Membrane Structure of Protein Kinase C and Calmodulin Binding Domain of Myristoylated Alanine Rich C Kinase Substrate Determined by Site-Directed Spin Labeling†

Zhihai Qin and David S. Cafiso*

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

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ABSTRACT: Cysteine-substituted peptides based on the membrane, calmodulin, and protein kinase C binding domain of the myristoylated alanine rich C kinase substrate (MARCKS) were synthesized and derivatized with a sulfhydryl reactive proxyl nitroxide. These spin-labeled peptides were used in combination with continuous wave power saturation electron paramagnetic resonance (EPR) spectroscopy to determine the position and structure of the peptide on membranes containing phosphatidylserine. These peptides bind at the membrane interface, with nitroxide side chains in the central and C-terminal regions lying several angstroms below the level of the head group. In contrast, the N-terminus of the peptide is extended out of the membrane interface so that the two N-terminal residues are positioned on the aqueous side of the head group. When bound to the membrane, the N-terminal segment of this peptide is sensitive to the membrane surface charge density. Higher charge densities decrease the amplitude of side chain motions at the N-terminus and bring this end of the peptide closer to the membrane interface. When the location of successive residues along the bilayer normal is compared, no helical trend is seen, and no evidence for aggregation of the peptide is found. The EPR spectra of double spin-labeled peptides also show no evidence for a helical structure. Thus, these basic peptides are in an extended configuration at the membrane interface with hydrophobic side chains oriented inward toward the membrane hydrocarbon.

Protein kinase C (PKC)[1] performs a number of critical functions in cell-signaling pathways; however, the activity and molecular function of many PKC substrates is not well-understood. In addition, the molecular interactions between PKC and its substrates are not well-characterized. The myristoylated alanine rich C kinase substrate (MARCKS) is one of the major PKC substrates in brain and a number of other tissues (Taniguchi & Manenti, 1993), and it is currently of interest in signal transduction because it appears to mediate interactions between PKC, and calmodulin (CaM), mediated signaling pathways.

A 25-amino acid peptide derived from MARCKS (residues 151−175) contains both a calmodulin binding domain and four sites that can be phosphorylated by PKC (McIlroy et al., 1991). In addition, this segment has been shown to associate electrostatically with membrane surfaces containing acidic lipids. This segment of MARCKS is highly basic, containing a total of 12 lysines and one arginine residue with the sequence KKKKKRFSFKKSFKLSGFSFKKNKK. Estimates of the electrostatic and hydrophobic binding energies of the intact MARCKS protein indicate that it likely requires both myristoylation and the electrostatic interaction of this basic domain to attach to the membrane interface and that neither interaction alone is sufficient (Kim et al., 1994). Phosphorylation of this MARCKS-derived peptide dissociates it from the membrane and also dramatically reduces its affinity for CaM (Kim et al., 1993). Thus, this segment of MARCKS is in equilibrium between the solution, the membrane, and a CaM-bound form. It has been proposed that MARCKS may act to control free CaM levels through PKC-mediated pathways (McIlroy et al., 1991). The membrane attachment of MARCKS presumably serves to make the CaM binding motif a good substrate of PKC, and phosphorylation of MARCKS then increases cellular levels of CaM. In essence, MARCKS appears to be a PKC-regulated calmodulin buffer.

The electrostatic attachment of proteins to membrane interfaces through a basic domain appears to be a general phenomena. In addition to MARCKS, other examples of proteins that may attach through a positively charged region include PKC (Mosior & McLaughlin, 1991; Newton, 1993), neuromodulin (Kim et al., 1994), the HIV gag protein (Zhou et al., 1994), and the Src family of nonreceptor tyrosine kinases (Buser et al., 1994). In the case of v-Src, attachment through a basic domain near its N-terminus appears to be necessary for cell transformation (Buser et al., 1994). In spite of the importance of this type of protein−membrane electrostatic interaction for cell signaling and control, relatively little molecular information is available on the interaction of these charged domains with membrane surfaces. Several studies on the electrostatic interaction of

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

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[1] Abbreviations: CaM, calmodulin; CD, circular dichroism; DMF, dimethylformamide; EPR, electron paramagnetic resonance; egg PC, egg yolk phosphatidylcholine; HIV, human immunodeficiency virus; MARCKS, myristoylated alanine rich C kinase substrate; MTSSL, *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl); NiEDDA, nickel ethylenediaminediacetic acid; MBHA, *p*-methylbenzhydrylamine; NMM, *N*-methylmorpholine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PC, phosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; PKC, protein kinase C; PS, phosphatidylserine; v-Src, pp60$^{v\text{-}src}$; TFA, trifluoroacetic acid; TFE, trifluoroethanol; CW, continuous wave.

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charged peptides with charged membrane interfaces have been carried out (de Kruijff et al., 1985; Roux et al., 1988; Kim et al., 1991; Mosior & McLaughlin, 1992). Peptides such as pentalysine bind strongly to membrane interfaces containing acidic lipids but show no binding to neutral membranes containing only phosphatidylcholine. The binding to membranes appears to be highly cooperative and can be qualitatively accounted for by the Gouy−Chapman theory (Kim et al., 1991). The highly charged nature of these peptides, the acidic lipid requirement for binding, and ^{2}H NMR data (Roux et al., 1988) have led to the conclusion that these peptides interact with the surface but do not penetrate into the membrane interface. Clearly, additional information on the molecular interactions of these highly charged domains with membranes would be highly desirable. This information would allow for more sophisticated modeling of peptide−membrane electrostatic interactions and would provide insight into the interaction of PKC with its substrates.

In this report, the synthesis and characterization of 15 cysteine-substituted peptides based on the CaM binding and PKC substrate site on MARCKS is described. These peptides are derivatized with nitroxide probes, and EPR spectroscopy is used to evaluate the conformation and position of these peptides when bound to membranes containing acidic lipids. The results indicate that this basic peptide is in an extended conformation at the interface except at the N-terminus where the peptide extends into the aqueous solution. The structure of the bound peptide is also observed to be sensitive to changes in the membrane surface charge density.

