# Membrane Interactions of the Sodium Channel S4 Segment and Its Fluorescently-Labeled Analogues†

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*Received May 14, 1992; Revised Manuscript Received July 1, 1992*

ABSTRACT: A 24-amino acid peptide corresponding to the S4 segment of the sodium channel was synthesized. In order to perform fluorescence energy transfer measurements and to monitor the interaction of the peptide with lipid vesicles, the peptide was selectively labeled with fluorescence probes at either its N- or C-terminal amino acids. The fluorescent emission spectra of 7-nitrobenz-2-oxa-1,3-diazol-4-yl- (NBD-)labeled analogues displayed blue shifts upon binding to small unilamellar vesicles (SUV), reflecting the relocation of the fluorescent probe to an environment of increased apolarity. The results revealed that both the N- and C-terminus of the S4 segment are located within the lipid bilayer. Titration of solutions containing NBD-labeled peptides with SUV was used to generate binding isotherms, from which surface partition constants, in the range of $10^4$ $M^{-1}$, were derived. The shape of the binding isotherms as well as fluorescence energy transfer measurements suggest that aggregation of peptide monomers within the membrane readily occurs in acidic but not in zwitterionic vesicles. Furthermore, the results provide good correlation between the incidence of aggregation in PC/PS vesicles and the ability of the peptides to permeate the vesicle's membrane. However, a transmembrane diffusion potential had no detectable effect on the location of the peptide within the lipid bilayer or on its aggregation state. Taken together, these results provide experimental support for a transmembranal localization for the sodium channel S4 segment as well as for its potential in forming part of the channel's lining, both properties in agreement with the "propagating helix" model, suggested by Guy and Conti [Guy, H. R., & Conti, F. (1990) *Trends Neurosci. 13*, 201–206].

Current models of voltage-gated ionic channels propose that such channels should contain charged structures referred to as voltage sensors, able to move within the lipid bilayer in order to sense the electric potential of the membrane. Sequences of one such channel, the sodium channel, have recently reported in several species (Noda et al., 1984, 1986; Kayano et al., 1988). In each case, the sodium channel molecule is predicted to be a large (>1800 amino acids), single-chain polypeptide containing four homologous domains. Examination of the amino acid sequences have led to the proposal of various models aimed at describing the sodium channel's structure. Most of these models predict that each homologous domain should contain six (Noda et al., 1986) to eight (Greenblat et al., 1985; Guy, 1988) transmembrane $\alpha$-helices, all of which are either hydrophobic or amphipathic except for the fourth transmembrane segment of each repeat, designated as S4 (Noda et al., 1986). This segment is highly conserved between sodium channels of different species; similar S4 sequences were reported in the putative membrane-spanning domains of other voltage-sensing proteins (Jan & Jan, 1990). S4 is unique in that every third amino acid is a positively charged Lys or Arg residue. Because of the unique structure of the S4 segment, it was proposed that this putative transmembrane segment may form part of the voltage sensor (Noda et al., 1984, 1986). Support for this hypothesis comes from site-directed mutagenesis studies in which alterations in the S4 sequence were shown to affect the voltage-dependent activation process (Stühmer et al., 1989; Auld et al., 1990). How the S4 segment achieves this voltage-sensing function is, however, unclear.

Various models have been proposed to explain the charge movements that result in the "gating-currents" observed upon sodium channel activation. The "helical screw" (Guy & Seetharamulu, 1986) or "sliding helix" (Catterall, 1986) models postulate that, at rest, the positive charges on S4 are balanced by negative charges of other transmembrane segments. Membrane depolarization causes the S4 helix to spiral toward the extracellular membrane surface; each 60° rotation causes a lateral movement of 4.5 Å. Other models argue in favor of conformational changes of the arginine side chains as responsible for the movements of charges. It is well possible that activation involves both conformational changes of the arginine side chains and movement of at least some S4 helices (Guy, 1988). Recently, Guy and Conti (1990) proposed a propagating helix model as a possible explanation for the channel-activating mechanism of S4. According to this model, only the central portion of S4 is in a helical conformation in the closed state, while the N terminus (external) and C terminus (internal) are in an extended or $\beta$-strand conformation. During activation, the $\alpha$-helical portion of S4 propagates toward the N terminus, shifting closer to the extracellular surface, thereby unblocking the central part of the channel. Site-directed mutagenesis (Pusch, 1990) and studies involving specific antibodies (Sammar et al., 1992) give experimental support for the shifting behavior of the S4 domain to the extracellular solution upon depolarization.

