Location of the Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Effector Domain in Negatively Charged Phospholipid Bicelles

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ABSTRACT The effector domain of the myristoylated alanine-rich C-kinase substrate (MARCKS-ED) is a highly basic, unstructured protein segment that is responsible for attaching MARCKS reversibly to the membrane interface. When attached to the interface, it also has the capacity to sequester phosphoinosities, such as PI(4,5)P_2, within the plane of the bilayer. Here, the position of the MARCKS-ED was determined when bound to phospholipid bicelles using high-resolution NMR methods. Two sets of data indicate that the phenylalanine residues of the MARCKS-ED are positioned within the membrane hydrocarbon a few angstroms from the aqueous-hydrocarbon interface. First, short-range nuclear Overhauser effects are detected between the aromatic side chains and the lipid acyl chain methylenes. Second, paramagnetic enhancements of nuclear relaxation, produced by molecular oxygen, are similar for the phenylalanine aromatic protons and those observed for protons in the upper portion of the acyl chain. The rates of amide-water proton exchange are fast and only slightly hindered when the peptide is bound to bicelles, indicating that the backbone does not lie within the membrane hydrocarbon. These results indicate that highly charged peptides such as the MARCKS-ED penetrate the membrane interface with aromatic amino acid side chains inserted into the hydrocarbon and the peptide backbone lying within the bilayer interface. This position may serve to enhance the electrostatic fields produced by this basic domain at the membrane interface and may play a role in the ability of the MARCKS-ED to sequester polyphosphoinositides.

INTRODUCTION

Highly basic regions of proteins, such as the effector domain of the myristoylated alanine-rich C-kinase substrate (MARCKS-ED), function to reversibly bind proteins to the membrane-solution interface (Kim et al., 1991). The electrostatic interaction between the 13 basic residues in the MARCKS-ED and the negatively charged cytoplasmic surface of the plasma membrane facilitates the membrane attachment of MARCKS. Phosphorylation of the MARCKS-ED reduces the magnitude of the electrostatic interaction resulting in the translocation of MARCKS from the membrane to the cytosol, thereby acting as an electrostatic switch (Kim et al., 1994; McLaughlin and Aderem, 1995; Seykora et al., 1996; Swierczynski and Blackshear, 1996). In addition to its role in membrane attachment, the MARCKS-ED also mediates the primary function of MARCKS, which appears to be the sequestration of highly phosphorylated inositol lipids, such as PI(4,5)P_2, within the plane of the bilayer (McLaughlin et al., 2002).

Several lines of evidence indicate that the MARCKS-ED sequesters PI(4,5)P_2 through a mechanism that is largely electrostatic: this domain will bind other similarly charged phosphoinositides, such as PI(3,4)P_2, with equal efficacy; its binding is reduced by high ionic strength; the structure of the MARCKS-ED at the membrane interface is not altered by the presence of PI(4,5)P_2; finally, when positioned on the membrane interface, electrostatic calculations indicate that the effector domain of MARCKS produces a region of positive potential on the membrane interface that could serve to bind or sequester polyvalent negatively charged lipids such as PI(4,5)P_2 (Rauch et al., 2002; Wang et al., 2001, 2002).

When localized at the membrane interface, site-directed spin labeling (SDSL) of the MARCKS-ED indicates that it is localized within the membrane interface with its phenylalanine residues placed ~5–10 Å below the level of the lipid phosphate. This conclusion assumes that the spin labeled side chain, because it is similar in hydrophobicity to phenylalanine (Russell et al., 1996), undertakes a similar position to that of a phenylalanine side chain (Qin and Cafiso, 1996). Removal of the five phenylalanine residues produces a peptide that also binds strongly to negatively charged membrane surfaces but behaves quite differently than the native peptide. Rather than penetrating the bilayer, a spin labeled side chain on the bound peptide is localized in the aqueous phase and the peptide appears to reside within the double layer (Victor et al., 1999). The shift in position of the peptide upon removal of phenylalanine is thought to be due to the balance between a long-range Coulombic attractive force and a repulsive dehydration force that opposes insertion of the peptide into the bilayer (Ben-Tal et al., 1996). Removal of the aromatic residues eliminates a hydrophobic contribution to the binding that would overcome dehydration at the membrane interface. This general conclusion is consistent with spin-label studies on model peptides based on lysine and phenylalanine, where the spin-label amino acid tetramethyl-piperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) is incorporated into the

Submitted May 17, 2003, and accepted for publication June 30, 2003.