EXPERIMENTAL PROCEDURES

Materials

Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), palmitoyloleoylphosphatidylglycerol (POPG), head group spin-labeled PC, and spin-labeled doxyl phosphatidylcholines [1-palmitoyl-2-steroyl(*n*-doxyl)phosphatidycholines, $n = 5$, 7, 10, or 12] were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The methanethiosulfonate spin-label, *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl) (MTSSL), was obtained from Reanal (Budapest, Hungary). Nickel ethylenediaminediacetic acid (NiEDDA) was the generous gift of Dr. Christian Altenbach. (Benzotriazolyloxy)tris(pyrrolidino)-phosphonium 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).

Methods

Synthesis and Labeling of MARCKS-Derived Peptides. A series of 25 residue peptides corresponding to the CaM binding and PKC substrate domain of MARCKS were synthesized on a Gilson Automated Multiple Peptide System (AMS 422) using a rink amide *p*-methylbenzhydrylamine (MBHA) resin. In addition to the native peptide, 12 peptides were synthesized with a single cysteine substitution at positions 2−5, 11−14, and 21−24. Three double cysteine-substituted peptides were also produced with cysteines at positions 12 and 14, 12 and 15, and 12 and 16. Following synthesis, the peptides were cleaved from the resin and protecting groups were removed by reaction with a solution of 89% trifluoroacetic acid, 3% thioanisole, 2% anisol, 3% dithioethanol, and 3% water for 5 h at room temperature. The peptides were precipitated in cold ether and then lyophilized.

The crude cysteine-substituted MARCKS peptides were derivatized with the sulfhydryl reactive MTSSL by dissolving approximately 2−3 mg of crude peptide in 1 mM MOPS (pH 7.0) containing a 5−10-fold molar excess of MTSSL. The label was allowed to react for approximately 4 h at room temperature, and the derivatized peptides were purified on a Poros perfusion reversed phase high-performance liquid chromatography (HPLC) column (PerSeptive Biosystem, Cambridge, MA) using a 14−30% water/acetonitrile gradient containing 0.1% trifluoroacetic acid. EPR spectroscopy and electrospray/ionization mass spectrometry were used to confirm the identity of the isolated peptides. Mass spectrometry yielded an *m/z* for the native MARCKS peptide of 3125, and for the spin-labeled derivatives K2C-SL, K3C-SL, K4C-SL, K5C-SL, K11C-SL, K14C-SL, K21C-SL, K22C-SL, and K24C-SL, the value of *m/z* was found to be 3285. For S12C-SL, F13C-SL, and N23C-SL, values for *m/z* of 3326, 3266, and 3299 were found, respectively. The double nitroxide-labeled peptides with derivatized cysteines at 12 and 14, 12 and 15, and 12 and 16 gave *m/z* values of 3486, 3501, and 3527, respectively.

Preparation of PS-Containing Membranes. Mixtures containing preweighted amounts of PC and PS were dried in a vacuum desiccator overnight and hydrated using a buffer of 1 mM MOPS and 100 mM KCl (pH = 7.0). The lipid suspension was then freeze−thawed five times and extruded through polycarbonate filters (500 Å pore size) using a mini LiposoFast extruder (Avestine, Ottawa, Canada) to produce unilamellar vesicles. Measurements of membrane binding of the spin-labeled MARCKS peptides were made directly from the EPR spectra by measuring amplitude changes in the $m_I = -1$ resonance as described previously (Archer et al., 1991). For these measurements, the total lipid concentration, which was approximately 150 mM, was accurately determined for each experiment using a modified Fiske−Subbarow phosphate assay (Bartlett, 1959). The PS concentration was varied from 10 to 40 mol % for these binding measurements, and the labeled peptide was added to a final concentration of 100−150 μM. The partition coefficients, β, reported here for spin-labeled peptides represent the ratio of the surface density of bound peptide to the aqueous peptide concentration. If λ represents the mole ratio of membrane bound to aqueous peptide, then β is related to λ by

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

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.

EPR Spectroscopy and cw Power Saturation. EPR spectra were recorded on a Varian E-line Centuries series spectrometer using an X-band loop gap resonator with a standard two-loop, one-gap configuration. Unless otherwise indicated, the microwave power was 2 mW and the peak-to-peak modulation amplitude was 1.25 g. Quartz capillary tubes, i.d. = 0.6 mm and o.d. = 0.84 mm (Vitro Dynamics, Inc., Rockaway, NJ), were used to hold nitroxide samples and were nominally filled with 5 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 MARCKS peptides that were totally membrane-bound to PC/PS lipid vesicles containing 25 mol % PS. Peptides were added at concentrations of approximately 100 μM to preformed vesicle mixtures with lipid concentrations of approximately 100 mM. The microwave power was increased from 0.1 to 100.0 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

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

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 in this case). The parameter Φ was calculated using eq 3 from the experimentally measured values of $\Delta P_{1/2}$ obtained in the presence of air and NiEDDA.

$$\Phi = \ln\left[\frac{\Delta P_{1/2}(O_2)}{\Delta P_{1/2}(\text{NiEDDA})}\right] \qquad (3)$$

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 was obtained for the parameter Φ as a function of depth within the bilayer using a series of nitroxide-labeled PCs. The parameter Φ was measured for the head group spin-labeled PC and spin-labeled PCs with doxyl nitroxides at positions 5, 7, 10, and 12 along the alkyl chain. These spin-labels were codissolved with PS and PC in chloroform before the lipids were dried, hydrated, and extruded to form vesicles. For these measurements, the lipid was at a total concentration of approximately 60 mM and spin-labels were incorporated at a concentration of about 0.5 mol %.

