Location and Dynamics of Basic Peptides at the Membrane Interface: Electron Paramagnetic Resonance Spectroscopy of Tetramethyl-Piperidine-*N*-Oxyl-4-Amino-4-Carboxylic Acid-Labeled Peptides

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ABSTRACT The attractive interaction between basic protein domains and membranes containing acidic lipids is critical to the membrane attachment of many proteins involved in cell signaling. In this study, a series of charged model peptides containing lysine, phenylalanine, and the spin-labeled amino acid tetramethyl-piperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) were synthesized, and electron paramagnetic resonance (EPR) spectroscopy was used to determine their position on the membrane interface and free energy of binding. When membrane-bound, peptides containing only lysine and TOAC assume an equilibrium position within the aqueous double layer at a distance of ~5 Å from the membrane interface, a result that is consistent with recent computational work. Substitution of two or more lysine residues by phenylalanine dramatically slows the backbone diffusion of these peptides and shifts their equilibrium position by 13–15 Å so that the backbone lies several angstroms below the level of the lipid phosphate. These results are consistent with the hypothesis that the position and free energy of basic peptides when bound to membranes are determined by a long-range Coulombic attraction, the hydrophobic effect, and a short-range desolvation force. The differences in binding free energy within this set of charged peptides is not well accounted for by the simple addition of free energies based upon accepted side chain partition free energies, a result that appears to be in part due to differences in membrane localization of these peptides.

INTRODUCTION

Many proteins that are involved in cell signaling are water-soluble but become attached to the membrane interface as a result of an electrostatic interaction between a basic domain in the protein and the negatively charged lipid interface. A number of peptides derived from these basic domains have been studied and include peptides from the N-terminal segment of the src tyrosine kinase (Buser et al., 1994; Sigal et al., 1994; Victor and Cafiso, 1998), the protein kinase C pseudosubstrate (Mosior and McLaughlin, 1991, 1992), neuromodulin (Kim et al., 1994; Wertz et al., 1996), and the myristoylated alanine rich C-kinase substrate (MARCKS) protein (Kim et al., 1994; Qin and Cafiso, 1996). These peptides generally exhibit strong binding to membranes containing acidic lipid and much weaker or no binding to zwitterionic lipid bilayers.

Several methods have been used to study the membrane interactions of basic peptides with membrane surfaces. For example, ^{2}H-NMR spectroscopy on the lipid headgroup provides evidence that pentalysine (Lys_5) does not penetrate the membrane interface when bound (Roux et al., 1988), a result that is also consistent with surface and zeta potential measurements (Kim et al., 1991). A purely electrostatic association for short lysine-containing peptides is also indicated by electron paramagnetic resonance (EPR) spectroscopy of spin-labeled lipids (Kleinschmidt and Marsh, 1997). Measurements using site-directed spin-labeling have been made on basic membrane-binding domains derived from MARCKS, neuromodulin, and src (Qin and Cafiso, 1996; Victor and Cafiso, 1998; Wertz et al., 1996). When bound to the membrane interface, these peptides assume extended structures. In the case of a peptide derived from the membrane-binding domain of MARCKS (MARCKS (151–175)), the peptide binds so that its phenylalanine side chains are positioned ~10 Å below the level of the lipid phosphates. In a derivative of MARCKS (151–175) where the five phenylalanine residues are replace by alanine, the equilibrium position of the peptide is shifted so that it resides within the aqueous double layer at a position several angstroms from the membrane interface (Victor et al., 1999). As expected, the substitution of these five phenylalanine residues by alanine reduces the membrane affinity of this peptide, but by much less than expected. Under the conditions of this experiment, the apparent binding energy lost by this substitution is only 0.2 kcal/mol per Phe residue, far less than the 1.3 kcal/mol expected (Wimley and White, 1996). Similar observations have been made on these peptides using different approaches (Arbuzova et al., 2000; Kim et al., 1994).

The results obtained with MARCKS (151–175) can be explained by an interplay of attractive and repulsive forces (Arbuzova et al., 2000; Ben-Tal et al., 1996; Murray et al., 1997; Qin and Cafiso, 1996; Victor et al., 1999). Attractive forces are thought to include a long-range Coulombic attraction and the hydrophobic effect. Repulsive forces include a short-range dehydration force that may be due in part to a Born image energy experienced near the membrane interface (Ben-Tal et al., 1996). The balance between long-range attractive and short-range repulsive forces determines

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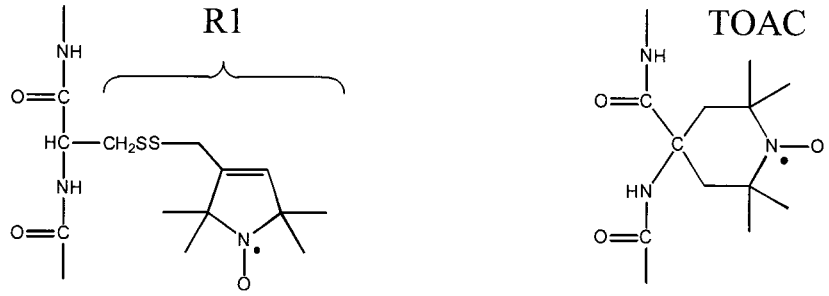

Scheme 1

the position of MARCKS (151–175) with respect to the water-membrane interface and its free energy of binding. In the absence of its five phenylalanine residues, MARCKS (151–175) residues within the ionic double layer, a result suggesting that the attractive Coulombic interaction is not sufficient to overcome the dehydration force. The small apparent 0.2 kcal/mol free energy contribution per phenylalanine residue seen when the two MARCKS analogs are compared is consistent with the idea that a significant portion of the energy gained from the binding of phenylalanine is expended in overcoming the dehydration energy.