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0006-3495/03/10/2442/07 $2.00

peptide (Victor and Cafiso, 2001). Here the spin label is locked into the peptide backbone and side-chain conformations of the spin label are not in doubt. Work on these peptides indicates that the backbone of simple lysine containing peptides are positioned within the double layer adjacent to the membrane interface when bound, whereas peptides containing phenylalanine are localized with the backbone of the peptide ~8 or 9 Å below the level of the lipid phosphate within the bilayer interior.

The localization of MARCKS at the membrane interface is an important issue for several reasons. First, the energetics of the binding of these domains is likely to be influenced by their membrane penetration, and the extent of dehydration of the peptide (Ben-Tal et al., 1997, 1996). Second, when localized at or within the membrane interface, protein segments such as MARCKS-ED may produce a larger electrostatic field in the plane of the membrane than when positioned within the aqueous double layer (McLaughlin, 1989). As a result, membrane insertion may play a role in the ability of the peptide to sequester charged lipids (such as $PI(4,5)P_2$). Indeed, recent work indicates that the derivative of the MARCKS-ED lacking phenylalanine is less effective at sequestering $PI(4,5)P_2$ than the native MARCKS-ED (A. Gambhir and S. McLaughlin, unpublished results). A number of measurements have already been made aimed at determining the position of the MARCKS-ED on the membrane interface. However, there is a great deal of uncertainty associated with any one measurement, making additional independent measurements of its position highly desirable.

In the study, we undertook high-resolution NMR measurements to determine the position of the MARCKS-ED when bound to lipid interfaces. To facilitate these high-resolution measurements, NMR experiments were carried out on the MARCKS-ED bound to isotropic lipid bicelles. Both nuclear Overhauser effects (NOEs) as well as the effects of molecular oxygen on the paramagnetic enhancements of nuclear relaxation indicate that the peptide penetrates the lipid interface, with its phenylalanine residues located several angstroms within the lipid hydrocarbon. The backbone of the peptide is likely to reside within the interfacial region of the bilayer with the charged side chains exposed to the aqueous phase. These results are consistent with a recent solid-state NMR study on this peptide (Zhang et al., 2003), and they are consistent with previous work utilizing site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy (Qin and Cafiso, 1996; Victor et al., 1999).

MATERIALS AND METHODS

Materials

All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). A peptide based on the MARCKS effector domain Ac-KKKKKRFSFKKSFKLSGFSFKKNKK-NH was synthesized and purified by the Biomolecular Research Facility at the University of Virginia, and its identity was confirmed by mass spectrometry. This peptide was blocked at both the N- and C-termini. In some measurements, we also employed a version of this peptide having a single cysteine substitution near the C-terminus, N23C (Qin and Cafiso, 1996). This peptide produced identical results to the native MARCKS effector domain.