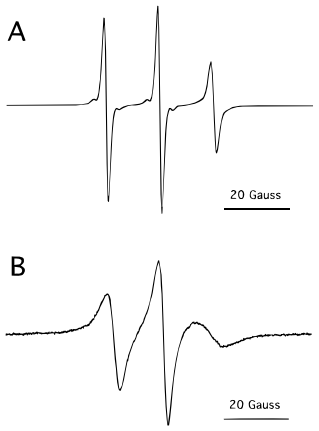

FIGURE 1: (A) EPR spectrum of approximately 60 μM K11C-SL in aqueous solution. (B) Spectrum of K11C-SL completely bound to membranes containing 25 mol % PS. The slower motion of the membrane-bound peptide is seen as an increase in the EPR line width and a change in the relative amplitudes of the nitroxide resonances.

Estimates of the rotational mobility of the nitroxide probes derivatized to peptides were made from the transverse relaxation rates measured from the EPR spectra as described elsewhere (Keith et al., 1970; Nordio, 1976; Archer et al., 1991). These estimates assume that the motion of the nitroxide is isotropic and are used only to provide a comparison of the motion between various peptide domains. They are not intended to represent a rigorous analysis of the motion for the labels studied here.

CD Measurements. CD measurements were made on a Jasco J-720 Spectropolarimeter using a 1 mm quartz cell at room temperature. Each spectrum was an average of 10 scans taken at 50 nm/min with a scan rate in steps of 1 nm. A band width of 1 nm and a time constant of 0.25 s were used. Samples were flushed for 20 min to eliminate any molecular oxygen in the buffer solution. PG was substituted for PS in these experiments in order to avoid interference from the absorption of PS that occurs at less than 200 nm. The measurements were made on samples of small sonicated vesicles, prepared as described previously (Castle & Hubbell, 1976), at a concentration of approximately 2 mM lipid to avoid light-scattering problems. Under these conditions, virtually all the peptide is membrane-associated.

RESULTS

The N-Terminus of MARCKS Exhibits More Motion Than the Middle or C-Terminus. Shown in parts A and B of Figure 1 are spectra for a peptide derived from MARCKS derivatized with a spin-label at position 11 (K11C-SL) in aqueous solution and in the presence of vesicles containing 25 mol % PS in PC. The nitroxide spectra of these labeled peptides are typical of other nitroxide-labeled peptides that are freely diffusing in solution. The spectra in solution correspond to isotropic correlation times of approximately 0.1 ns. In the presence of membranes containing PS, the EPR spectra are dramatically different, consistent with a label that is more dynamically restricted and undergoing anisotropic motion. For this spectrum, the peptide is completely membrane-associated. In more dilute membrane suspensions, this peptide partitions between the membrane surface and the aqueous phase, leading to a composite EPR spectrum. In this case, the partitioning of the peptide to charged lipid

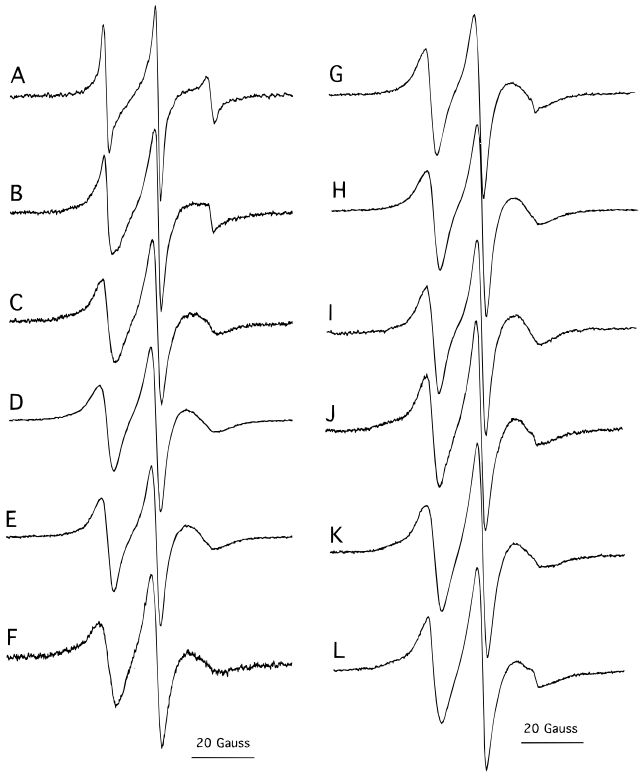

FIGURE 2: EPR spectra corresponding to membrane-bound peptides labeled at 12 different positions on the MARCKS-derived peptide. A−D correspond to labels on residues 2−5, respectively; E−H correspond to labels on residues 11−14, respectively; and I−L correspond to labels on residues 21 and 22, respectively. The membranes were at a total concentration of approximately 200 mM lipid containing 25 mol % PS, and the nitroxide concentrations ranged from 100 to 150 μM.

vesicles can be extracted from the composite spectrum (Archer et al., 1991). No binding of these peptides to membranes that consisted only of PC was detected. The EPR spectra for completely membrane-associated spin-labeled MARCKS peptides bound to PS/PC vesicles containing 25 mol % PS are shown in Figure 2.[2] These nitroxide-labeled peptides include labels at positions 2−5, 11−14, and 21−24.

A comparison of these spectra shows a similar degree of motion and anisotropy throughout the central portion of the peptide as well as throughout the peptide C-terminus. However, spin-labels placed at positions 2 and 3 yield spectra that reflect a higher degree of mobility than the rest of the peptide. A comparison of several parameters obtained from these MARCKS-derived peptides is shown in Table 1. Table 1 lists the correlation times for the spin-label that are obtained if the label motion is assumed to be isotropic as well as the hyperfine splittings obtained for each labeled position. The label motion is clearly anisotropic, and the numbers listed here are only used to provide a rough comparison of motion between labels located at different side chain positions. Except for the second and third positions from the N-terminus, all the labeled positions show a similar motional rate and have roughly equivalent hyperfine splittings, indicating that these segments lie roughly in an environment of similar polarity. The N-terminal residues are, however, both more mobile and more exposed to the aqueous phase than the rest of the peptide. The hyperfine splitting (A_0) for K2C-SL is close to that obtained for nitroxides in bulk water, while the values for nitroxides placed in the central and C-terminal domains are close to the values usually obtained for nitroxides in the normal alkanols (Morrisett, 1976).