The work described here was carried out to address several questions regarding the interaction of charged peptides with the membrane interface. First, we wanted to determine the equilibrium position of highly basic peptides when bound to the membrane interface. Although previous work using ^{2}H-NMR indicated that Lys_5 failed to penetrate the interface, it provided little quantitative information regarding the position of Lys_5 at the membrane interface (Roux et al., 1988). Second, because of the profound effect of phenylalanine on the position of the MARCKS-derived peptide, we wanted to investigate the effect of adding phenylalanine to the equilibrium position of highly basic peptides. And finally, we wanted to compare the membrane binding of model peptides containing lysine and phenylalanine to obtain an estimate of the contributions that these residues make to the free energy of peptide binding. To address these questions, a series of model peptides that incorporate the spin-labeled amino acid 2,2,4,4-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) (Scheme 1) was synthesized. These peptides are shown in Table 1 and are variations of pentalysine and hexalysine, where lysine residues are substituted with two, three, or four phenylalanine residues. The spin-labeled amino acid TOAC lacks the rotatable bonds found in the spin-labeled side chain R1 (Scheme 1). This label is fixed to the peptide backbone, and it is highly sensitive to peptide backbone dynamics. The nitrogen *p*-orbital on TOAC lies ~2.4 Å from the C_α carbon, and as a result distances estimated by EPR using this label should provide a less ambiguous estimate of the membrane-bound peptide position.

In the present study we show that basic peptides lacking phenylalanine reside in the aqueous double layer adjacent to the membrane interface at a position that is consistent with computational work (Murray et al., 1999). The incorporation of phenylalanine dramatically alters the equilibrium binding position and the backbone diffusion rates. Finally, the apparent contributions made to the free energy of binding by specific amino acid side chains is remarkably varied, making it difficult to predict the affinity of these peptides based upon standardized side chain free energies.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), headgroup spin-labeled PC, and spin-labeled doxyl phosphatidylcholines [1-palmitoyl-2-stearoyl (*n*-doxyl) phosphatidylcholines, $n = 5, 7, 10, 12$] were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Nickel (II) ethylenediaminediacetic acid (NiEDDA) was synthesized using a procedure obtained from Christian Altenbach. All N_α-Fmoc L-amino acids and (benzotriazolyloxy)tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (La Jolla, CA). *N*-methylmorpholine (NMM), piperidine, pyridine, peptide synthesis grade dichloromethane (DCM), and dimethyl formamide (DMF) were obtained from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Applied Biosystems (Foster City, CA), and acetonitrile was obtained from Mallinckrodt Chemicals (Chesterfield, MO).

Peptide synthesis

The Fmoc-TOAC used during the solid phase peptide synthesis of the TOAC model peptides was produced from 4-oxo-TEMPO and 9-fluorenyl-

TABLE 1 TOAC-containing peptides

Peptide	Sequence
K_5	Ac-KKxKKK-NH_2
K_6	Ac-KKKxKKK-NH_2
K_3F_2	Ac-KFxKFK-NH_2
K_4F_2	Ac-KFKxKFK-NH_2
K_2F_3	Ac-KFxFFK-NH_2
K_2F_4	Ac-KFFxFFK-NH_2

Derivatives of pentalysine and hexalysine into which the spin-labeled amino acid 2,2,4,4-tetramethyl-peperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) was incorporated (x ≡ TOAC).

methyl-oxycarbonyl (Fmoc) by using previously published procedures (del Milton et al., 1987; Lapatsanis et al., 1983; Marchetto et al., 1993; Rassat and Rey 1967; Smythe et al., 1995; Ten Kortenaar et al., 1986). The TOAC model peptides were synthesized on a Gilson automated multiple peptide system (AMS 422) using a rink amide *p*-methylbenzhydrylamine resin. The reaction time allowed for the coupling between each activated amino acid and the nascent peptide chain was set at 60 min. The long coupling time was needed because of the slow coupling reaction between the activated amino acid and a nascent peptide chain with a TOAC residue at its N-terminus. The source of this reduced reactivity may be the low nucleophilicity of the TOAC amino group on the peptide chain (Marchetto et al., 1993). Once the synthesis was complete, the dried resin wafers were swollen with pyridine and then washed repeatedly with DMF. A solution containing 65% DMF, 20% anhydrous pyridine, and 15% acetic anhydride was then added to each resin wafer at 3× molar excess of acetic anhydride to alkylate the N-terminus of the nascent peptides. Next, a solution of 90% trifluoroacetic acid and 10% water was used as a cleavage bath for 5 h at room temperature to separate the acylated peptides from the resin. The use of thioanisole, anisol, and dithioethanol was avoided to protect the nitroxide moiety of the TOAC residue. A cold ethyl ether bath precipitated these peptides, which were then lyophilized and redissolved several times in water. The TOAC peptides were then purified by using a reverse-phase C4 column from Vydac (cat. no. 214TP510). Solvent A, double-distilled water, and solvent B, acetonitrile, both contained 0.085% TFA. Because of the large differences of polarity among this set of peptides, the solvent gradient profile varied significantly between each TOAC peptide. For the peptide KKKxKKK (K_6), a linear gradient was run beginning with 3% solvent B and increasing to 50% after 4 min. For the more hydrophobic peptide KFFxFFK (K_2F_4) the gradient began with 30% solvent B and increased to 42% after 18 min. Each peptide underwent two complete rounds of HPLC and its identity was subsequently confirmed by electrospray ionization mass spectrometry.

Lipid vesicle preparation for EPR

Lipid mixtures containing the desired mole ratio of PC and PS were produced by mixing the appropriate lipid solutions in chloroform, removing the chloroform by vacuum desiccation overnight, and then hydrating the resulting lipid film by the addition of a buffer containing 100 mM KCl, 10 mM MOPS, pH 7.0. Unilamellar vesicles were produced by freeze-thawing this lipid suspension five times followed by extrusion of the mixture through 1000 Å polycarbonate filters (Poretics, Livermore, CA) using a LiposoFast extruder (Avestine, Ottawa, Canada).