Sample preparation

In all cases, the sample buffer used was 10 mM sodium phosphate, 150 mM NaCl, 10% (v/v) D_2O, pH 6.6. Samples containing MARCKS-ED bound to negatively charged bicelles were prepared as follows. A concentrated solution of DCPC (dicaproylphosphatidylcholine) in buffer was prepared in a glove bag filled with dry nitrogen. Appropriate amounts of dry dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were added to the DCPC solution, the volume was adjusted with buffer, and the mixture was freeze thawed five times. A concentrated solution of MARCKS-ED in buffer was then added to the bicelles and the mixture was freeze thawed three more times; the final concentrations of MARCKS and lipid were 1 mM and 20% (w/v), respectively. The molar ratio of lipids in bicelle samples was 0.53 DCPC:0.27 DMPC: 0.2 DMPG (the peptide:lipid molar ratio was 1:356). Some samples containing MARCKS bound to bicelles were degassed by using the freeze-pump-thaw method and then pressurized to 17 atm of O_2 or N_2. A sample containing 5 mM MARCKS-ED, 10 mM sodium phosphate, 150 mM NaCl, 10% (v/v) D_2O, pH 6.6 was prepared by dissolving the appropriate amount of MARCKS-ED in the same buffer used for the bicelle samples. In some cases, partial aggregation of the MARCKS-ED bicelle samples was observed after 1–3 days at 40°C. However, this aggregation had no effect on spectral lineshapes or relaxation measurements. NMR intensity from the aggregated material is likely to be too broad to be observable and much of the aggregated material sedimented to the bottom of the NMR tube and was outside the NMR spectrometer probe observe/receive coil.

NMR spectroscopy

Varian (Palo Alto, CA) UnityPlus and Inova 500 MHz spectrometers were used to obtain NMR spectra. e-SNOB pulses (Kupce et al., 1995) were used for selective excitation of the amide and aromatic regions of 1D ^1H spectra. The PENCE sequence containing an r-SNOB selective pulse (Kupce et al., 1995) was used for selective excitation of the 6.5–10 ppm spectral region in ω_2-selective NOESY experiments (Seigneuret and Levy, 1995). Standard pulse sequences from Varian were used to obtain the NOESY and ROESY spectra used for measurement of amide-water proton exchange rates. Exchange rates were obtained by measuring the slope of the initial linear portion of the amide-water crosspeak intensity versus mixing time plots. The sample temperature during all NMR experiments was 40°C.

RESULTS

Amide and aromatic ^1H resonances are resolved in bicelle bound MARCKS-ED

Shown in Fig. 1 are the amide and aromatic regions of the ^1H spectra of the MARCKS-ED in two environments: bound to negatively charged bicelles (Fig. 1 *A*) and in aqueous solution (Fig. 1 *B*). Under the conditions of this experiment, we expect that all the MARCKS-ED will be bound to micelles based on its high reciprocal molar binding constant (Kim et al., 1994), and the significant line broadening seen in Fig. 1 *A* is consistent with this expectation. The amide chemical shifts vary between 7.9 and 8.3 ppm for MARCKS-ED in solution and between 7.9 and 8.5 ppm for MARCKS-ED bound to bicelles. The phenylalanine aromatic chemical

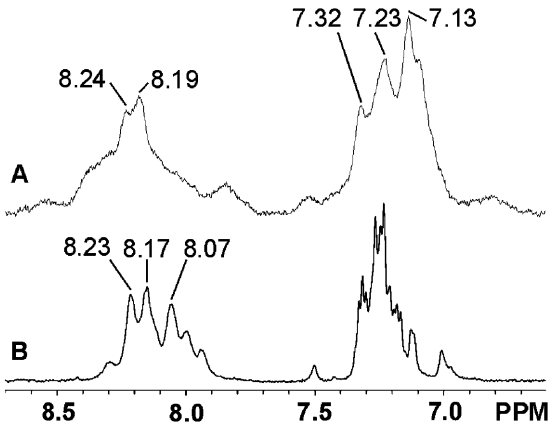

FIGURE 1 One dimensional 500 MHz ^{1}H NMR spectra showing the amide and aromatic regions of the MARCKS-ED in bicelles (*A*) and solution (*B*).

shifts vary from 7.1 to 7.3 ppm for the MARCKS-ED in solution and bound to bicelles. The small amide and aromatic chemical shift ranges and consequent extensive resonance overlap are consistent with the fact that in both environments MARCKS-ED has no regular secondary or tertiary structure (Qin and Cafiso, 1996; Wang et al., 2001).