[2] The spectra shown in Figure 2 originate entirely from membrane-bound peptide, and there is no free peptide present in these samples. The lipid concentrations used are at least 1 order of magnitude higher than is needed to completely bind the peptide, and further increases in lipid concentration do not alter the spectra shown in Figure 2. The more mobile component that appears in the spectra of labels at positions 2 and 3 does not correspond to the spectrum of free peptide or the free proxyl nitroxide.

Table 1: Correlation Times (τ_c) and Averaged Hyperfine Coupling Constants (A_0) for Spin-Labeled Membrane-Associated MARCKS Peptides

peptide	τ_c (ns)	A_0 (G)
K2C-SL	1.1	16.9
K3C-SL	2.0	16.4
K4C-SL	3.5	16.0
K5C-SL	3.6	16.0
K11C-SL	3.0	16.0
S12C-SL	3.9	15.8
F13C-SL	3.3	16.0
K14C-SL	3.2	15.6
K21C-SL	2.8	16.0
K22C-SL	3.8	15.8
N23C-SL	4.3	15.8
K24C-SL	3.6	16.1

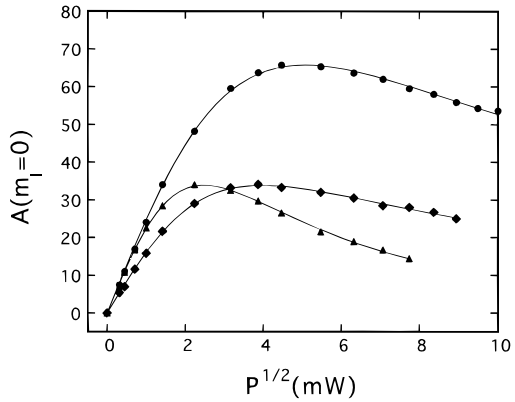

FIGURE 3: Power saturation curves obtained for K11C-SL. Amplitudes of the $m_I = 0$ line in the absence of any paramagnetic reagent (▲), in the presence of air (●), and in the presence of 40 mM NiEDDA (◆). The solid lines represent fits to the data using eq 2.

MARKCS Derived Peptides Are in an Extended Structure Near the Membrane Interface with the N-Terminus Lying in the Aqueous Phase above the Interface. Hydrophobic and hydrophilic paramagnetic agents have been used previously in egg PC membranes to calculate the bilayer depth of protein and alkyl chain-labeled nitroxides (Altenbach et al., 1994). This approach was used here to examine the depth of side chains associated with peptides based on the PKC and calmodulin binding domain of MARCKS. Shown in Figure 3 are power saturation curves for the MARCKS-derived peptide K11C-SL in the absence of any paramagnetic species and in the presence of O_2 or NiEDDA. The presence of these paramagnetic species enhances the power required to produce saturation of the electron spin, and the measured $P_{1/2}$ values are related to the frequency of collisions with these reagents. Because the system used here contains a lipid mixture different than that used previously (Altenbach et al., 1994), the values of Φ obtained as a function of depth were calibrated using a head group nitroxide and a series of chain-

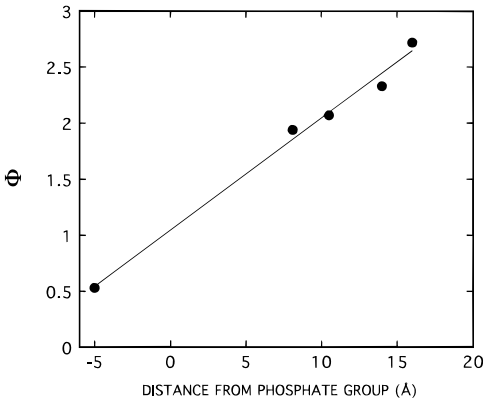

FIGURE 4: Depth calibration for the values of Φ, calculated using eq 2, versus the known depths of the nitroxide labels. Values for the depths of the *n*-doxyl PCs with $n = 5$, 7, 10, and 12 were obtained from Dalton et al. (1987). The head group-labeled PC was assigned to a position of −5 Å (see text).

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

peptide	$\Delta P_{1/2}$ (air)[a]	$\Delta P_{1/2}$ (NiEDDA)[a]	Φ
K2C-SL	8.47	18.4	−0.78
K3C-SL	16.5	8.82	0.63
K4C-SL	24.3	4.16	1.8
K5C-SL	22.3	6.50	1.2
K11C-SL	29.5	3.99	2.0
S12C-SL	30.6	4.83	1.9
F13C-SL	29.0	5.85	1.6
K14C-SL	29.2	5.16	1.7
K21C-SL	26.9	4.76	1.7
K22C-SL	23.5	4.57	1.6
N23C-SL	24.3	6.48	1.3
K24C-SL	25.7	4.26	1.8

[a] Values for $\Delta P_{1/2}$ are given in milliwatts. Air contains approximately 20% O_2 which is a hydrophobic paramagnetic species; NiEDDA was added to a final concentration of about 40 mM.

labeled PC nitroxides. Figure 4 shows a plot of the values of Φ obtained for head group-labeled tempo-PC, and for the *n*-doxyl PCs where $n = 5$, 7, 10, and 12 as a function of the previously determined depths for these nitroxide labels (Dalton et al., 1987). As described previously (Farahbakhsh et al., 1992), the position of the head group-labeled PC was assigned to a distance of −5 Å, which places it into the aqueous phase. As seen in Figure 4, the nitroxide depths are approximately linear as a function of Φ over the range examined. We did not use doxyl PCs labeled near the end of the acyl chain as these labels have been shown to deviate significantly from the linear relationship shown in Figure 4 (Altenbach et al., 1994).