Partition coefficient measurements using continuous-wave (cw) EPR

The membrane binding affinity of the TOAC-containing peptides was determined using EPR in a manner similar to that described previously (Archer et al., 1991). Briefly, a 50–100 μl sample of spin-labeled peptide was titrated with lipid while measuring the first derivative EPR resonance amplitude. A Varian E-line Centuries series spectrometer and an X-band loop-gap resonator (Medical Advances, Milwaukee, WI) with a standard two-loop, one-gap configuration were used to make the binding measurements. Using a stainless steel plunger, ~10 μl of sample could be loaded into or removed from a round (0.5 I.D. × 0.7 O.D.) quartz capillary (VitroCom, Mt. Lakes, NJ) that was secured within the loop-gap resonator. The peak-to-peak amplitude of the $m_I = -1$ EPR resonance ($A_{pp}(-1)$) of the peptide was then measured as a function of the concentration of lipid as illustrated in Fig. 1. For each EPR spectrum obtained during the lipid titration, the fraction of membrane bound peptide, f_b, was then determined from the peak-to-peak amplitude of the high-field EPR resonance using Eq. 1 (Cafiso and Hubbell, 1981).

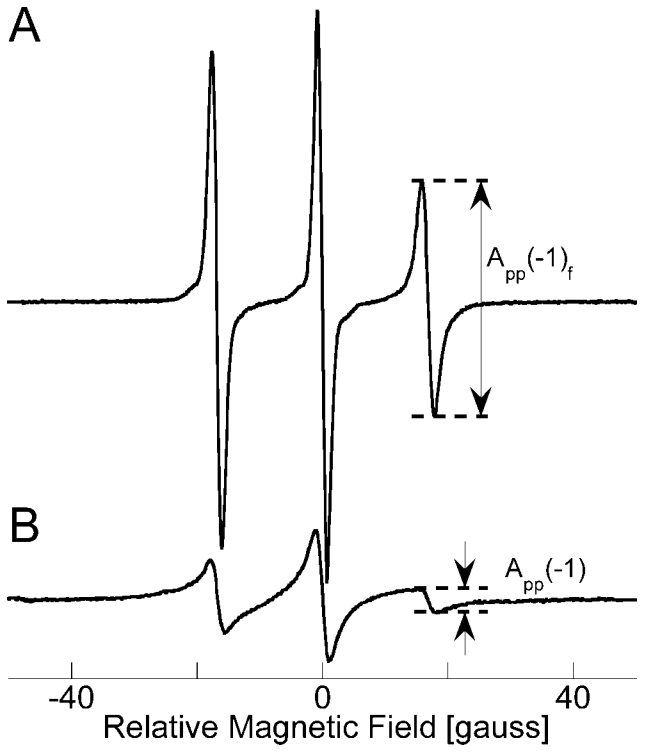

FIGURE 1 EPR spectra for K_6 in (*A*) aqueous solution and (*B*) in the presence of PC membranes containing 33 mol % PS. The signal intensities of these spectra have been normalized against their second integral. The peak-to-peak intensity of the high-field resonance for the aqueous $A_{pp}(-1)_f$ and composite $A_{pp}(-1)$ peptide spectra are used to determine the aqueous-membrane partitioning of the peptide as given in Eq. 1.

$$f_b = \frac{N_b}{N_{tot}} = \frac{A_{pp}(-1)_f - A_{pp}(-1)}{A_{pp}(-1)_f - A_{pp}(-1)_b} \quad (1)$$

Here $A_{pp}(-1)_f$ and $A_{pp}(-1)_b$ represent the peak-to-peak amplitude of the high-field resonance of the EPR spectrum obtained with the spin-labeled peptide in aqueous solution or fully bound to the lipid vesicle membranes, respectively. The molar partition coefficient, K_p (units of M^{-1}) was determined from f_b by assuming:

$$f_b = \frac{K_p[\text{Lipid}]}{1 + K_p[\text{Lipid}]} \quad (2)$$

where [Lipid] represents the molar concentration of accessible lipid in the sample. The externally added peptide is accessible only to the outer vesicle monolayer; as a result, the accessible lipid is taken as half the total lipid concentration (Kim et al., 1994).

A determination of f_b from Eq. 1 requires that the high-field resonance amplitude of the fully bound lineshape, $A_{pp}(-1)_b$, be known. However, there is an upper limit on the concentration of lipid vesicles that can be used in these binding studies, and $A_{pp}(-1)_b$ cannot be directly measured for peptides with partition coefficients on the order of 100 M^{-1} or less. In this case, both $A_{pp}(-1)_b$ and K_p were allowed to vary during the least-squares analysis preformed on the experimental data of $A_{pp}(-1)_f$ versus [Lipid]. The value of $A_{pp}(-1)_b$ that is obtained in this manner provides a check on how reasonable the analysis is because the value of the parameter should be bound between 0 and the value of $A_{pp}(-1)$ obtained at the highest lipid concentration used.

The partition coefficient of the TOAC peptides studied here is strongly dependent upon the membrane surface charge density. Therefore, to avoid perturbing the surface charge density, the total peptide charge during a titration experiment was always kept below a level that represented 2% of the accessible lipid surface charge.

EPR cw power saturation measurements

Continuous-wave power saturation measurements were performed in a manner similar to that previously described (Victor and Cafiso, 1998). In these measurements, 30–100 μM peptide in the presence of lipid vesicles at a total phospholipid concentration of 100–250 mM was placed into TPX capillary tubes (Medical Advances). These high concentrations of lipid were used to maximize the fraction of membrane-bound peptide; however, peptides with relatively small partition coefficients yielded spectra that were a composite of both an aqueous and a membrane-bound population. In these cases the contribution due to the aqueous peptide population is easy to distinguish and was subtracted from each experimental spectrum to obtain $A_{pp}(0)$. The dependence of $A_{pp}(0)$ on the incident microwave power, P, was then fit to the expression:

$$A_{pp}(0) = I\sqrt{P}\left[1 + (2^{\epsilon} - 1)\frac{P}{P_{1/2}}\right]^{-\epsilon} \quad (3)$$