Intermolecular NOEs are observed between peptide and bicelle lipid

We obtained NOESY spectra of the MARCKS-ED in bicelles in an attempt to observe short range distances between MARCKS-ED and bicelle protons. Selective excitation of the amide and aromatic region in ω_2 was employed so that crosspeaks between this region and the aliphatic region of the spectrum would not be obscured by the very intense lipid diagonal peaks (Seigneuret and Levy, 1995). Fig. 2 is an ω_2-selective NOESY spectrum with a 100-ms mixing time showing all of the NOE crosspeaks involving the amide and aromatic protons of MARCKS-ED with the water exchange crosspeaks suppressed. Similar crosspeaks were observed in an ω_2-selective NOESY with a 50-ms mixing time. The crosspeaks in Fig. 2 labeled *B–E* are due to intraresidue interactions (see figure legend). Crosspeaks in the box labeled *A* in Fig. 2 are due to close approach of the phenylalanine aromatic protons to the lipid acyl chain methylenes 4–13. These crosspeaks are the only intermolecular interactions observed in the NOESY spectrum and indicate that when MARCKS-ED is bound to the bicelles the phenylalanine aromatic protons reside in the hydrocarbon region of the bilayer.

Peptide position from the effect of molecular oxygen on peptide spin-lattice relation rates

Further evidence that the phenylalanine aromatic rings are located in the hydrocarbon region of the bicelle bilayers was

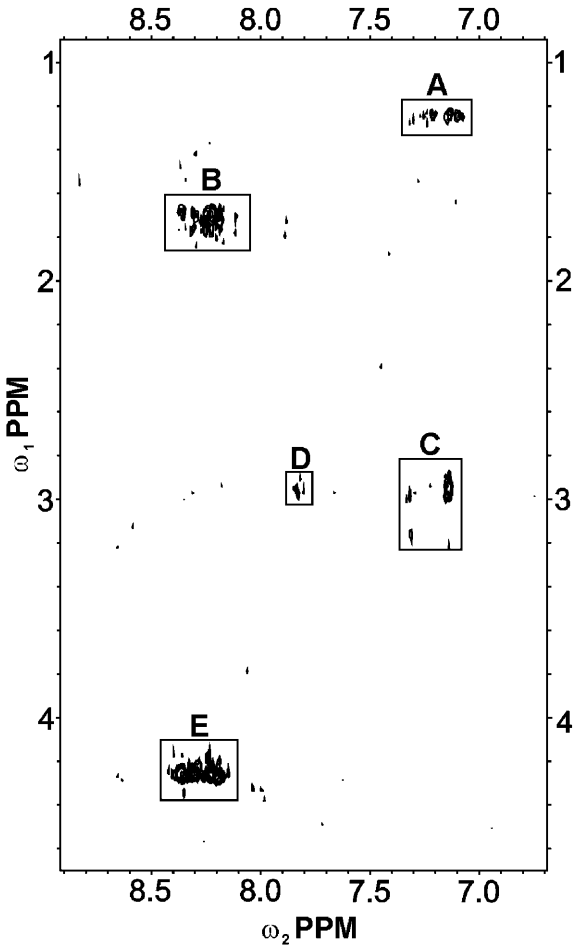

FIGURE 2 Crosspeaks from an ω_2-selective NOESY spectrum (mixing time = 100 ms) of MARCKS-ED in bicelles. (*A*) F aromatic—lipid acyl chain $(CH_2)_{4-13}$; (*B*) amide—K, R, L H$_\beta$; (*C*) F aromatic—F H$_\beta$; (*D*) K $(CH_2)_\varepsilon$—K εNH_3; (*E*) amide—K, R, L H$_\alpha$.

