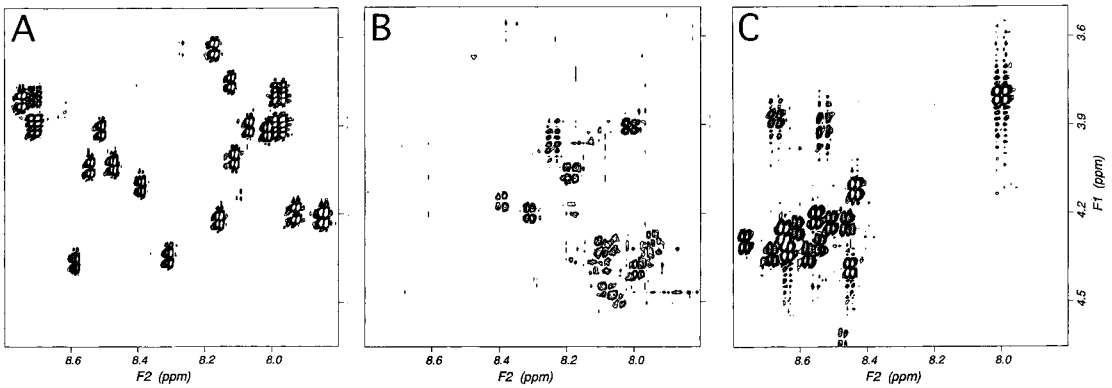

FIGURE 1: Double quantum filtered COSY spectra of the NH−Cα region for the neuromodulin based peptide in (A) methanol, (B) aqueous solution, and (C) deuterated SDS micelles.

nm/min in steps of 1 nm. A band width of 1 nm and a time constant of 0.25 s were used. Samples were flushed with N_2 for 20 min to eliminate any molecular oxygen in the buffer solution, and the data were not smoothed. PG was substituted for PS in these experiments in order to avoid interference from the absorption of bovine brain PS that occurs at less than 200 nm. The measurements were also made in small sonicated vesicles at a concentration of 1 mM lipid to avoid light scattering problems. The CD spectra were then analyzed for secondary structure content using the program PROSEC (Chang et al., 1978).

RESULTS

Solution and Micelle Structure of a Neuromodulin Based Peptide by NMR. Shown in Figure 1 are double quantum filtered COSY spectra of the neuromodulin peptide in methanol, aqueous solution, and SDS micelles. Resonances are well separated in methanol and less well resolved in SDS, and there is much less chemical shift dispersion and much more overlap in aqueous solution. From these spectra and the NOESY spectra of the peptide in methanol and SDS, assignments were made as described above. Nuclear Overhauser effects were relatively weak in SDS, but were not detected for the peptide in aqueous solution using a range of mixing times from 50 to 300 ms. A partial set of assignments for this peptide in aqueous solution were obtained.

From these assignments, we determined the chemical shift index for the α-carbon protons as described previously (Wishart et al., 1992), and these are shown in Figure 2. Four or more consecutive resonances with indices less than −0.1 are indicative of helix, whereas three or more consecutive resonances greater than +0.1 are indicative of a β-sheet. The data shown in Figure 2 provide a strong indication that the peptide is helical in methanol, but the chemical shifts suggest that the peptide is largely unstructured in water and SDS micelles, an observation that is consistent with the weak NOEs and poor chemical shift dispersion in these solvents. The helical assignment in methanol was confirmed by examining the intraresidue NOEs for the neuromodulin peptide, and the interresidue connectivities are shown in Figure 3. The presence of HN−HN(i,i+1), Hα−HN(i,i+3), and Hα−HN(i,i+4) seen in Figure 3 is strongly indicative of an α-helical secondary structure.

Binding of Spin-Labeled Peptides to PS Containing Membranes. Binding measurements were made on the spin-labeled neuromodulin based peptides to determine the