where I is a scaling factor, $P_{1/2}$ is the microwave power required to reduce the resonance amplitude to half its unsaturated value, and ϵ is a measure of the homogeneity of the saturation of the resonance (Altenbach et al., 1994). By allowing I, ϵ, and $P_{1/2}$ to be adjustable parameters in a fit of the data to Eq. 3, a characteristic $P_{1/2}$ was obtained. Values for $P_{1/2}$ were then generated for each sample under three different sets of conditions: 1) equilibrated with N_2, 2) equilibrated with air (20% O_2), and 3) equilibrated with N_2 in the presence of 20 mM NiEDDA. A collision parameter, Π^{oxy}, was calculated from the values of $P_{1/2}$ using:

$$\Pi^{oxy} \equiv \frac{\Delta P'_{1/2}(O_2)}{P'_{1/2}(DPPH)} = \frac{P_{1/2}(O_2)/\Delta H_{pp}(O_2) - P_{1/2}(N_2)/\Delta H_{pp}(N_2)}{P_{1/2}(DPPH)/\Delta H_{pp}(DPPH)} \quad (4)$$

where ΔH_{pp} represents the peak-to-peak width of the $m_{I=0}$ resonance (Farahbakhsh et al., 1992). A corresponding calculation for Π^{NiEDDA} was also performed. For each labeled peptide a depth parameter, Φ, was determined from the two collisional parameters using Eq. 5.

$$\Phi \equiv \ln\left[\frac{\Pi^{oxy}}{\Pi^{NiEDDA}}\right] \quad (5)$$

The parameter Φ is directly related to the difference in the standard state chemical potentials of O_2 and NiEDDA, which vary as a function of depth in the lipid bilayer. As a result, Φ provides an estimate of the nitroxide depth in the lipid bilayer (Altenbach et al., 1994).

To convert Φ values into an estimate of distance (x) with respect to the lipid phosphate, a calibration curve ($\Phi(x)$) was generated. Previously, both nitroxide-labeled membrane proteins (Altenbach et al., 1994) and lipids (Qin and Cafiso, 1996) had been used to establish this dependence, and a roughly linear dependence of Φ upon distance is found within the bilayer interior. However, the linear dependence of $\Phi(x)$ must break down near the aqueous interface, and at some distance above the water-membrane interface Φ must reach a constant value characteristic of the bulk aqueous solution. For this reason, a hyperbolic tangent function was chosen to describe $\Phi(x)$. Assuming this functional dependence, a calibration curve was generated by combining EPR power saturation data obtained from spin-labeled sites on a membrane-bound C2 domain of known structure (Frazier et al., 2000) and nitroxide-labeled lipids. From these data we estimate that $\Phi(x)$ is described by:

$$\Phi = 3.3\tanh[0.11(x - 7.6)] + 0.9 \quad (6)$$

where x is defined as the distance along the bilayer normal relative to the lipid phosphate (positive numbers indicate a location within the bilayer, negative numbers indicate an external location). Equation 6 indicates that Φ reaches 95% of its bulk aqueous value at 10 Å ($x = -10$) from the membrane surface.

RESULTS

Localization and dynamics of basic TOAC peptides bound to PC/PS bilayers

Shown in Fig. 2 are EPR spectra of the six peptides listed in Table 1 in aqueous solution, and bound to PC/PS (73:27) membranes. In solution, the EPR spectra are characteristic of a nitroxide undergoing rapid rotation with a correlation time of ~0.3–0.5 ns, consistent with that expected for a small peptide in solution. When bound to PC/PS (73:27) membranes, the EPR spectra are broadened. For the two peptides exclusively containing lysine, Ac-KKxKKK-NH_2 (K_5) and Ac-KKKxKKK-NH_2 (K_6), the spectra have correlation times that are approximately four times longer than the peptide in solution. Membrane-bound spectra for the four peptide derivatives containing phenylalanine are dramatically different from the spectra for K_5 and K_6. Spectra for the phenylalanine peptides are significantly broader and exhibit correlation times of 10 ns or longer. Fig. 3 *A* shows the scaled mobilities, M_s, of the nitroxide on these TOAC peptides while free in solution or bound to PC or to PC/PS (73:27) lipid vesicles. The value of M_s (see legend to Fig. 3) provides an approximate measure of the dynamic state of the protein-associated spin-labeled side chain (Hubbell et al., 2000). Values near 1 correspond to side chains on highly mobile flexible protein segments, and values near 0 correspond to highly restricted motion usually seen in buried protein sites. As expected, the values of M_s for the TOAC peptides are near 1 when they are present in aqueous solution, indicating that they are as mobile as the most mobile protein-associated nitroxides. When membrane-bound, the values of M_s for K_5 and K_6 are not significantly reduced relative to the aqueous phase. However, the values of M_s for the TOAC label on the four peptides containing phenylalanine are close to those seen for the most rigid protein-associated nitroxides. Thus, the addition of phenylalanine to these sequences dramatically alters the dynamics of these bound peptides. The EPR spectra indicate that the peptides containing phenylalanine in this set (Table 1) interact quite differently with the membrane interface than do peptides containing only lysine.

Changing the membrane surface charge density alters the lineshape of peptides K_5 and K_6, but does not significantly alter the spectra for the other TOAC peptides. Shown in Fig.