Shown in Table 2 are the $\Delta P_{1/2}$ values obtained in the presence of O_2 and NiEDDA for 12 spin-labeled derivatives of the MARCKS-derived peptide. Also shown in Table 2 are the values of Φ calculated from eq 3. For residues 4, 5, 11−14, and 21−24, the nitroxides show a similar accessibility to O_2 and NiEDDA; however, for residues 2 and 3, there is a decreased accessibility to O_2 and an increased accessibility to NiEDDA. The values of Φ are not strongly affected by local atom density around the nitroxide and provide a more accurate reflection of depth than do the $\Delta P_{1/2}$ values alone (Altenbach et al., 1994). For Φ, all the positions labeled yielded similar values except positions 2 and 3, which clearly exhibit significantly smaller values than other regions of the peptide.

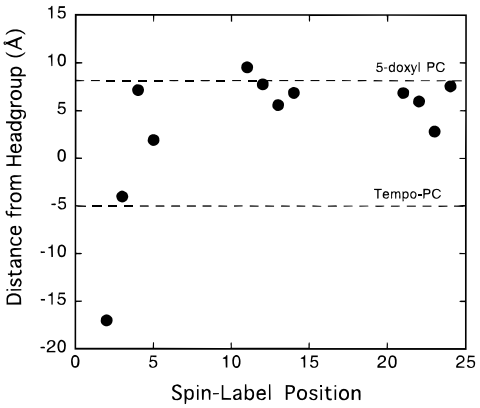

FIGURE 5: Nitroxide distances from the membrane solution interface as a function of the position of the label on the MARCKS peptide sequence. The distances are obtained using the Φ values for the spin-labeled MARCKS peptides shown in Table 2 and the depth calibration shown in Figure 4.

When the values for Φ obtained from Table 2 are compared with the positions defined by the calibration curve in Figure 4, it can be seen that all residues except 2 and 3 appear to lie below the level of the head group phosphate. Most of the residues are slightly shallower than the 5-doxyl PC label. Residue 3 resides near tempocholine PC, and residue 2 is positioned even further out into the external aqueous space. This observation regarding the N-terminus is qualitatively consistent with the line widths and hyperfine coupling constants obtained above. For positions 4, 5, 11−14, and 21−24, the Φ values indicate that these side chains take up similar positions in the bilayer. Shown in Figure 5 is a plot of the nitroxide depths, determined from the Φ values in Table 2 and the calibration curve in Figure 4, plotted as a function of the residue position. The nitroxide at position 2 lies significantly outside the limits of our calibration curve and was determined by extrapolation from the linear behavior shown in Figure 4. Because the paramagnetic species change little in concentration at significant distances from the interface, the uncertainty in the position of this residue may be quite high. For residues within the interface, the error is much lower. On the basis of the error in the power saturation measurements, the position for residues 4, 5, 11−14, and 21−24 is accurate to within 2−3 Å. It can be seen from Figure 5 that there is a significant distance separation between positions 2 and 3 (>10 Å). A large separation between these residues is consistent with the positions of nitroxides on an extended structure which turns out of the membrane. From Figure 5, the total variation in nitroxide depths among positions 4−24 is relatively small (within 5−6 Å), and their is no indication for differences between sequential residues that would be consistent with a helical structure. When combined with the observation that line widths and coupling constants also show no helical periodicity, these data suggest that this peptide is in an extended conformation on the membrane surface. The peptides examined here appear to be monomeric as there is no evidence for spin−spin exchange or other interactions that would accompany aggregation (Barranger-Mathys & Cafiso, 1994).

As a check on the membrane conformation of this basic region of MARCKS, we synthesized three additional doubly labeled peptides. Pairs of residues were switched to cysteines and labeled with the sulfhydryl specific label MTSSL. This

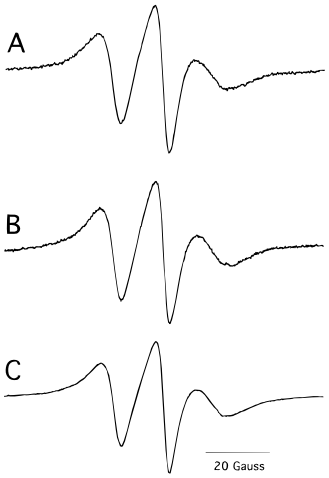

FIGURE 6: EPR spectra of double spin-labeled MARCKS-derived peptides bound to membrane vesicles containing 25 mol % PS in PC. Proxyl nitroxides are derivatized onto (A) positions 12 and 14, (B) positions 12 and 15, and (C) positions 12 and 16. There is very little spin exchange in these samples, which is roughly equivalent for each of the labeled peptides. This result is consistent with this peptide being present in an extended configuration.

included positions 12 and 14, 12 and 15, and 12 and 16, and the spectra of these peptides bound to vesicles containing 25 mol % PS are shown in Figure 6. The spectra of these peptides reveal $i-i+2$, $i-i+3$, and $i-i+4$ side chain interactions and are shown in parts A−C of Figure 6, respectively. These spectra show only weak evidence for spin exchange and are very similar in shape. This is in contrast to spectra which are observed for doubly labeled peptides which are in helical conformations (Millhauser, 1992). An α-helical conformation would have shown strong spin exchange as a result of interactions between labeled side chains in the $i-i+3$ and $i-i+4$ positions. The lack of these interactions and the similarity of these three spectra are most easily explained if this peptide is in an extended conformation.

This result is in agreement with the results of CD measurements on the MARCKS-derived peptide. Shown in Figure 7 are CD spectra of the native (unlabeled) MARCKS-derived peptide (A) in trifluoroethanol (TFE) and (B) bound to small sonicated vesicles composed of PC and 25 mol % POPG. The spectra obtained in TFE resemble those obtained in methanol, and the strong negative bands in these spectra are those expected from a helical secondary structure. The spectrum shown in Figure 7B is similar to that obtained in aqueous solution and closely resembles that expected for a random coil. CD spectra were taken on several of the spin-labeled peptides,, and they produced results identical to that of the native MARCKS-derived peptide. It should be noted that the CD measurements require much higher membrane densities of peptide, primarily because light scattering limits the concentration of lipid that can be used.