obtained by examining the effect of O_2 on the ^{1}H spin-lattice relaxation of the phenylalanine aromatic and bicelle phospholipid protons. Shown in Fig. 3 are the ^{1}H spin-lattice relaxation rates due to the presence of 17 atm of O_2. The phospholipid protons on the *x* axis of Fig. 3 are plotted in order of increasing distance from the bilayer surface from left to right, and the observed increase in relaxation rate as one proceeds from the bilayer surface to the center is due to the O_2 concentration gradient in bilayers. This gradient allows one to establish a relationship between the effect of O_2 on ^{1}H spin-lattice relaxation and bilayer depth. This relationship, along with measurement of the O_2 effect on ^{1}H spin-lattice relaxation of the phenylalanine aromatic protons of MARCKS-ED, allows one to determine the aromatic proton position with respect to the bicelle phospholipid. Also shown in Fig. 3 is the average oxygen relaxation enhancement obtained for the aromatic protons from the five phenylalanine residues. The relaxation enhancement experienced by the phenylalanine aromatic protons is similar to

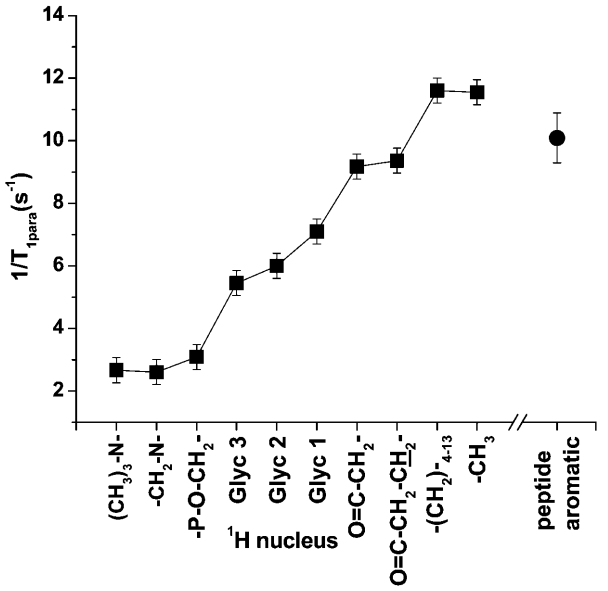

FIGURE 3 Paramagnetic enhancements of the ^{1}H spin-lattice relaxation rates due to the presence of 17 atm molecular oxygen. The paramagnetic enhancements ($1/T_{1para}$) are shown for bicelle phospholipid protons and MARCKS-ED phenylalanine aromatic protons. Phospholipid resonances are arranged (*left to right*) from the bicelle surface to the hydrocarbon interior.

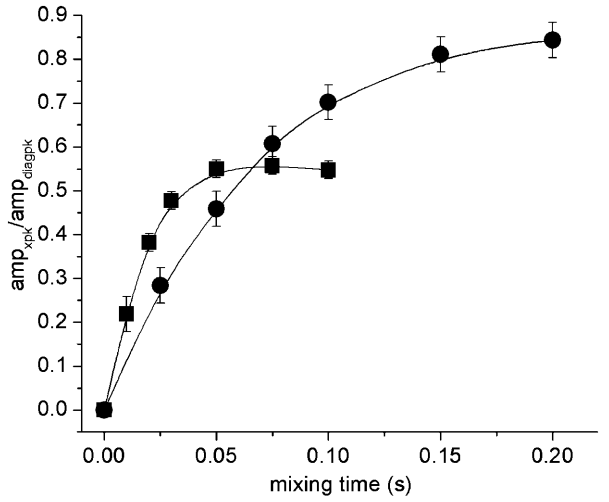

FIGURE 4 Intensities of the ^{1}H NOESY amide-water crosspeak as a function of the mixing time, τ_m. Data is shown for the amide peak at 8.19 ppm from the MARCKS-ED in bicelles (●), and the amide peak at 8.23 ppm from MARCKS-ED in solution (■). ROESY crosspeaks measured over the same mixing time range yielded the same rates (see text).

that observed for protons in the upper portion of the acyl chain, indicating that the average position of the phenylalanine aromatic protons is in the bilayer hydrocarbon region a few angstroms from the aqueous-hydrocarbon interface.