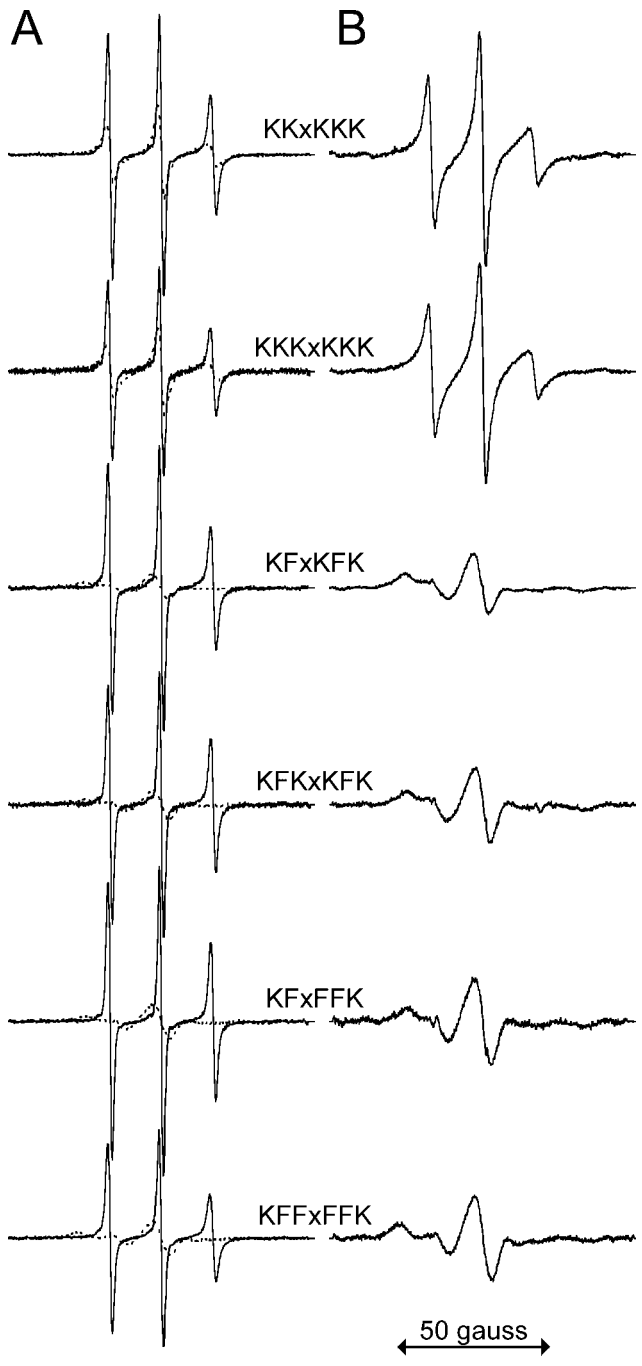

FIGURE 2 EPR spectra of the TOAC model peptides listed in Table 1. In (*A*) are unbound and membrane-bound spectra (*dashed lines*) that have been normalized by their second integral. (*B*) The membrane-bound spectra are on an expanded scale to more clearly reveal their spectral features. The aqueous solution contained 100 mM KCl and 10 mM MOPS, pH 7.0. The membrane-bound spectra were taken in the presence of 1000 Å diameter PC/PS (73:27) lipid vesicles at a total lipid concentration of 250 mM. The spectra were taken at 25°C with a scan width of 100 G, a scan time of 480 s, and a modulation amplitude of 1.0 G.

3 *B* are scaled mobilities for K_5 in solution and fully membrane-bound at several concentrations of PS. As the charge density is increased the mobility of the peptide decreases, and a similar behavior is seen for the K_6 peptide (data not shown). The parameter Φ, which provides an indication of position from the interface, also decreases, indicating that K_5 and K_6 are positioned closer to the membrane interface as the surface charge density is increased.

To estimate the position of the TOAC peptides, their EPR spectra were power-saturated both in solution and when bound to lipid vesicles. Shown in Table 2 are the power saturation data and spectral parameters for these peptides in solution and bound to lipid bilayers containing PC and 27 mol % PS. When bound to lipid bilayers, peptides K_5 and K_6 have depth parameters, Φ, close to those obtained for the peptide in bulk solution. We estimate the position of the nitroxide label on these peptides (Table 2) to be 5 Å from the membrane-solution interface. As the mol % of PS is increased, these two peptides move closer to the membrane surface, which accounts for the reduced peptide mobility seen in Fig. 3 *B*. The remaining four peptides are localized within the lipid bilayer, and our calibration curve places the TOAC spin-label for these peptides at distances of 8–9 Å below the level of the phosphate.

There are several sources of error in this distance estimate. Although Φ can be determined with quite good precision ($\sim\pm0.1$) the associated error in x grows exponentially as Φ approaches its bulk value. As a result, distances on the aqueous side of the membrane-solution interface, particularly at distances greater than 5 Å, are not well determined. For a value of x that is 3 Å on the aqueous side of the membrane surface the error is ~1 Å. In addition, there is an error associated with the accuracy of Eq. 6 that is just as large. Despite this uncertainty, it is clear that the peptides studied here are either localized within the double layer several angstroms from the lipid phosphate or buried below the phosphate, depending upon the presence of aromatic residues in the sequence.

The difference in interfacial localization of these peptides is consistent with the differences between their EPR spectra. The rotational rate of the TOAC spin label is tied to the rotational rate of the peptide backbone, and attachment of the peptide to the membrane is expected to dramatically reduce this diffusional rate. This is apparently the case for peptides containing phenylalanine, as they are essentially immobile on the EPR time scale (that is, correlation times for rotational diffusion of the backbone are on the order of tens of nanoseconds or greater). The lysine-containing peptides K_5 and K_6 are localized within the double layer, and give relatively isotropic motionally averaged spectra. Apparently rapid rotational diffusion is allowed for these peptides when membrane-associated.