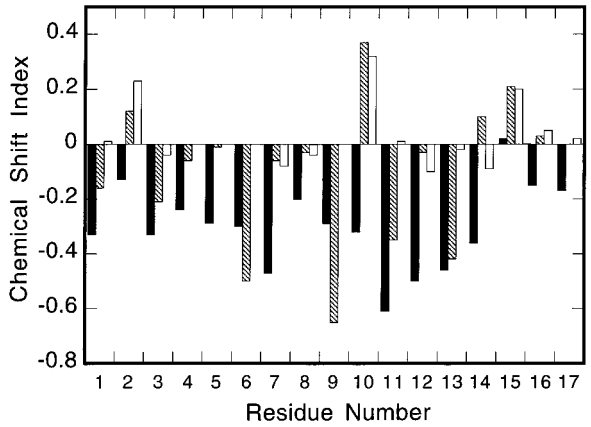

FIGURE 2: A summary of the αH chemical shift indices (Wishart et al., 1992) for the neuromodulin peptide in methanol (solid bars), SDS micelles (striped bars), and aqueous solution (open bars). Spectra were obtained using solutions of pH 4.3 at 25 °C using a GE Omega 500 NMR spectrometer.

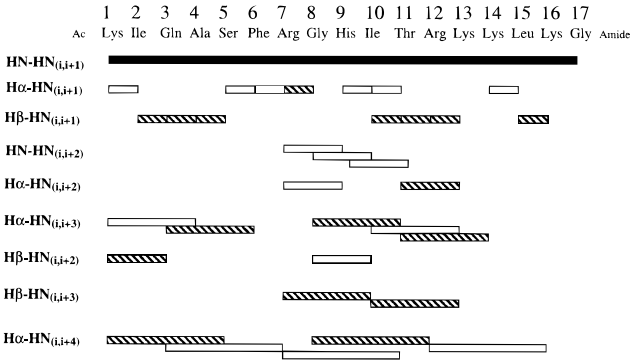

FIGURE 3: A summary of the NOE data for the neuromodulin based peptide (5 mM) in CD_3OH at 25 °C at a pH of 4.3 using a GE Omega 500 NMR spectrometer operating at a frequency of 500.13 MHz. Strong NOEs are represented by solid bars, weak NOEs are represented by clear bars, and medium NOEs are represented by shaded bars.

conditions under which all the peptide would be membrane bound for the EPR and CD measurements described below, and to ensure that nitroxide substitutions did not make dramatic changes in the membrane binding of this peptide. The binding of spin-labeled peptides based on neuromodulin was determined by EPR spectroscopy as described above. No binding of these peptides to neutral PC membranes was detected, but these peptides did bind strongly to membranes containing some negatively charged lipid such as PS or PG. In 25 mol % PS, these peptides bound with partition coefficients, β, of between 0.1 and 1 μm, close to that

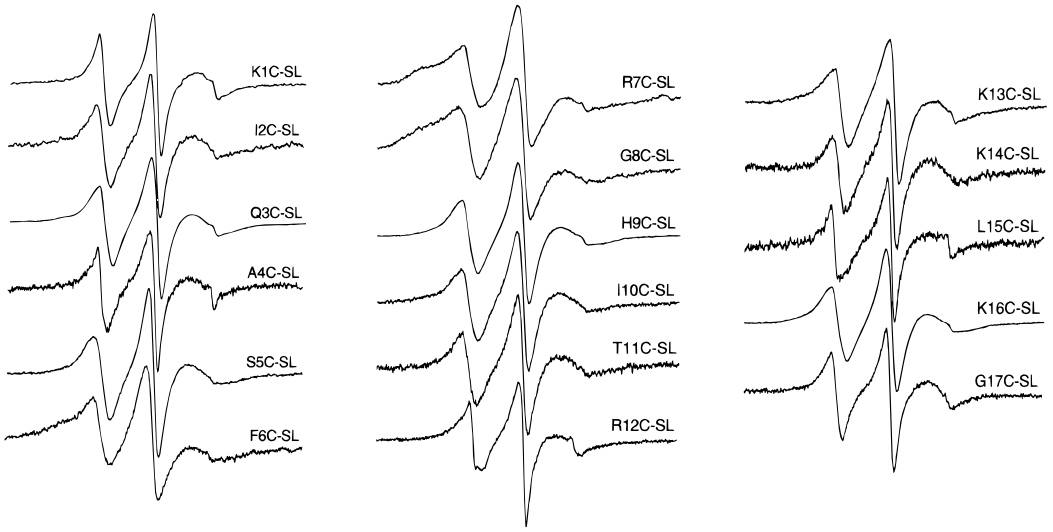

FIGURE 4: EPR spectra of spin-labeled neuromodulin peptides (20−80 μM) in PC/PS (25:75) vesicles of 500 Å in 100 mM KCl/1 mM MOPS, pH = 7 buffer. Lipids were at a concentration of 50 mM. Spectra were taken at 25 °C with a scan width of 100 G for 8 min.

observed for the native unlabeled neuromodulin derived peptide under similar conditions (Kim et al., 1994). With the exception of the first position, replacing a charged residue with a nitroxide reduced the binding of the peptide to PS containing membranes by a factor of 5- to 10-fold compared to the native peptide. Generally, substitution of hydrophobic residues with the nitroxide label produced the smallest changes in binding from that of the native peptide. This is not surprising, since the nitroxide substitution is expected to be nearly as hydrophobic as residues such phenylalanine (Qin & Cafiso, 1996).