Amide-water NOEs in bicelle-bound MARCKS-ED are due to proton exchange

To obtain information on the environment of the amide protons of the MARCKS-ED bound to the bicelles, amide-water proton exchange of the MARCKS-ED in solution and bound to bicelles were compared. Under appropriate conditions large amide-water crosspeaks were present in the NOESY spectra of both solution MARCKS-ED and MARCKS-ED bound to bicelles. Representative examples of the dependence of crosspeak intensity on mixing time are shown in Fig. 4. These crosspeaks are most likely dominated by amide-water proton exchange. However other contributions to these crosspeaks are possible, including: direct NOEs between water and amide protons, exchange relayed NOEs involving water-amine exchange followed by amine-amide NOEs, and finally NOEs between amide and α-carbon protons when the α-carbon protons have the same chemical shift as water.

Contributions to the amide-water crosspeaks other than those due to amide-water proton chemical exchange can be ruled out. First, the protons of the MARCKS-ED in solution have a rotational correlation time that is roughly the inverse of the NMR spectrometer Larmor frequency used here. As a result, NOE crosspeaks should be very small or absent, and we have observed this experimentally. Second, we used both NOESY and ROESY spectra to measure amide-water proton exchange rates of the MARCKS-ED in bicelles and obtained very similar results from the two sets of spectra. NOE crosspeaks have opposite signs in NOESY and ROESY spectra whereas exchange crosspeaks are positive in both types of spectra (Cavanagh et al., 1996). The similarity of the amide-water proton exchange rate measured from NOESY and ROESY spectra for the MARCKS-ED bound to bicelles and the very small intensity of NOESY crosspeaks in solution for the MARCKS-ED suggests that contributions to amide-water proton crosspeaks in NOESY spectra, other than chemical exchange, are absent or minor and would have no effect on the interpretation of the measured proton exchange rates.

Proton exchange rates are not slowed for bicelle bound MARCKS-ED

Overall, amide-water proton exchange rates are fast but only slightly diminished by MARCKS-ED binding to bicelles. After selectively irradiating the water peak for 1 s, only 14% and 33% of the amide proton intensity remains in spectra of MARCKS-ED in solution and in bicelles, respectively. The amide-water proton exchange rates obtained from NOESY spectra for the two and three largest amide peaks in spectra of MARCKS-ED bound to bicelles and solution MARCKS-ED respectively are shown in Table 1. These peaks represent over 60% of the total amide intensity for both samples. The two peaks seen in the bicelle sample have rates that are very similar to each other and to one of the peaks in the solution

TABLE 1 Amide-water proton exchange rates

Sample	Amide ^{1}H δ (ppm)	Exchange rate (s^{-1})
MARCKS-ED in solution	8.23	22 ± 2
	8.17	13 ± 1
	8.07	21 ± 2
MARCKS-ED in bicelles	8.24	15 ± 2
	8.19	13 ± 2

sample. The other two rates for the solution sample are very similar to each other and 1.6-fold higher than the bicelle rates. Thus the bicelle has little or no effect on amide-water proton exchange of MARCKS-ED. Consequently when MARCKS-ED is bound to bicelles the amide protons must be located on the bicelle surface or in an interfacial region accessible to water because the bicelle does little to hinder amide-water proton exchange (see Discussion).

DISCUSSION

In the work presented here, we used three NMR techniques to obtain information on the location of the aromatic and amide protons in MARCKS-ED in phospholipid bicelles. First, the effect of O_2 on the ^{1}H spin-lattice relaxation rates of both the bicelle lipid and the phenylalanine aromatic protons of MARCKS-ED bound to bicelles was determined. Second, intermolecular NOEs between peptide aromatic protons and bicellar lipid were observed. Finally, the rates of amide-water proton exchange for solution and bicelle bound peptide were measured.