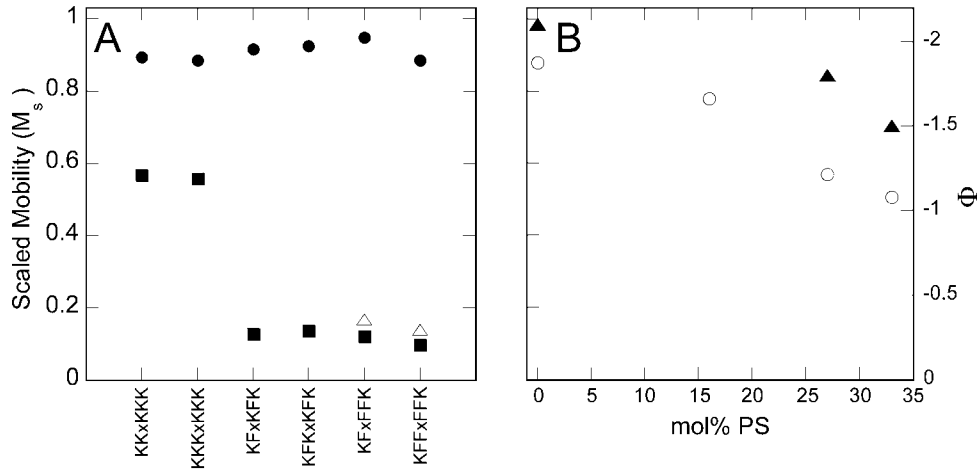

FIGURE 3 (*A*) Scaled mobility parameter, M_s, for the TOAC peptides free in solution (●), membrane-bound to vesicles formed from PC (△), and PC/PS (73:27) (■). The scaled mobility is defined as $M_s = (\delta^{-1} - \delta_i^{-1})/(\delta_m^{-1} - \delta i^{-1})$ (Hubbell et al., 2000, where δ is the linewidth of the central $m_I = 0$ resonance, and δ_m and δ_i represent the linewidths of the most mobile and immobile protein-associated linewidths observed. In (*B*) are shown the values of M_s (○) for K_5 as a function of the membrane concentration of PS. In the absence of PS the peptide is aqueous, but for all other points shown the data correspond to a peptide that is fully associated with the membrane. Also shown are depth parameters, Φ (▲), determined at several concentrations of PS (see Eq. 5).

Free energy of binding of TOAC peptides

The membrane partitioning of the basic TOAC peptides in Table 1 was determined using EPR spectroscopy as described above. Shown in Fig. 4 are binding plots obtained for several of the peptides shown in Table 1, and Table 3 shows the molar partition coefficients for these peptides. The binding affinity for these peptides was well within the detection limits afforded by EPR for most of the lipid conditions examined. As seen in Table 3, the binding of each peptide is increased by the addition of PS to the bilayer. In addition, only peptides having phenylalanine bind to membranes in the absence of negatively charged lipid.

From Table 3 one can extract free energy differences between pairs of peptides. When K_5 and K_6 are compared, addition of a single lysine makes the binding energy more favorable by 0.3 to 0.5 kcal/mol. This is consistent with the energies that have been reported for the interaction of a single lysine residue to PS bilayers (Kim et al., 1991), and with recent calculations on the binding of Lys_5 and Lys_7 (Murray et al., 1999). However, when K_3F_2 and K_4F_2 are compared, the addition of a single lysine is unfavorable. In this case a single lysine addition lowers the peptide binding to PC membranes by ~0.4 kcal/mol. Although this is a small energy difference, it is reproducible and the trend is seen for five different lipid compositions. Among the pentamers, substitution of two or three phenylalanines for lysine in K_5 makes relatively little difference in the binding free energies to membranes containing PS. Substitution of the first two Phe residues for Lys lowers the partition free

TABLE 2 EPR spectral parameters and power saturation data

	K_5	K_6	K_3F_2	K_4F_2	K_2F_3	K_2F_4
Aqueous Peptide						
ΔH_{pp} (G)	1.4	1.4	1.4	1.4	1.4	1.4
Π^{NiEDDA}	2.44	2.53	2.4	2.3	2.42	2.46
Π^{oxy}	0.36	0.36	0.35	0.33	0.37	0.42
Peptide bound to PC/PS (73:27) lipid vesicles						
ΔH_{pp} (G)	2.1	2.1	5.0	4.8	5.1	5.5
Π^{NiEDDA}	1.50	1.52	0.08	0.08	0.05	0.05
Π^{oxy}	0.25	0.25	0.28	0.28	0.26	0.18
Φ	−1.8	−1.8	1.2	1.2	1.7	1.4
Distance (Å)	−5 ± 2	−5 ± 2	8 ± 1	8 ± 1	9 ± 1	8 ± 1

Spectral parameters are determined according to Eqs. 4 and 5. ΔH_{pp} represents the unsaturated peak-to-peak width of the $m_I = 0$ EPR resonance; Π^{NiEDDA} is obtained in the presence of 20 mM NiEDDA; Π^{oxy} is obtained in the presence of air (20% O_2). The estimated distance is taken from the nitrogen *p*-orbital of the TOAC label to the lipid phosphate. Positive numbers indicate a location within the bilayer interior whereas negative numbers indicate a location on the aqueous side of the membrane interface. The error in the distance estimate includes experimental error in the measurement of $P_{1/2}$ and in the fit of Eq. 6 to the calibration data. The error does not include uncertainty in the placement of the doxyl calibration standards used to generate Eq. 6.

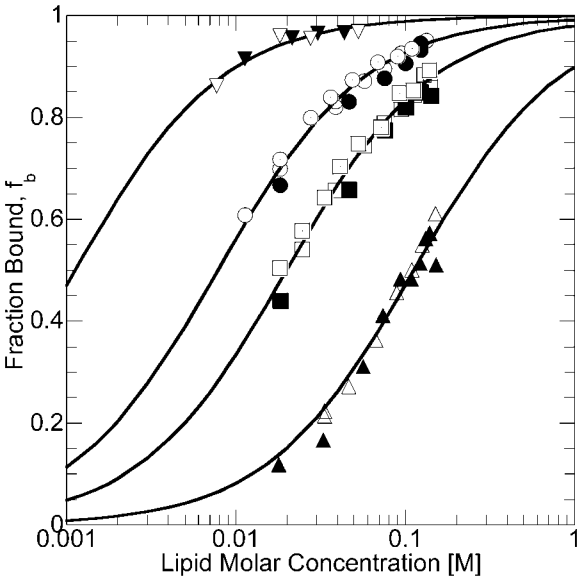

FIGURE 4 Binding curves obtained for peptide K_2F_4 with PC/PS (84:16) (▼), peptide K_3F_2 with PC/PS (73:27) (●), peptide K_5 with PC/PS (73:27) (■), and peptide K_5 with PC/PS (84:16) (▲). The shape of the symbols corresponds to a particular peptide and lipid vesicle composition, while the different shadings of the symbols represent data obtained during independent experiments. The partition coefficients, K_p, were extracted from curves (shown) that represent a least-squares fit of Eq. 2 to all of the data from the multiple experiments.

energy by ~0.3 kcal/substitution, and substitution of a third Lys slightly increases ΔG. For the hexamers, substitution of two Lys by Phe in K_6 also has a small effect, slightly raising ΔG in PS-containing membranes. Substitution of two more Lys residues by Phe produces a bigger effect in the opposite direction, decreasing ΔG by 1 kcal/mol or more. The uncertainty in these free energy differences is estimated to be 0.1 kcal/mol (see Table 3, legend), and they demonstrate that the same amino acid substitutions in two different peptides do not yield the same binding free energy differences.