Membrane Structure and Topography. Shown in Figure 4 are the membrane bound EPR spectra of 17 spin-labeled analogs of the neuromodulin based peptide encompassing the PKC and calmodulin binding domains of the protein. These spectra exhibit motional broadening relative to the aqueous peptide, with effective correlation times of approximately 3 ns. Most of the labeled peptides exhibit spectra that are quite similar to each other and spectra found for other membrane bound peptides (Altenbach et al., 1989; Qin & Cafiso, 1996); however, at several, particularly at positions 6, 7, and 8, nitroxides are more restricted in their motion. This could reflect either differences in backbone dynamics in this portion of the peptide or additional tertiary contacts of the labeled side chain. In this sequence of spectra there are no clear patterns in label motion as a function of side chain position that would indicate the presence of either α-helical or aggregated sheet structures.

To characterize these labels further, the power-saturation behavior of the EPR signal was examined to provide an estimate of the nitroxide depths. Shown in Figure 5 are power-saturation curves for K13C-SL in the presence of N_2, air, and NiEDDA. The power required to saturate the nitroxide spectrum increases in the presence of air or NiEDDA. As described elsewhere, this is a result of the enhanced electron relaxation rate (R_{1e}) that occurs as a result of collisions between paramagnetic oxygen or Ni with the nitroxide. Shown in Table 1 are the $\Delta P_{1/2}$ values determined for each nitroxide labeled peptide in air and in the presence of NiEDDA. From Table 1, the accessibility to oxygen appears to similar for most of the labels, except that nitroxides on the N-terminus generally have higher $\Delta P_{1/2}$ values than do the C-terminus. The $\Delta P_{1/2}$ values for NiEDDA are also fairly uniform, except at the N- and C-termini where they are higher. The power-saturation data

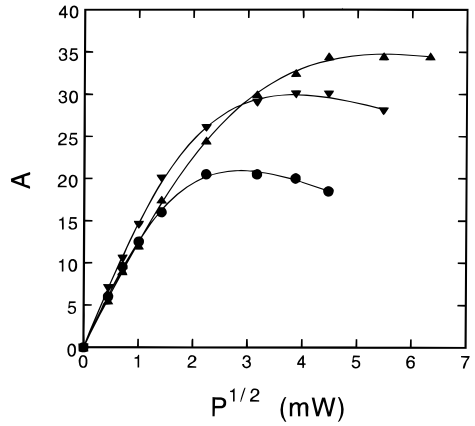

FIGURE 5: Power saturation curves for spin-labeled K13C-SL (50 μM) bound to 25% PS in PC vesicles at 25 °C in 100 mM KCl/1 mM MOPS, pH = 7. Nitrogen (●), air (▲), and NiEDDA (▼) curves are shown. Solid lines represent fits to the data using eq 1.

Table 1: Values of $\Delta P_{1/2}$ and Φ for Spin-Labeled Membrane Bound Neuromodulin Peptides

peptide	air $\Delta P_{1/2}$[a]	NiEDDA $\Delta P_{1/2}$[a]	Φ
K1C-SL	22.8	17.7	0.25
I2C-SL	21.3	5.0	1.4
Q3C-SL	33.6	3.2	2.35
A4C-SL	37.6	8.7	1.5
S5C-SL	28.8	8.7	1.2
F6C-SL	29.9	9.9	1.1
R7C-SL	42.6	2.7	2.8
G8C-SL	28.6	8.9	1.2
H9C-SL	26.2	5.1	1.6
I10C-SL	22.4	4.2	1.7
T11C-SL	19.1	2.9	1.9
R12C-SL	23.0	6.5	1.3
K13C-SL	20.0	5.4	1.3
K14C-SL	7.5	2.3	1.2
L15C-SL	15.9	9.0	0.6
K16C-SL	18.5	10.1	0.6
G17C-SL	29.1	15.1	0.7

[a] Values for $\Delta P_{1/2}$ are given in mW. Air contains approximately 20% O_2 which is a hydrophobic paramagnetic species; NiEDDA was added to a final concentration of about 20 mM.