Previous work has shown that the S4 segment can form high conductance pores in planar lipid bilayers, implying bilayer association (Tosteson et al., 1989). However, questions of membrane affinity, the influence of membrane potential on the segment's orientation within the membrane, and the correlation between membrane permeabilization and formation of S4 aggregates remain unanswered. In order to

† This work was supported in part by the Basic Research Foundation administered by the Israel Academy of Sciences and Humanities, by the MINERVA Foundation, Munich, Germany, and by the Israel Cancer Research Fund. Y.S. is an Incumbent of the Adolpho and Evelyn Blum Career Development Chair in Cancer Research.

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0006-2960/92/0431-8868$03.00/0 

investigate these questions, we have synthesized and fluorescently labeled a peptide with a sequence corresponding to that of the S4 segment of the first internal repeat of the *Electrophorus electricus* electric organ sodium channel (amino acids 210–233, eel channel numbering) and have characterized its interactions with phospholipid bilayers.

We find that the peptide can associate, albeit with a moderate surface partition coefficient, with lipid bilayers. Both terminals of the peptide were found to be located within the hydrocarbon core of the membrane. The results reveal a good correlation between the ability of the peptide to significantly permeate PC/PS, but not PC vesicles, and capability to aggregate within a PC/PS but not within a PC vesicular bilayer. No detectable effects of transmembrane diffusion potential on the location of the peptide within the lipid bilayer or on its aggregation state could be observed. Taken together, these results provide experimental support for a transmembranal localization for the S4 segment of the sodium channel and give support to the potential role of this segment in forming part of the channel's lining, as suggested in the propagating helix model of Guy and Conti (1990).

## EXPERIMENTAL PROCEDURES

*Materials.* *tert*-Butyloxycarbonyl-Glu(*O*-Benzyl)-PAM[1] resin was purchased from Applied Biosystems (Foster City, CA). *tert*-Butyloxycarbonyl amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis were obtained from Sigma, as was 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl). Calcein was purchased from Hach Chemical Co. (Loveland, CO). Egg phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (sodium salt-grade I) were purchased from Lipid Products (South Nutfield, U.K.). Cholesterol (extra pure), purchased from Merck (Darmstadt, Germany), was recrystallized twice from ethanol. 3,3′-diethylthiadicarbocyanine iodide (diS-$C_2$-5), the 5-(and 6-)carboxyfluorescein succinimidyl ester (Flu-Su), the 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester (Rh-Su) were obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buffers were prepared using double glass-distilled water.

*Peptide Synthesis and Purification.* The peptide was synthesized by a solid phase method on PAM-amino acid resin (0.15 mequiv) (Merrifield et al., 1982), as previously described (Shai et al., 1987, 1990). Coupling was carried out with freshly prepared hydroxybenzotriazole active esters of *tert*-butyloxycarbonyl amino acids. The synthetic peptide was purified to a chromatographic homogeneity of >95% by reverse-phase HPLC on a $C_4$ column using a linear gradient of 25–80% acetonitrile in 0.1% TFA, in 40 min. The peptide was subjected to amino acid analysis in order to confirm its composition.

*Chemical Modification and Fluorescent Labeling of Peptides.* $N^1$-Acetyl-$C^1$-aminoethanol-S4 peptide was synthesized as follows: Resin-bound peptide (30 mg resin) was treated with 150 μL of aminoethanol in dimethylformamide (1:1 v/v) for 5 h, resulting in transamidation of the carboxylate group of the alanine residue located at the C-terminus of the peptide. By this procedure, a peptide analogue was obtained in which all protecting groups were maintained, but in which the C-terminal residue was modified by an aminoethanol group. This protected peptide was extracted from the resin by washing with dimethylformamide and precipitation with dry ether. The precipitate was first washed with ether and then with dry acetone before being reacted with TFA (50% v/v in methylene chloride), in order to remove the *tert*-butyloxycarbonyl protecting group from the N-terminal amine group of the attached peptide. The peptide was then reacted with a mixture of acetic anhydride and *N*-hydroxysuccinimide (1:1 mol/mol) in 0.1 M $NaHCO_3$, pH 8.0, resulting in the formation of the desired peptide analogue. The product, precipitated with ether, was subjected to HF cleavage.