TABLE 3 Partition coefficients, K_p, determined for the TOAC-containing peptides in PC and PC/PS lipid vesicles

Peptide	PC only	16 mol% PS	20 mol% PS	27 mol% PS	33 mol% PS
K_5	0	20	30	100	300
K_6	0	40	70	200	500
K_3F_2	<10	80	100	300	600
K_4F_2	<10	30	30	70	70
K_2F_3	20	70	70	100	150
K_2F_4	90	~10^3	~10^3	~10^3	~10^3

Values of K_p are in units of M^{-1}. Partition coefficients that fall within the range $10 < K_p < 1000$ are estimated to have a precision of ±10% based upon the results of multiple independent experiments. Given this precision, free energy comparisons among this set of peptides will have an uncertainty of ~100 cal/mol. Because these partition coefficients strongly depend upon the PS concentration, the predominant source of this error arises from the experimental uncertainty of determining the PC/PS ratio. For partition coefficients that fall outside of this range, the error associated with the measurement significantly increases as a result of the range of total lipid concentrations that can be used.

DISCUSSION

Basic peptides containing the spin-labeled amino acid TOAC were investigated here with several goals in mind. First, we wanted to obtain information about the position of peptides that interact electrostatically with the membrane interface by localizing the position of the peptide backbone. Second, we wanted to examine the influence of peptide composition on their equilibrium position when bound to bilayers. Finally, we were interested in determining whether a simple addition of side chain free energies could account for the differences in binding affinity among a set of related charged peptides.

The data presented here demonstrate that basic peptides lacking phenylalanine, such as K_5 and K_6, bind to membranes but are localized in the aqueous phase several angstroms from the interface, as illustrated in Fig. 5. This location is consistent with previous observations on basic peptides. For example, peptides such as Lys_5 do not produce changes in the headgroup angle upon binding to PC/PS-containing membranes as determined by ^{2}H-NMR (Roux et al., 1988). Because the headgroup angle is known to be sensitive to membrane surface charge density, these peptides do not appear to reside at the interface when bound. Changes in surface pressure are also not seen when Lys_5 is bound to monolayers (Ben-Tal et al., 1996), indicating that this peptide does not penetrate the interface when bound. Finally, removal of the aromatic phenylalanine residues from the MARCKS effector domain shifts the membrane bound position of this peptide to the aqueous double layer (Victor et al., 1999). As indicated above, our estimate of the membrane position of K_5 and K_6 places the backbone of these peptides in the aqueous phase at a distance of ~5 Å from the level of the lipid phosphate, and this distance is in good agreement with computational estimates for the position of Lys_5 made using a finite difference Poisson-Boltzmann method (Ben-Tal et al., 1996).

The available evidence suggests that peptides such as K_5 and K_6 fail to contact the bilayer because of a dehydration force that is experienced near the membrane interface. This dehydration force likely includes a Born repulsion due to the proximity of charge near the interface and a loss in entropy of the peptide as it is constrained at the membrane interface. As expected, raising the attractive Coulombic interaction by increasing the membrane surface charge density shifts the position of these two peptides closer to the water-membrane interface, an effect that was also observed with the effector domain from MARCKS (Qin and Cafiso, 1996; Victor et al., 1999). The substitution of two or more phenylalanine residues into the K_5 or K_6 peptides alters their

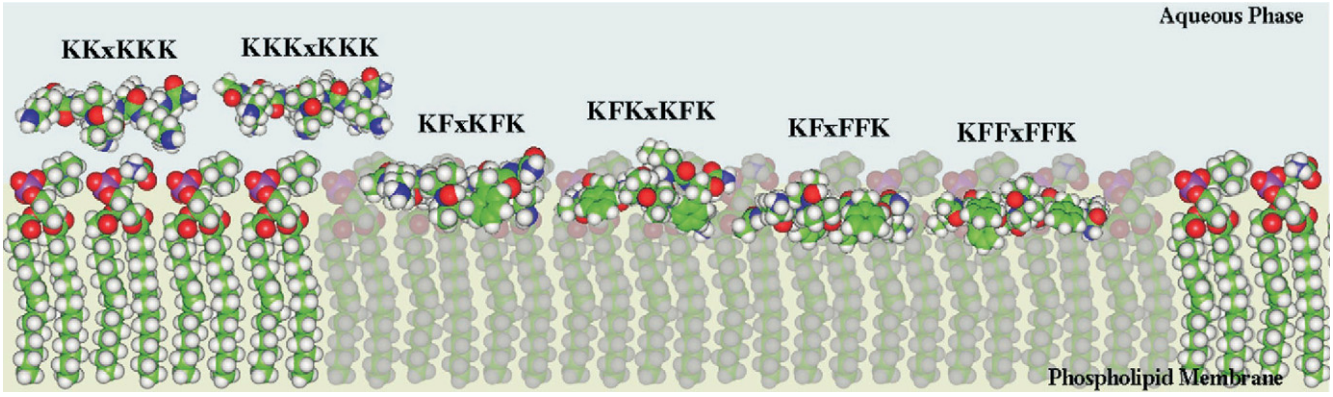

FIGURE 5 CPK models for the TOAC peptides bound to lipid bilayers based upon the experimental results that are listed in Table 2 for the peptides in PC/PS (73:27) vesicles. Peptides K_5 and K_6 lie on the aqueous side of the membrane-solution interface. All other peptides containing phenylalanine reside within the bilayer interior.

equilibrium position so that they lie below the level of the lipid phosphate, and this is likely the result of the increased hydrophobicity and reduced Born repulsion of these peptides (see Fig. 5). Among the peptides studied here, equilibrium positions were found to lie several angstroms on the aqueous side of the membrane interface or several angstroms on the hydrocarbon side of the membrane interface. Although a more extensive set of peptides might reveal a different or intermediate behavior, this general finding is consistent with other EPR measurements on charged peptides from MARCKS (151–175) and the N-terminal end of the src tyrosine kinase (Victor and Cafiso, 1998; Victor et al., 1999).