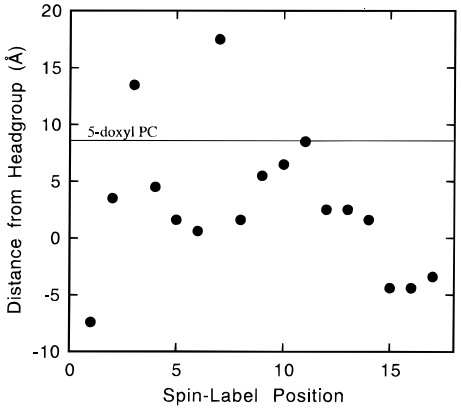

FIGURE 6: Nitroxide distances from the membrane solution interface as a function of the position of label on the neuromodulin peptide sequence. The distances are obtained using Φ values for the spin-labeled neuromodulin peptides shown in Table 1 and a depth calibration obtained previously for Ni(II)EDDA in PC/PS (Qin & Cafiso, 1996).

for positions 6, 7, and 8 show that these nitroxide labels have similar accessibilities to oxygen and paramagnetic metals as do the more mobile nitroxides. Therefore, it is unlikely that the slower motion seen for these positions is a result of aggregation or contacts of the nitroxide with other side chains (Altenbach et al., 1994).

Table 1 also lists the depth parameter, Φ, for these labels, which was calculated using eq 2 from the $\Delta P_{1/2}$ values. Using a depth calibration obtained previously for air and NiEDDA in PS/PC membrane mixtures (Qin & Cafiso, 1996), distances were obtained for the nitroxides on the spin-labeled neuromodulin peptides from the lipid phosphate, and these depths are shown in Figure 6. Nitroxides at the ends of the peptide lie on the aqueous side of the membrane−solution interface, and nitroxides at most other positions are near or slightly within the interface. At positions 3 and 7 the nitroxides are deep in the bilayer hydrocarbon domain. As discussed below, this may be a result of the substitution of a polar or charged residue for a spin-label in a region that is relatively hydrophobic. Unlike peptides that form helical structures on membrane surfaces (Altenbach et al., 1989), there is no evidence for a helical pattern from the power saturation and depth data shown in Table 1 and Figure 6.

Evidence for an Extended Structure on Membranes. The lineshapes and power-saturation data provide a strong indication that the peptide is in an extended conformation with most of the nitroxide substitutions lying slightly below the membrane−solution interface. As an additional check on the secondary structure, a series of double spin-labeled peptides were synthesized. Shown in Figure 7A are spectra from double labeled peptides at positions 4−6, 4−7, and 4−8 bound to the membrane. When nitroxides on these side chains are in close proximity, they can interact through a collisional or dipole−dipole mechanisms, a process that has been shown to provide an indication of peptide secondary structure (Miick et al., 1992; Millhauser, 1992). In an α-helical configuration, *i*,*i*+3 and *i*,*i*+4 side chain interactions are equally likely, and greater than *i*,*i*+2 interactions. In 3_{10} configurations, *i*,*i*+3 interactions are much stronger than either *i*,*i*+2 or *i*,*i*+4. From the data in Figure 7A each of the three labeled positions produces identical spectra, and there was no evidence for collisional or dipole−dipole exchange that would be expected for a helical structure (*i*,*i*+2 ≈ *i*,*i*+3 ≈ *i*,*i*+4). Similar spectra are also obtained for double-labeled peptides at positions 10−14 and 11−14. Thus, the peptide when bound to membranes appears to be in an extended conformation in both the N- and C-terminal domains. Spectra of double labeled peptides in water also show no helical pattern and appear to be in an extended structure. In comparison, the spectra for peptides labeled at positions 4−6, 4−7, and 4−8 in methanol are shown in Figure 7B. The exchange pattern in methanol clearly provides evidence for a helical structure, and these spectra are very similar to those seen for the peptides in trifluoroethanol (*i*,*i*+2 < *i*,*i*+3 ≈ *i*,*i*+4). These EPR results are qualitatively identical to those found using NMR described above.