On the basis of residue surface areas, a nitroxide-labeled cysteine has a hydrophobicity similar to that of phenylalanine. One concern regarding the use of these spin-labels is the possibility that nitroxide substitutions might perturb the position of the peptide, for example, yielding a deeper binding position than actually occurs for the native peptide. To test for this possibility, we generated membrane binding curves for several spin-labeled peptides. Because the nitroxide at position 2 remains in the aqueous phase, the free

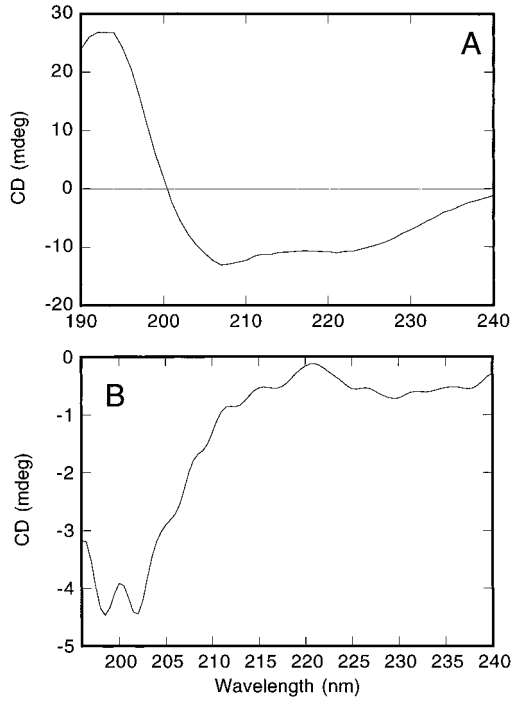

FIGURE 7: CD spectrum of the native MARCKS-derived peptide (A) at a concentration of 30 μM in TFE and (B) in the presence of sonicated PC vesicles containing 25 mol % POPG in 10 mM Tris and 100 mM KCl (pH 7.4). The spectra indicate a largely α-helical structure in TFE, but a random structure when membrane-associated. These measurements are in qualitative agreement with those obtained using EPR spectroscopy.

energy for the partitioning of this peptide should be quite different than that for peptides substituted in the central or C-terminus if these nitroxides are displacing the peptide into the membrane interface. In all cases that were examined, similarly substituted residues yielded similar partition coefficients. For example, for the second position, K2C-SL, a partition coefficient of 1.6×10^{-4} cm was found at 25 mol % PS. For the peptide labeled at position 11, K11C-SL, a partition coefficient of 1.5×10^{-4} cm was obtained. This pair provides a comparison of a nitroxide position which lies outside the membrane−solution interface with a position where the nitroxide is buried within the interface. The difference in the free energy of partitioning for these two peptides is insignificant and is less than 100 cal/mol. Therefore, the placement of the nitroxide has little effect on the partition free energy of the peptide. The simplest interpretation of this result is that the presence of a nitroxide does not significantly alter the position of the peptide.

The N-Terminal Peptide Conformation Is Dependent on Membrane Charge Density. The association of this MARCKS-derived peptide with membranes is strongly driven by electrostatic interactions with the membrane−solution interface, and variations in membrane charge density might affect the position or dynamics of the peptide on the membrane surface. To test for this possibility, the spectra obtained for membrane-associated spin-labeled peptides were compared over a PS concentration from 10 to 40 mol %. Spectra were obtained in the presence of high lipid concentrations so that only signals associated with membrane-bound peptide were observed. Shown in Figure 8 is a comparison of spectra obtained at 10 and 40 mol % PS for labels at positions 2, 3, and 13. Almost no change in line shape is seen for position 13 (K13C-SL) over this range of charge

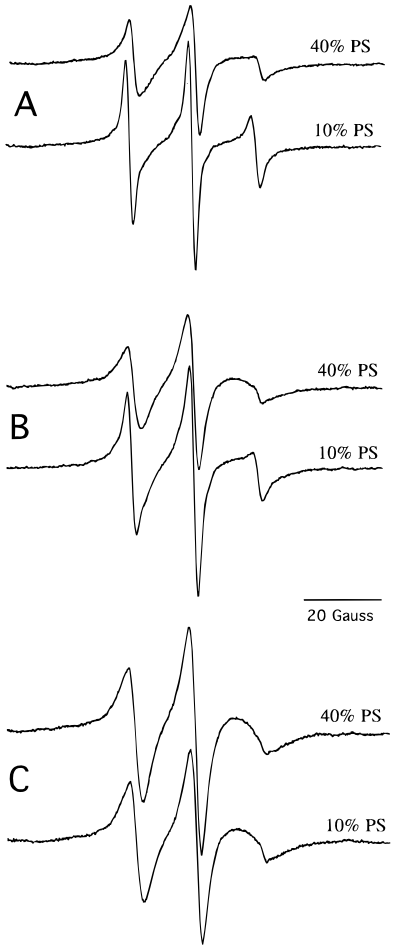

FIGURE 8: EPR spectra for membrane-associated MARCKS-derived peptides labeled at (A) position 2, (B) position 3, and (C) position 13 in PC membranes containing PS. Two spectra for each position are shown, one at 10 mol % PS and the other at 40 mol % PS in PC. Positions 2 and 3 are unique in that they show a decreased spectral amplitude and increased line width with an increase in charge density. All other positions are similar to position 13 in that they show little or no change as the membrane charge density is varied. For these spectra, lipid concentrations were 150 mM at 10 mol % and 160 mM at 40 mol %. Spin-labels were at a concentration of approximately 100 μM.