The concentration of O_2 in phospholipid bilayers is known to vary as a function of bilayer depth, where the O_2 concentration is lowest at the bilayer surface and increases toward the bilayer center (Windrem and Plachy, 1980). This gradient has been exploited for positioning spin-labeled segments on proteins using EPR (Altenbach et al., 1994), and it has more recently been used to localize small molecules and proteins in micelles, bicelles, and small unilamellar phospholipid vesicles with NMR (Ellena et al., 2002; Luchette et al., 2002; Prosser et al., 2000, 2001). The effects of O_2 on bilayer lipids and other molecules residing in bilayers can be directly compared and interpreted in terms of the location of membrane bound molecules relative to membrane lipid. Here, we observed clear differences in the effect of 17 atm of O_2 on the headgroup, glycerol, acyl chain C_2 and C_3, and acyl chain $C_{4–14}$ protons (Fig. 3). Unfortunately the acyl chain $C_{4–13}$ protons were not resolvable. The effect of 17 atm of O_2 on the three main MARCKS-ED aromatic resonances was the same within experimental error consistent with similar intramembrane locations for all five phenylalanine aromatic rings. The average phenylalanine aromatic ring location is deeper than the acyl chain C_3 position but shallower than the average position for acyl chain segments $C_{4–14}$. Therefore the average phenylalanine aromatic ring depth inferred from this paramagnetic effect is likely between acyl chain C_4 and C_9.

The appearance of intermolecular NOEs between peptide and bicelle lipid also provides information on position. NOEs are observed between the MARCKS-ED phenylalanine protons and methylene positions 4–13 on the lipid acyl chains at relatively short mixing times (50 ms and 100 ms). The presence of these intermolecular crosspeaks at short mixing times suggests close proximity (<5 Å) of some or all of the acyl chain methylene positions 4–13 to some or all of the phenylalanine aromatic protons. Thus this result is in agreement with the phenylalanine position inferred from the effect of molecular oxygen.

The third NMR approach used was measurement of the amide-water proton exchange rates for MARCKS-ED both in solution and bound to bicelles. Although amide-water proton exchange rates of membrane bound peptides and proteins are primarily determined by the extent and stability of intramolecular hydrogen bonding (Dempsey, 2001), the exchange rates are also sensitive to the bilayer binding location of the exchangeable amides (Dempsey and Butler, 1992; Spyracopoulos and O'Neil, 1994). Amide protons residing in the bilayer hydrocarbon region have been observed to exchange up to 25 times more slowly than those located at the bilayer surface or interfacial region. The MARCKS-ED has no regular secondary or tertiary structure even when bound to membranes (Qin and Cafiso, 1996; Wang et al., 2001); therefore, one would expect amide-water proton exchange to be fast, and we have observed this in saturation transfer and NOE experiments. As presented above (see Results), the bicelle slightly slows the MARCKS-ED amide-water proton exchange rates. The similarity of all the measured rates (Table 1) and the fact that the bicelle has little effect on the rates suggests that all or most of the MARCKS-ED amides, when bound to the bicelles, must reside in a region that is accessible to water. In bilayers (Wiener et al., 1991) and in micelles (see MacKerell, 1995, and references therein), water appears to be present in the interfacial region and is absent only from the hydrocarbon core; as a result, the exchange data suggest that the backbone of the MARCKS-ED must reside on the bicelle surface or within the interfacial region.

The measurements made here are consistent with previous studies using EPR indicating that spin labels on the central portion of the MARCKS-ED are buried within the bilayer interface, ~5–10 Å below the level of the lipid phosphate (Qin and Cafiso, 1996; Victor et al., 1999). Thus, despite the fact that nitroxide labels are attached to the peptide in these EPR experiments, the label location appears to correctly reflect the position of the phenylalanine residues in the peptide. Evidence for membrane penetration of the MARCKS-ED is also indicated by its effect on the deuterium NMR spectrum of headgroup labeled lipids, and by the effect of the peptide upon monolayer surface pressure (Arbuzova et al., 2000; Victor et al., 1999), although it is difficult from these measurements to assess the depth of penetration. Finally, the results obtained here are in agreement with