Labeling of the N-terminus of the peptide was achieved as previously described (Rapaport & Shai, 1991, 1992). Briefly, 30–70 mg of resin-bound peptide (10–25 μmol) was treated with TFA (50% v/v in methylene chloride), in order to remove the *tert*-butyloxycarbonyl protecting group from the N-terminal amine group of the linked peptide. The resin-bound peptide was then reacted with either (i) NBD-Cl (3–4 equiv) in dimethylformamide (containing 2.5% v/v diisopropylethylamine) or (ii) the 5-(and 6-)carboxyfluorescein succinimidyl ester (Flu-Su), or (iii) the 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester (Rh-Su) (5–7 equiv), the latter two in dimethylformamide (containing 5% v/v triethylamine). The three reactions led to the formation of resin-bound $N^1$-NBD-S4, $N^1$-Flu-S4, or $N^1$-Rh-S4, respectively. After 24 h, the mixtures were washed thoroughly with methylene chloride and the peptides were then cleaved from the corresponding resins by HF, extracted with TFA, and finally precipitated with ether.

$N^1$-Acetyl-$C^1$-NBD-S4 peptide was synthesized as follows: Resin-bound peptide (30 mg of resin) was treated with 150 μL of ethylenediamine in dimethylformamide (1:1 v/v), resulting in transamidation of the carboxylate group of the alanine residue located at the C-terminus of the peptide. This protected peptide was extracted and precipitated as above and then reacted with NBD-Cl (3–4 equiv) in dimethylformamide (containing 2.5% v/v diisopropylethylamine) at room temperature, for 3–4 h. Further processing was as above.

All peptides were purified by reverse-phase HPLC on a $C_4$ Vydac column (300 Å pore size). The column was eluted in 40 min, at a flow rate of 0.9 mL/min, using a linear gradient of 25–80% acetonitrile in water in the presence of 0.1% TFA (v/v).

*Preparation of Small Unilamellar Vesicles.* Small unilamellar vesicles (SUV) were prepared from PC or an equimolar mixture of PC/PS by sonication. Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1 v/v) such that the mixture contained 10% (w/w) cholesterol. The solvents were evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were resuspended in buffer by vortex mixing. The resulting lipid dispersion was sonicated (~30 min) in a bath-type sonicator (G1125SP1 sonicator; Laboratory Supplies Company Inc., NY) until turbidity had cleared. The lipid concentration of the solution was then determined by phosphorus analysis (Barlett, 1959). Vesicles were visualized by electron microscopy (JEOL, Tokyo, Japan), and shown to be unilamellar with a diameter of 20–40 nm.

*Intrinsic Fluorescence Measurements.* Changes in the intrinsic fluorescence of NBD-labeled peptides were measured upon binding to vesicles. NBD-labeled peptide (0.1 μM) was added to 2 mL of buffer containing 80 μL (580 μg) of SUV

[1] Abbreviations: diS-$C_2$-5, 3,3′-diethylthiadicarbocyanine iodide; Flu, 5-(and 6-)carboxyfluorescein; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PAM, phenylacetamidomethyl; PC, egg phosphatidylcholine; PS, phosphatidylserine; Rh, 5-(and 6-)carboxytetramethylrhodamine; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

composed of either PC or PC/PS to establish a lipid:peptide ratio in which all the peptide is bound to lipid. After a 2-min incubation, emission spectra were recorded with a SLM-8000 spectrofluorometer (SLM Instruments, Urbana, IL), with excitation at 468 nm (4-nm slit).

*Binding Experiments.* The degree of peptide association with lipid vesicles was measured by adding SUV (prepared as described above) to a fixed amount of NBD-labeled peptide (0.1 $\mu$M) at 24 °C. The fluorescence intensity was measured as a function of the lipid:peptide molar ratio on a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 468 nm, using a 10-nm slit, and emission set at 530 nm, using a 5-nm slit. In order to determine the extent of the lipids' contribution to any given signal, the readings observed when unlabeled S4 was titrated with lipid vesicles were subtracted as background from the recorded fluorescence intensities.