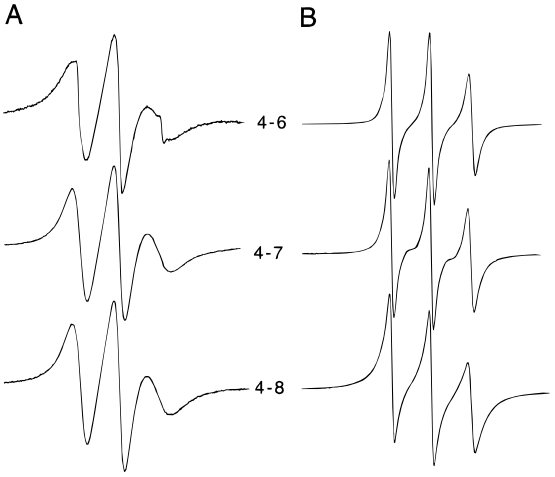

FIGURE 7: EPR Spectra for the double labeled neuromodulin derived peptides 4−6, 4−7, and 4−8 at a concentration of 50 μM in (A) 500 Å vesicles composed of PS/PC (25:75) in a buffer of 100 mM KCl/1 mM MOPS, pH = 7, and (B) methanol. Spectra were taken at 25 °C using a scan width of 100 G.

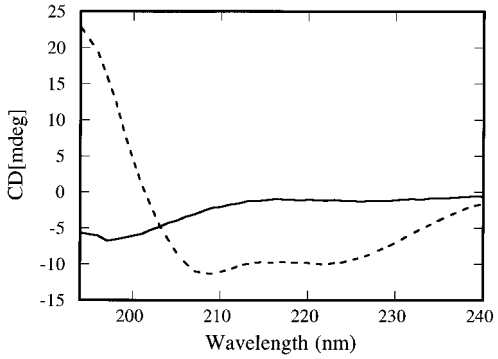

FIGURE 8: Circular dichroism spectra of the 30 μM neuromodulin peptide in methanol (dashed line) and aqueous solution (100 mM KCl/1 mM Tris, pH = 7.2) (solid line). Measurements were made on a Jasco J-720 spectropolarimeter using a 1 mm quartz cell, a band width of 2 nm, and a response time of 0.25 s.

The CD spectra of the native neuromodulin derived peptide were also obtained in membranes and in the solvent systems described above. Shown in Figure 8 are CD spectra in methanol and in buffer. Table 2 lists the secondary structure analysis of CD spectra obtained using the program PROSEC (Chang et al., 1978) in these solvents, in buffer, and bound to bilayers. In methanol and trifluoroethanol, the secondary structure appears to be primarily helical. In aqueous solution, the secondary structure is largely random coil or extended as judged by CD. The spectra and structural analysis in sonicated vesicles containing PC and PG indicate that the

Table 2: Estimate of Peptide Secondary Structure from CD[a]

solvent	% helix	% sheet	% β-turn	% random coil
methanol	64.1	6.0	0.0	29.9
trifluoroethanol	62.5	0.0	0.0	37.5
buffer	0.0	10.2	18.1	71.7
PG vesicles	9.6	19.1	5.2	66.1

[a] Secondary structure was estimated from the CD spectra in Figure 8 using the program PROSEC. Errors in the percent structure content are estimated to range from ±5% to ±15% based on the reproducibility of the spectra. The buffer contains 100 mM KCl/1 mM MOPS, pH = 7.

peptide also has a low helical content when bound to lipid bilayers.

DISCUSSION

The solution and membrane bound structure of a basic peptide based on the PKC and calmodulin binding domain of neuromodulin has been examined using several different spectroscopic approaches. NMR, site-directed spin-label EPR, and CD measurements described above are all in agreement and indicate that this peptide is in an extended structure in aqueous solution, but can adopt helical configurations in methanol or trifluoroethanol. EPR experiments on single and double nitroxide labeled peptides provide information on the position and secondary structure of the nitroxide when the peptide is membrane bound. The data are consistent with an extended peptide structure, where the ends of the peptide appear to lie just outside the membrane in the aqueous solution. CD measurements in PG containing membranes also indicate that the peptide adopts a nonhelical secondary structure when membrane bound, as do NMR measurements in SDS micelles.