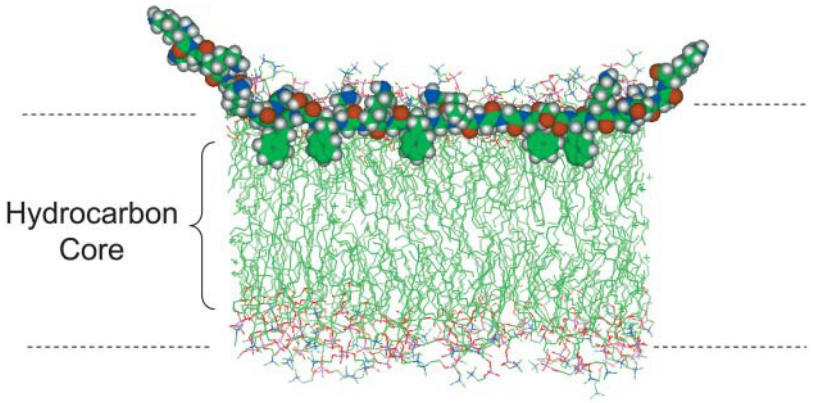

FIGURE 5 Model for the structure and position of the MARCKS-ED on the membrane surface. The five phenylalanines are inserted into the membrane hydrocarbon, and the peptide backbone is localized in the membrane interface. The charged lysine residues likely extend toward the bulk aqueous phase to reduce the electrostatic Born energy. The peptide is shown superimposed on a model for a fully hydrated segment of DPPC bilayer obtained by molecular dynamics simulation (Tieleman and Berendsen, 1996). The dashed lines indicate the approximate position of the lipid phosphates.

a recent solid state NMR study of the MARCKS-ED (Zhang et al., 2003) that also observed nuclear Overhauser effects between phenylalanine protons and lipid protons. These results were carried out in lipid bilayers, and the similarity of these results to those obtained here indicates that studies of membrane interacting domains using membrane mimetic systems may be a appropriate approach for high-resolution NMR studies in some systems.

Shown in Fig. 5 is a model for the position of the MARCKS-ED domain on the membrane surface. The position of the five phenylalanines within the bilayer interior is based on the magnetic resonance data presented here and elsewhere (Qin and Cafiso, 1996; Zhang et al., 2003). This allows the backbone of the MARCKS-ED to be positioned within the bilayer interface, in a region where water-amide proton exchange should occur. Although we have no direct evidence for the placement of the lysine side chains, we expect that they will ''snorkel'' toward the aqueous phase, as is believed to occur for lysine residues in some amphipathic peptides (Mishra et al., 1994; Segrest et al., 2002; Strandberg et al., 2002). Each end of the peptide is highly charged and is shown in Fig. 5 extended off the membrane surface. The localization of the N-terminus in the aqueous phase is based upon data obtained from the MARCKS-ED and pentalysine using site-directed spin labeling and NMR (Qin and Cafiso, 1996; Roux et al., 1988; Victor et al., 1999; Victor and Cafiso, 2001) as well as electrostatic computations (Ben-Tal et al., 1996). We do not have direct evidence from EPR that the C-terminus of the peptide lies off the membrane surface.

In summary, the O_2-induced spin-lattice relaxation and ω_2-selective NOESY experiments indicate that the MARCKS-ED phenylalanine aromatic groups reside in the bilayer hydrocarbon region and the amide-water proton exchange experiments indicate that the amide groups reside at or near the bilayer surface. The locations of these regions of the MARCKS-ED are in agreement with the results of other spectroscopic methods. This position may be significant and it may facilitate the action of MARCKS by bringing positively charged side chains on the effector domain in proximity to the membrane interface thereby enhancing the electrostatic interaction with multivalent polyphosphoinositols such as $PI(4,5)P_2$.

We thank Stuart McLaughlin for helpful discussions during the course of this work.

This work was supported by National Institutes of Health grant GM62305 to D.S.C.

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