Table 3: Dependence of Rotational Correlation Times (τ_c) and Averaged Hyperfine Coupling Constants (A_0) on Membrane Charge Density[a]

peptide	τ_c (10% PS)	τ_c (40% PS)	A_0 (10% PS)	A_0 (40% PS)
K2C-SL	0.13	0.27	17.0	16.6
K3C-SL	0.22	0.35	16.5	16.0
K13C-SL	0.35	0.39	16.0	15.9

[a] Values of τ_c are given in nanoseconds, and values of A_0 are given in gauss.

density (Figure 8C), and this result is reproduced for other positions in the central portion of the peptide as well as for the C-terminal domain of the peptide. However, for positions 2 and 3, significant changes in line width and amplitude are seen. In both cases, an increase in the charge density results in a decrease in signal amplitude and a broadening of the EPR line widths. Shown in Table 3 are rotational correlation times and hyperfine splittings associated with positions 2, 3, and 13 at 10 and 40 mol % PS. Except for position 13, which shows little change in τ_c or A_0 at the two extreme charge densities, positions 2 and 3 exhibit an increased τ_c and a decreased A_0 as the charge density is increased. This is consistent with the N-terminal domain being drawn closer to the membrane interface and experiencing a more restricted motion at higher charge densities.

DISCUSSION

Previous work on a peptide derived from the PKC substrate and CaM binding domain of the MARCKS protein has shown that it binds strongly to bilayers containing acidic lipids as a result of electrostatic interactions (Kim et al., 1993). The data obtained here provide evidence that this peptide lies near the membrane−solution interface in an extended, nonhelical configuration with the N-terminus bending out into the aqueous phase. In addition, the N-terminal domain of the peptide is sensitive to the density of negative charge at the membrane interface. The N-terminus moves closer to the interface, and it becomes more restricted in its motion as the surface charge density becomes more negative. Although these peptides are extended when bound to the membrane, they apparently take up a helical configuration when bound to CaM (Z. Qin and D. S. Cafiso, unpublished). This is also the case for a related peptide we have studied based on the PKC and CaM binding domain of neuromodulin (S. Wertz and D. S. Cafiso, unpublished).

Approaches similar to the one used here have been used to examine the configuration and placement of membrane-bound peptides. For example, the membrane structure of the cytochrome oxidase signal sequence was recently examined using site-directed spin labeling (Yu et al., 1994). This peptide was also found to be in an extended configuration parallel to the membrane interface in the presence of acidic lipids. An earlier EPR study found a quite different conformation for melittin, where clear evidence for an α-helical configuration was obtained from an analysis of both the line widths and the power saturation data of spin-labeled peptides (Altenbach et al., 1989).

Previous work utilizing ^{2}H NMR has indicated that charged peptides such as pentalysine do not produce changes in the quadrupolar splitting of head group-labeled PCs in mixtures of PC and PS (Roux et al., 1988). Even though these peptides affect the quadrupolar splitting of ^{2}H-labeled PS, these results were taken as evidence that these charged peptides do not penetrate within the head group when associated with the membrane. Although these measurements are indirect, the placement of these highly basic peptides outside the membrane solution interface appears to be reasonable because of the high energy associated with burying charges within a low dielectric domain. This conclusion is consistent with surface pressure measurements which do not show any indication for the penetration of pentalysine into PC/PS monolayers upon binding (V. Boguslavsky and S. McLaughlin, unpublished).

The 12 lysines and one arginine in the MARCKS-derived peptide studied here make it a highly positively charged peptide (+13) at neutral pH. However, unlike pentalysine, the MARCKS-derived peptide contains a number of hydrophobic residues including 5 phenylalanines. As seen above in Figure 5, each labeled position, with the exception of the N-terminus, shows bilayer depths that are several anstroms below the membrane−solution interface. This result can be interpreted in two different ways. First, these residues may be buried because the peptide backbone itself lies below the level of the membrane−solution interface. Alternatively, the

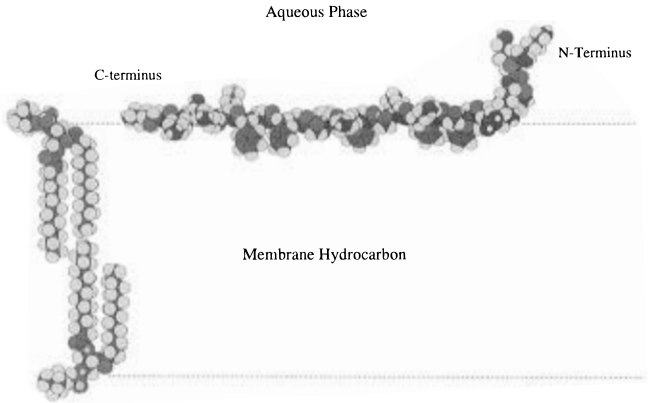

FIGURE 9: A CPK model showing the MARCKS-derived peptide in an extended structure with its N-terminus turned perpendicular to the membrane interface. Two molecules of dimyristoylphosphatidylcholine are shown for comparison. The dashed lines indicate the position of the membrane−solution interface as defined by the position of the phosphate groups. The fully extended peptide is approximately 70 Å in length. Nitroxide substitutions on this peptide are likely to take up a position similar to that seen for the phenylalanine residues.

nitroxide side chains may be twisted and inserted into the membrane interface, leaving the backbone and charged side chains at the interface. The distances in Figure 5 are not inconsistent with this possibility, and we believe that this latter possibility is the correct one for the MARCKS-derived peptide. Measurements in PC/PS monolayers yield only small surface pressure changes upon the binding of the MARCKS peptide (V. Boguslavsky and S. McLaughlin, unpublished). These small changes are consistent with the insertion of several phenylalanine residues but are not consistent with the insertion of the entire peptide into the interface. Because this peptide is in an extended configuration, the peptide backbone and side chains are relatively unrestricted in their motion. It is entirely reasonable that more hydrophobic residues, such as phenylalanine or a nitroxide side chain, are twisted inward toward the membrane hydrocarbon with the charged hydrophilic residues lying at the membrane interface. As indicated above, an estimate of the accessible surface area of the nitroxide side chain indicates that it should have a hydrophobicity similar to that of phenylalanine. Thus, the nitroxide is likely to probe the position that a phenylalanine or other hydrophobic residue would have on this peptide.