*Resonance Energy-Transfer Measurements.* Fluorescence spectra were obtained at room temperature in a SLM-8000 spectrofluorometer, with the excitation monochromator set at 470 nm with a 4-nm slit width. Measurements were performed in a 1-cm pathlength quartz cuvette in a final reaction volume of 2 mL. Although the excitation maximum for fluorescein is 490 nm, a lower wavelength was chosen in order to minimize the excitation of tetramethylrhodamine (Harris et al., 1991; Rapaport & Shai, 1992). In a typical experiment, the desired amount of donor–peptide was added to a dispersion of SUV (prepared as described above) in buffer (50 mM $Na_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8), followed by the addition of acceptor–peptide either as a single dose or in several sequential doses. Fluorescence spectra were obtained before and after the addition of the acceptor. Any changes in the fluorescence intensity of the donor due to processes other than energy transfer to the acceptor were determined by substituting unlabeled peptide in place of the acceptor.

*Resonance Energy-Transfer Calculations.* The efficiency of energy transfer ($E$) was determined by measuring the decrease in the quantum yield of the donor as a result of the addition of acceptor. $E$ was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence ($I_{da}$) and in the absence ($I_d$) of the acceptor at the donor's emission wavelength, after corrections were made for membrane light scattering and the contribution of acceptor emission. The percentage of transfer efficiency ($E$) is given in

$$E = (1 - I_{da}/I_d) \times 100 \quad (1)$$

The correction for light scattering was made by subtracting the signal obtained when unlabeled analogues were added to vesicles containing the donor molecule. Correction for the contribution of acceptor emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

### *Membrane Permeability Studies*

*Valinomycin-Mediated Diffusion Potential Assay.* Pore-mediated diffusion potential collapse was detected fluorometrically as previously described (Sims et al., 1974; Loew et al., 1983, 1985; Shai et al., 1990). Briefly, 4 $\mu$L (28.8 $\mu$g) of a liposome suspension, prepared in 50 mM $K_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8 ($K^+$ buffer), was added to 1 mL of an isotonic buffer containing 50 mM $Na_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8 ($K^+$ free buffer), in a glass tube to which the dye diS-$C_2$-5 ($M_r$ 492) was then added. Subsequent addition of a valinomycin solution (final concentration $10^{-9}$ M) created a negative diffusion potential inside the vesicles by a selective afflux of $K^+$ ions, resulting in a quenching of the dye's fluorescence. Once the fluorescence had stabilized, S4 analogues were added, leading to an increased permeability toward other ions in the solution, resulting in a dissipation of the diffusion potential, monitored as an increase in the dye's fluorescence. Fluorescence was monitored on a Perkin Elmer LS-5 spectrofluorometer set for excitation at 620 nm and for emission at 670 nm. The percentage of fluorescence recovery ($F_t$) was defined by

$$F_t = [(I_t - I_0)/(I_f - I_0)] \times 100 \quad (2)$$

where $I_t$ = fluorescence observed after addition of peptide at time $t$, $I_0$ = fluorescence after addition of valinomycin, and $I_f$ = total fluorescence prior to addition of valinomycin.

*Calcein Release from Vesicles.* Calcein ($M_r$ 623), entrapped in liposomes at a self-quenching concentration, increases its fluorescence upon leakage, due to a dilution into the surrounding buffer solution (Allen & Cleland, 1980). Liposomes formed from PC or PC/PS phospholipids were prepared in buffer (10 mM HEPES, 150 mM NaCl, pH 7.4), containing 60 mM calcein, a concentration previously shown to cause self-quenching. Nonencapsulated calcein was removed from the liposome suspension by gel filtration on a Sephadex G-50 (Pharmacia) column connected to a low-pressure LC system (Pharmacia). Typically, 20 $\mu$L of the liposome suspension was applied to the column and then eluted in 10 mM HEPES, 150 mM NaCl, pH 7.4. The eluted vesicles peak, detected by UV spectroscopy ($\lambda$ = 280 nm), was collected and diluted to 2 mL with the elution buffer. Peptides were then added to 1 mL of a stirring vesicle suspension containing 2.5 $\mu$M liposomes. The resulting increase in fluorescence was monitored at room temperature on a Perkin-Elmer LS-5 spectrofluorometer set at an excitation wavelength of 490 nm and an emission wavelength of 526 nm. The percentage of fluorescence recovery is defined as