The results obtained here indicate that charged peptides having strong electrostatic interactions with membrane surfaces do not necessarily adopt helical secondary structures when membrane bound. An extended membrane bound structure was recently found for a highly basic peptide based on the PKC and CaM binding domain of MARCKS (Qin & Cafiso, 1996), and for a basic peptide based on the cytochrome oxidase signal sequence (Yu et al., 1994). It is not presently clear whether an extended secondary structure is important to facilitate the interactions of PKC with substrates such as neuromodulin or MARCKS. Although the peptide described here is extended on the membrane interface, it does have the capacity to adopt a helical structure in methanol or TFE and it adopts a helical configuration when bound to calmodulin as determined using EPR (Qin et al., 1996).

Many peptides that associate with membranes appear to take on helical secondary structures. This is not unexpected, because the low dielectric and diminished water activity in the membrane should promote internal hydrogen bonding within the peptide (Engleman et al., 1986). Melittin is a charged peptide that interacts electrostatically with negatively charged membrane interfaces, and it assumes a regular helical structure when membrane bound (Altenbach et al., 1989). However, melittin also forms a strongly laterally amphipathic helix that interacts hydrophobically with the membrane interface. This is not the case for peptides derived from the PKC substrate and CaM binding domains of MARCKS and neuromodulin, which are not strongly amphipathic in a helical form. Because they are not buried deeply in the interface, an extended structure is possible for these peptides, and at the interface, hydrogen bonding to water or other polar groups in the interface is possible. An additional factor which could clearly drive these peptides into an extended conformation is electrostatic repulsion between lysine and arginine amino acid side chains. An extended structure would minimize these electrostatic forces, which would be enhanced at the membrane interface.

From the power-saturation data presented here, most of the labeled positions are at or near the membrane−solution interface, which is consistent with a location expected for a highly charged peptide. Locations deeper in the interface would not be expected due to the large Born charging energy required to place charged side chain within the low dielectric of the membrane interior. Several nitroxide positions such as those at 9, 10, and 11 are deeper and appear to be buried within the interface. This likely reflects the position that would be taken up by a more hydrophobic residue, as the nitroxide is comparable in hydrophobicity to more apolar amino acids such as leucine (Yu et al., 1994). In the case of MARCKS, the peptide backbone lies on the membrane interface with only its more hydrophobic residues buried within the interface (Qin & Cafiso, 1996), and this is likely the case with neuromodulin as well. Nitroxide substitutions at positions 3 and 7 are unusually deep and not consistent with the depths that are obtained at adjacent positions. These residues are unlikely to be buried within the hydrocarbon in the native peptide, and substitution of the more hydrophobic nitroxide for a polar or charged residue has likely altered the position of the peptide backbone. This effect of nitroxide substitution is not seen at the C-terminus or in peptides derived from MARCKS which are more highly charged than the N-terminus of neuromodulin.

It is well-known that basic, hydrophilic peptides bind to membranes containing acidic lipids (Dufourcq et al., 1981; de Kruijff et al., 1985; Roux et al., 1988; de Kroon et al., 1990; Kim et al., 1991; Mosior & McLaughlin, 1992). Generally this interaction can be accounted for by a combination of mass action and the Gouy−Chapman−Stern theory (Kim et al., 1991; Thorgeirsson et al., 1995), and more sophisticated theoretical approaches are being taken that include the molecular structures of the peptides and the membrane interface (Ben-Tal et al., 1996).

A number of these basic peptides, including those derived from MARCKS and neuromodulin, fail to bind to zwitterionic membranes and bind only to membranes containing some acidic lipid. One conclusion from this observation is that the interaction of these peptides is mainly due to electrostatic interactions with the membrane interface. This seems to be case for peptides such as pentalysine, which appear to lie on the aqueous side of the interface, and do not interact hydrophobically with the membrane (Roux et al., 1988; Kim et al., 1991; Montich et al., 1993). However, the work carried out here and elsewhere (Ito et al., 1993; Yu et al., 1994; Qin & Cafiso, 1996) clearly indicates that, for some of these peptides, the hydrophobic side chains can interact with the membrane interior. In these cases, the binding must include significant hydrophobic as well as electrostatic contributions.