In general, the free energy of binding of charged peptides to membranes containing acidic lipid can be approximated using the Gouy-Chapman model. However, the Gouy-Chapman model does not include the repulsive energy that results from peptide dehydration near the membrane interface. Although this repulsive energy is likely to represent only a small fraction of the total peptide binding energy, it should be more pronounced for peptides that penetrate the membrane interface. Simple electrostatic calculations utilizing the Gouy-Chapman model along with a Born repulsion term and a hydrophobic interaction do a reasonable job of approximating the membrane binding of the peptides studied here (Victor and Cafiso, unpublished results).

In the measurements made here, peptides containing phenylalanine were found to reside with the TOAC label 8–9 Å below the level of the lipid phosphate groups. Because the nitrogen on the TOAC label is 2.4 Å from the peptide backbone, this would place the backbone ~6 Å below the lipid phosphate if the TOAC label faced the hydrocarbon. Previous EPR work on the membrane binding domain from MARCKS placed the spin-labeled side chain R1 (see Scheme 1) ~10 Å below the level of the lipid phosphate (Qin and Cafiso, 1996). If we assume that R1 has a conformation similar to that found on proteins from crystallography (Langen et al., 2000), the nitrogen on R1 would lie ~5–8 Å from the C_α carbon. If we assume that R1 is directed toward the hydrocarbon, this would place the backbone of MARCKS (151–175) 2–5 Å below the level of the lipid phosphate. Although there is considerably more uncertainty regarding the position the R1 side chain, the data suggest that MARCKS (151–175) may assume a position that is within a few angstroms of the position found here for the basic phenylalanine-containing peptides. It should be noted that the spectra of TOAC bound to these peptides is remarkably different from that obtained for R1 for membrane-associated peptides. This almost certainly arises because the diffusion of TOAC is tied to the diffusion of the peptide backbone, whereas the R1 side chain undergoes rapid rotation about the two bonds adjacent to the pyrrolidine ring (Langen et al., 2000).

Previous work that was carried out on two MARCKS-derived peptides determined the effect of substituting each phenylalanine by alanine (Victor et al., 1999). This substitution reduced the free energy of binding of this peptide, but did so by only 0.2 kcal/mol per Phe, much less than the 1.3 kcal/mol expected based upon the interfacial free energy of transfer for phenylalanine (Wimley and White, 1996). Given that the two analogs assume different equilibrium positions at the interface, one explanation for this result is that some of the free energy gained from the hydrophobic interaction of phenylalanine was expended in overcoming a dehydration energy required to position these peptides deeper in the membrane interface. It should be noted that the comparison of K_2F_3 and K_2F_4 (Table 3) yields an apparent binding free energy for a single phenylalanine on the order of −1.0 to −1.6 kcal/mol for PC or PC/PS membranes, close to the free energy determined previously (Wimley and White, 1996). This result clearly contrasts to that seen previously for MARCKS; however, unlike the MARCKS-derived peptides, K_2F_3 and K_2F_4 assume a similar location

in the bilayer, and they should exist in a similar dehydration state.

A comparison of the binding free energy differences between the peptides studied here yielded some surprising findings. For example, when K_5 and K_6 were compared, the contribution made by lysine was seen to be favorable and consistent with previous results (Kim et al., 1991). However, the apparent contribution of lysine was found to be unfavorable when K_3F_2 and K_4F_2 were compared. One important difference between these two sets of peptides is that the peptide backbone of K_5 or K_6 lies on the aqueous side of the membrane interface, whereas K_3F_2 and K_4F_2 reside within the bilayer below the level of the lipid phosphate. Clearly, any number of factors could contribute to the difference in the apparent free energy contribution made by lysine. For example, K_3F_2 and K_4F_2 may hydrogen-bond differently within the interface, there may be differences in side chain conformation between these two peptides, the additional lysine may be placed close enough to the membrane interior to contribute a significant Born energy, or the hexamer K_4F_2 may experience an additional backbone desolvation energy compared to the pentamer K_3F_2 (Wimley and White, 1996). In any case, the unexpected binding differences seen here and previously for MARCKS (Arbuzova et al., 2000; Victor et al., 1999) demonstrate the difficulty in using standard free energy values for amino acid side chains to predict the free energy of binding for this class of peptides. As indicated by the work carried out here, at least a part of this difficulty is due to the fact that the equilibrium position of these peptides with respect to the membrane interface is influenced by lipid composition and varies dramatically as a function of amino acid sequence.

In summary, the spin-labeled amino acid TOAC provides an excellent probe to detect the membrane attachment and position of membrane active peptides. It provides more accurate information on the position of the peptide backbone than side chain nitroxides and it is highly sensitive to backbone dynamics. For positively charged peptides, their energy and position when associated with acidic phospholipid membrane surfaces depends upon a long-range Coulombic attraction, the hydrophobic effect, and a short-range desolvation effect. When membrane-bound, the position with respect to the membrane interface can be altered by changing the composition of charged (lysine) and hydrophobic (phenylalanine) residues. Peptides lacking phenylalanine are localized within the double layer $\sim$5 Å above the level of the lipid phosphate. Peptides containing phenylalanine localize within the bilayer several angstroms below the level of the phosphate. For basic peptides, a comparison of the differences in the free energy of binding do not yield consistent free energy contributions for specific amino acid side chains. As a consequence, it is difficult to predict the membrane affinity of basic peptides based upon a simple addition of the free energy contributions expected from amino acid side chains.

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