$$R_t = [(I_t - I_0)/(I_f - I_0)] \times 100 \quad (3)$$

where $I_0$ = initial fluorescence, $I_f$ = total fluorescence observed upon complete calcein release, after introduction of Triton X-100 (0.1% final concentration), and $I_t$ = fluorescence observed after adding peptide, at time $t$. Leakage in the absence of peptide was less then 1% in 60 min.

## RESULTS

*Synthesis and Fluorescence Labeling of Peptides.* In the present study, a peptide with a sequence corresponding to the S4 segment of the first internal repeat of the eel sodium channel (residues 210–233) was synthesized and subjected to a variety of chemical modifications (see Table I for sequence and peptide designations). In order to block the charges found at the peptides' terminals, acetylation of the N terminus and aminoethanol blockage of the C terminal were performed (peptide 2, Table I). Fluorescently-labeled analogues were prepared by modifying the peptide with an NBD probe at either the N- or C-terminal amino acid residues of the peptide. Other analogues were prepared by labeling the N terminus with the fluorophores carboxyfluorescein (Flu), to later serve as an energy donor, or tetramethylrhodamine (Rh), to later serve as an energy acceptor.

*NBD Fluorescence Studies.* The fluorophore NBD can be utilized for polarity and binding studies as its fluorescence spectrum reflects the environment in which it is found. Accordingly, the fluorescence emission spectra of two NBD-labeled peptides and of NBD-aminoethanol, serving as a control, were monitored in aqueous solutions or in the presence of vesicles composed of either PC or equimolar mixture of

Table I: Amino Acid Sequences of Fluorescent-Labeled and Modified S4[a]

| Peptide no. | Peptide Designation | Sequence |
|---|---|---|
| 1 | S4 | $H_2N$-RTFRVLRALKTITIFPGLKTIVRA-COOH |
| 2 | Ac-S4-EtOH | **Ac**-RTFRVLRALKTITIFPGLKTIVRA-CO-**NH-$CH_2CH_2$-OH** |
| 3 | N-NBD-S4 | **NBD**-RTFRVLRALKTITIFPGLKTIVRA-COOH |
| 4 | Ac-S4-C-NBD | **Ac**-RTFRVLRALKTITIFPGLKTIVRA-CO-**NH-$CH_2CH_2$-NH-NBD** |
| 5 | Flu-S4 | **Flu**-RTFRVLRALKTITIFPGLKTIVRA-COOH |
| 6 | Rh-S4 | **Rh**-RTFRVLRALKTITIFPGLKTIVRA-COOH |

[a] Modifications are marked in bold type.

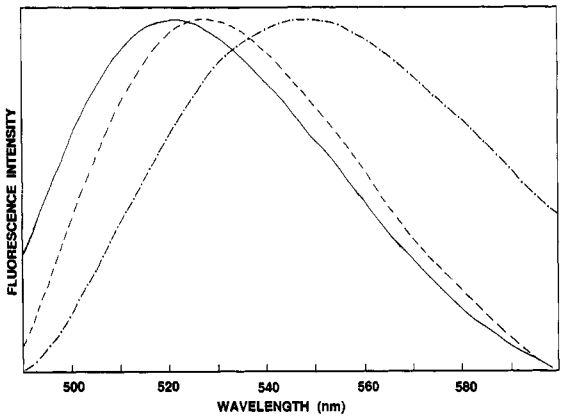


FIGURE 1: Fluorescence emission spectra of 0.1 $\mu$M NBD-labeled molecules. Spectra were determined in the presence of 350 $\mu$M PC/PS vesicles in buffer composed of 50 mM $Na_2SO_4$, 25 mM HEPES-$SO_4^{2-}$, pH 6.8. The excitation wavelength was set at 468 nm. Emission was scanned from 490 to 600 nm. Symbols: N-NBD-S4, continuous line; Ac-S4-C-NBD, dashed line; NBD-aminoethanol, dashed-dotted line.