If these peptides interact hydrophobically, why do they fail to bind membranes containing zwitterionic lipids? We speculate that they fail to bind neutral membranes because the hydrophobic interactions alone are insufficient to overcome a large Born repulsion that is experienced as they

approach a membrane interface. The Born repulsion results from the approach of charge to a boundary separating a low dielectric domain (Parsegian, 1969), and it can be overcome for these peptides by the addition of a favorable electrostatic attraction between the peptide and the interface (that is, by the addition of negatively charged lipid). Indeed, recent modeling of short lysine containing peptides identifies this repulsive force as an important component in the membrane association of charged peptides, and it correctly predicts the behavior of these peptides (Ben-Tal et al., 1996).

Recent work has demonstrated that the spin-labeled side chain used here is strongly influenced by backbone dynamics (Mchaourab et al., 1996). As described above, the motion of the nitroxide at several positions (e.g., 6, 7, and 8) is slower than that at other positions along the peptide, and power-saturation data indicate that this is not the result of tertiary contacts with other side chains. Because of its extended structure, hydrogen bonding interactions between the peptide backbone and lipids or water within the membrane interface should be possible, and these interactions may in part account for differences in label motion seen here. This result is in contrast to EPR spectra obtained previously for a charged peptide derived from MARCKS where all the nitroxide spectra show identical motion as a function of position (Qin & Cafiso, 1996).

Double spin-labeling techniques have recently been employed with peptides to distinguish secondary structure. This method, which relies on collisional and dipole exchange between two nitroxide labels, has been used to distinguish 3_{10} from α-helical secondary structures (Miick et al., 1992; Millhauser, 1992). Measurements of secondary structure were made here using this approach as well as CD and NMR for the neuromodulin based peptide in methanol and aqueous solution. In the limited cases examined here, the three approaches are in agreement. An advantage of the double spin-labeling approach is that it can be utilized to examine peptides that are bound to large macromolecules. It provides a means to investigate secondary structure in cases where CD is not feasible and where NMR may be limited by immobilization on a membrane or high molecular weight protein.

In conclusion, the structure of a peptide derived from the PKC and CaM binding domain of neuromodulin has been examined in solution and in membranes using site-directed spin-labeling. This basic peptide is in an extended structure in solution and bound to charged membranes, but it can adopt helical configurations in some solvents like methanol and TFE. When membrane bound, it is localized at the interface with the ends of the peptide extended into the aqueous solution and the hydrophobic side chains in the central portion of the peptide buried within the interface. Thus, this peptide interacts hydrophobically as well as electrostatically with the membrane interface. EPR spectroscopy indicates that the dynamics of the peptide backbone is not constant along its length, which may reflect hydrogen bond interactions between the peptide and the lipid bilayer interface.

ACKNOWLEDGMENT

We would like to thank Jeffrey Ellena for help with the NMR spectroscopy of the solution and micelle structures of the neuromodulin based peptide. We would also like to thank Stuart McLaughlin for helpful discussions during the course of this work.

SUPPORTING INFORMATION AVAILABLE

Tables containing the ^{1}H chemical shift assignments for the neuromodulin derived peptide in methanol, SDS micelles, and aqueous solution are available (3 pages). Ordering information is given on any current masthead page.