On the basis of the above considerations, a rough model for the MARCKS-derived peptide at the membrane−solution interface in an extended structure is shown in Figure 9. Phospholipids are also shown for comparison. In a completely extended structure, this peptide is over 70 Å long. In Figure 9, phenylalanine residues are turned into the membrane. It should be noted that this peptide may assume a different configuration than the corresponding segment in the intact protein. Nonetheless, the peptide examined here interacts with membranes, PKC, and CaM in a manner similar to that for the native MARCKS, and it therefore provides a useful model for the molecular function and interactions of MARCKS (Kim et al., 1994). In addition, relatively little structural information is available on the interaction of basic peptides with membrane interfaces, and this peptide provides a good model for the membrane−electrostatic association of this important class of peptides. In the case of MARCKS, the substrate domain may interact with PKC in an extended conformation and it is even possible that the membrane may function to present this substrate to the enzyme in this conformation. The interfacial location for this domain suggests that the PKC catalytic site may also be localized at the interface, since this position would optimize the enzyme for substrate reactivity.

The N-terminal sequence of the MARKCS-derived peptide begins with six positively charged residues, and this end of the peptide has the highest density of charge. Electrostatic image forces may in part account for the aqueous exposure and enhanced motion at this terminus. As seen in Figure 8, only the N-terminal domain shows changes in dynamics and position as a function of membrane charge density. The Gouy−Chapman theory does an excellent job of accounting for the energy of a range of charged amphiphiles at the membrane interface (Winiski et al., 1986; Hartsel and Cafiso, 1986; McLaughlin, 1989; Langner et al., 1990). In this theory, the electric field from the interface does not extend into the bilayer but resides only across the external aqueous solution. As a result, charged segments lying in the external solution and in the ionic double layer should be more strongly affected by surface charge density than charged segments lying at or within the membrane interface. This is consistent with the finding that only the N-terminal end of the peptide, which lies outside the membrane−solution interface, is affected by surface charge density. Unlike the central portion of the MARCKS-derived peptide, there are no phenylalanine residues at the N-terminus. These residues may help stabilize the peptide on the membrane interface by providing a small hydrophobic contribution to the binding.

In summary, a series of spin-labeled derivatives of the highly basic CaM and PKC binding domain of MARCKS have been used to determine its structure bound to lipid bilayers. This peptide is in an extended but nonaggregated conformation at the membrane interface, with its hydrophobic side chains buried below the level of the head group. The N-terminus of this peptide extends into the aqueous phase, but its dynamics and position are dependent upon the membrane charge density. In our laboratory, we are now using these spin-labeled derivatives to examine the structure and dynamics of these peptides bound to CaM and PKC.

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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.
Archer, S. J., Ellena, J. F., & Cafiso, D. S. (1991) *Biophys. J. 60*, 389−398.
Barranger-Mathys, M., & Cafiso, D. S. (1994) *Biophys. J. 67*, 172−176.
Bartlett, G. R. (1959) *J. Biol. Chem. 243*, 466−468.
Buser, C. A., Sigal, C. T., Resh, M. D., & McLaughlin, S. A. (1994) *Biochemistry 33*, 13093−13101.

Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry 15*, 4818−4831.
Dalton, L. A., McIntyre, J. O., & Fleischer, S. (1987) *Biochemistry 26*, 2117−2130.
de Kruijff, B., Rietveld, A., Telder, N., & Vaandrager, B. (1985) *Biochim. Biophys. Acta 820*, 295−304.
Farahbakhsh, Z. T., Altenbach, C., & Hubbell, W. L. (1992) *Photochem. Photobiol. 56*, 1019−1033.
Hartsel, S. C., & Cafiso, D. S. (1986) *Biochemistry 25*, 8214−8219.
Keith, A., Bulfield, G., & Snipes, W. (1970) *Biophys. J. 10*, 618−629.
Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. A. (1991) *Biophys. J. 60*, 135−148.
Kim, J., Blackshear, P. J., Johnson, J. D., & McLaughlin, S. (1993) *Biophys. J. 64*, A59.
Kim, J. K., Blackshear, P. J., Johnson, D. J., & McLaughlin, S. A. (1994) *Biophys. J. 67*, 227−237.
Langner, M., Cafiso, D., Marcelja, S., & McLaughlin, S. (1990) *Biophys. J. 57*, 335−349.
McIlroy, B. K., Walters, J. D., Blackshear, P. J., & Johnson, J. D. (1991) *J. Biol. Chem. 266*, 4959−4964.
McLaughlin, S. A. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 113−136.
Millhauser, G. L. (1992) *Trends Biochem. Sci. 17*, 448−452.
Morrisett, J. D. (1976) in *Spin labeling, theory and applications* (Berliner, L., Ed.) pp 273−338, Academic Press, New York.
Mosior, M., & McLaughlin, S. A. (1991) *Biophys. J. 60*, 149−159.
Mosior, M., & McLaughlin, S. A. (1992) *Biochemistry 31*, 1767−1773.
Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1−25.
Nordio, P. L. (1976) in *Spin labeling, theory and applications* (Berliner, L., Ed.) pp 5−52, Academic Press, New York.
Roux, M., Neumann, J.-M., Bloom, M., & Devaux, P. F. (1988) *Eur. Biophys. J. 16*, 267−273.
Taniguchi, H., & Manenti, S. (1993) *J. Biol. Chem. 268*, 9960−9963.
Winiski, A. P., McLaughlin, A. C., McDaniel, R. V., Eisenberg, M., & McLaughlin, S. A. (1986) *Biophys. J. 25*, 8206−8214.
Yu, Y. G., Thorgeirsson, T. E., & Shin, Y.-K. (1994) *Biochemistry 33*, 14221−14226.
Zhou, W., Parent, L. J., Wills, J. W., & Resh, M. D. (1994) *J. Virol. 68*, 2556−2569.

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