REFERENCES

Altenbach, C., Froncisz, W., Hyde, J. S., & Hubbell, W. L. (1989) *Biophys. J. 56*, 1183−1191.
Altenbach, C., Greenhalgh, D. A., Khorana, H. G., & Hubbell, W. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1667−1671.
Andreasen, T. J., Luetje, C. W., Heidman, W., & Storm, D. R. (1983) *Biochemistry 22*, 4615−4618.
Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., & Storm, D. R. (1990) *Biochemistry 29*, 2330−2335.
Archer, S. J., Ellena, J. F., & Cafiso, D. S. (1991) *Biophys. J. 60*, 389−398.
Bartlett, G. R. (1959) *J. Biol. Chem. 243*, 466−468.
Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 63*, 207−213.
Benowitz, L. I., & Lewis, E. R. (1983) *J. Neurosci. 3*, 2153−2163.
Ben-Tal, N., Honig, B., Peitzsch, R. M., Denisov, G., & McLaughlin, S. (1996) *Biophys. J.* (in the press).
Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521−528.
Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry 15*, 4818−4831.
Chan, S. Y., Murakami, K., & Routtenburg, A. (1986) *J. Neurosci. 6*, 3618−3627.
Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13−31.
de Kroon, A. I., Soekarjo, M. W., de Gier, J., & de Kruijff, B. (1990) *Biochemistry 29*, 8229−8240.
de Kruijff, B., Rietveld, A., Telders, N., & Vaandrager, B. (1985) *Biochim. Biophys. Acta 820*, 295−304.
Dufourcq, J., Faucon, J. F., Maget-Dana, R., Pileni, M. P., & Helene, C. (1981) *Biochim. Biophys. Acta 649*, 67−75.
Engleman, D. M., Steitz, T. A., & Goldman, A. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 321−353.
Flewelling, R. F., & Hubbell, W. L. (1986) *Biophys. J. 49*, 531−540.
Griesinger, C., Otting, G., Wüthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870−7872.
Houbre, D., Duportail, G., Deloulme, J.-C., & Baudier, J. (1991) *J. Biol. Chem. 266*, 7121−7131.
Ito, A. S., Castrucci, A. M. L., Hruby, V. J., Hadley, M. E., Krajcarski, D. T., & Szabo, A. G. (1993) *Biochemistry 32*, 12264−12272.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546−4553.
Katz, F., Ellis, L., & Pfenninger, K. H. (1985) *J. Neurosci. 5*, 1402−1411.
Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. A. (1991) *Biophys. J. 60*, 135−148.
Kim, J. K., Blackshear, P. J., Johnson, D. J., & McLaughlin, S. A. (1994) *Biophys. J. 67*, 227−237.
Liu, Y., & Storm, D. R. (1989) *J. Biol. Chem. 264*, 12800−12804.
Macura, S., & Ernst, R. R. (1980) *Mol. Phys. 41*, 95−117.
Mchaourab, H., Lietzow, M., Hideg, K., & Hubbell, W. (1996) *Biochemistry 35*, 7692−7704.
McLaughlin, S., & Aderem, A. (1995) *Trends Biochem. Sci. 20*, 272−276.
Miick, S. M., Martinez, G. V., Fiori, W. R., Todd, A. P., & Millhauser, G. L. (1992) *Nature 359*, 653−655.
Millhauser, G. L. (1992) *Trends. Biochem. Sci. 17*, 448−452.
Montich, G., Scarlata, S., & McLaughlin, S. (1993) *Biochim. Biophys. Acta 1146*, 17−24.
Mosior, M., & McLaughlin, S. A. (1992) *Biochemistry 31*, 1767−1773.
Nelson, R. B., Linden, D. J., & Routtenberg, A. (1989) *Brain Res. 497*, 30.
Parsegian, A. (1969) *Nature 221*, 844−846.
Peitzsch, R. M., & McLaughlin, S. M. (1993) *32*, 10426−10443.

Piantini, U., Sorensen, O. W., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800−6801.
Qin, Z., & Cafiso, D. S. (1996) *Biochemistry 35*, 2917−2925.
Qin, Z., Wertz, S. L., & Cafiso, D. S. (1996) *Biophys. J. 70*, A59.
Rance, M. (1987) *J. Magn. Reson. 74*, 557−564.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wuthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
Rodnight, R. (1982) *Prog. Brain Res. 56*, 1−25.
Roux, M., Neumann, J.-M., Bloom, M., & Devaux, P. F. (1988) *Eur. Biophys. J. 16*, 267−273.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53−56.
Skene, J. H. P., & Willard, M. (1981) *J. Cell Biol. 89*, 96−103.
Thorgeirsson, T. E., Yu, Y. G., & Shih, Y.-K. (1995) *Biochemistry 34*, 5518−5522.
Towler, D. A., Gordon, J. I., Adams, S. P., & Glaser, L. (1988) *Annu. Rev. Biochem. 57*, 69−99.
van Dongen, C. J., Zwiers, H., De Graan, P. N. E., & Gispen, W. H. (1985) *Biochem. Biophys. Res. Commun. 128*, 1219−1227.
van Hoof, C. O. M., De Graan, P. N. E., Oestreicher, A. B., & Gispen, W. H. (1988) *J. Neurosci. 8*, 1789−1795.
Wishart, D. S., Sykes, B. D., & Richards, F. M. (1992) *Biochemistry 31*, 1647−1651.
Wüthrich, K. (1986) *NMR of proteins and nucleic acids*, John Wiley and Sons, New York.
Yu, Y. G., Thorgeirsson, T. E., & Shin, Y.-K. (1994) *Biochemistry 33*, 14221−14226.
Zwiers, H., Weigant, V. M., Schotman, P., & Gispen, W. H. (1978) *Neurochem. Res. 3*, 455−463.

BI961248X