PC/PS. SUVs were used in all of the fluorometric studies in order to minimize differential light scattering effects (Mao & Wallace, 1984). The peptides all exhibited fluorescence emission maxima at 549 ± 1 nm in buffer, in agreement with previously reported emission maximum wavelengths for NBD derivatives (Rajarathnam et al., 1989; Rapaport & Shai, 1991). However, upon addition of the peptides to a solution (pH 6.8) of vesicles composed of either PC or PC/PS, blue shifts in the fluorescence emission maxima of the peptides, relative to their spectra in buffer, were observed. No such shift could be detected using the control molecule NBD-aminoethanol. The changes in spectra reflect relocation of the NBD group into a more hydrophobic environment. Note that in these experiments the lipid:peptide molar ratio was consistently elevated (>3000:1) so that spectral contributions of free peptides could be considered negligible. The fluorescence emission spectra of the NBD-labeled molecules, in the presence of PC/PS SUV, are given in Figure 1. Results obtained from PC vesicles were similar to those reported for PC/PS vesicles and as such are not shown. Attachment of the NBD probe at the peptide's N terminus resulted in a larger blue shift than when the NBD moiety was linked to the C terminus of the peptide: The maximal emission wavelengths were 522 ± 2 nm and 528 ± 2 nm, respectively.

In order to study the influence of diffusion potential on the location of the peptide within the lipid bilayer, we performed the following experiment: 60 $\mu$L of a liposome suspension (PC or PC/PS), prepared in $K^+$ buffer, was added to 2 mL of $K^+$-free buffer (both defined in the Experimental Procedures section). The NBD-labeled peptides (0.1 $\mu$M), N-NBD-S4 or Ac-S4-C-NBD, were then added to the mixture. Addition of a valinomycin solution (final concentration 4 nM) created a negative diffusion potential inside the vesicles by a selective afflux of $K^+$ ions. Formation of a negative diffusion potential was confirmed by the quenching of the voltage-sensitive dye, diS-$C_2$-5, under identical experimental conditions. Fluorescence emission spectra, recorded as described above, were recorded at time 0 and again after 10 min of exposure to valinomycin. The establishment of a diffusion potential had no effect on the spectra of the peptides, regardless of the SUV type chosen. Note that at the low peptide:lipid ratio used in this experiment the S4 peptide was unable to permeate the vesicles, thus dissipating the diffusion potential existing within them (see Figure 5).

*Characterization of Binding Isotherms.* The fluorescent properties of the NBD moiety enabled us to generate binding isotherms from which partition coefficients could be calculated. NBD-labeled peptides, at a concentration of 0.1 $\mu$M, were titrated with either PC or PC/PS SUV, as described in the Experimental Procedures section. Increases in the fluorescence intensities of the NBD-labeled peptides, at pH 6.8, were plotted as a function of the lipid:peptide molar ratios for PC or PC/PS vesicles and are reflected as traces A in Figures 2 and Figure 3, respectively. SUV vesicles were used in this assay in order to minimize light scattering effects (Mao & Wallace, 1984). The concentration of peptide used was low enough to cause minimal aggregation in the aqueous phase and was assumed not to disrupt the bilayer structure, as shown for the bee venom melittin, a well-known membrane-interacting peptide (Hider et al., 1983). Control experiments were performed by titrating unlabeled S4 with lipids up to the maximal concentration used in the assay. The fluorescence intensities of these solutions (after subtracting the contribution of the vesicles) remained unchanged.

The binding isotherms were analyzed as a partition equilibrium (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using the following formula:

$$X_b = K_p C_f \quad (4)$$

where $X_b$ is defined as the molar ratio of bound peptide per total lipid, $K_p$ corresponds to the partition coefficient, while $C_f$ represents the equilibrium concentration of free peptide in the solution. In order to calculate $X_b$, we extrapolated $F_\infty$ (the fluorescence signal obtained when all the peptide is bound to lipid) from a double-reciprocal plot of $F$ (total peptide fluorescence) versus $C_L$ (total concentration of lipids) as previously suggested by Schwarz (Schwarz et al., 1986). Knowing the fluorescence intensities of unbound peptide, $F_0$,

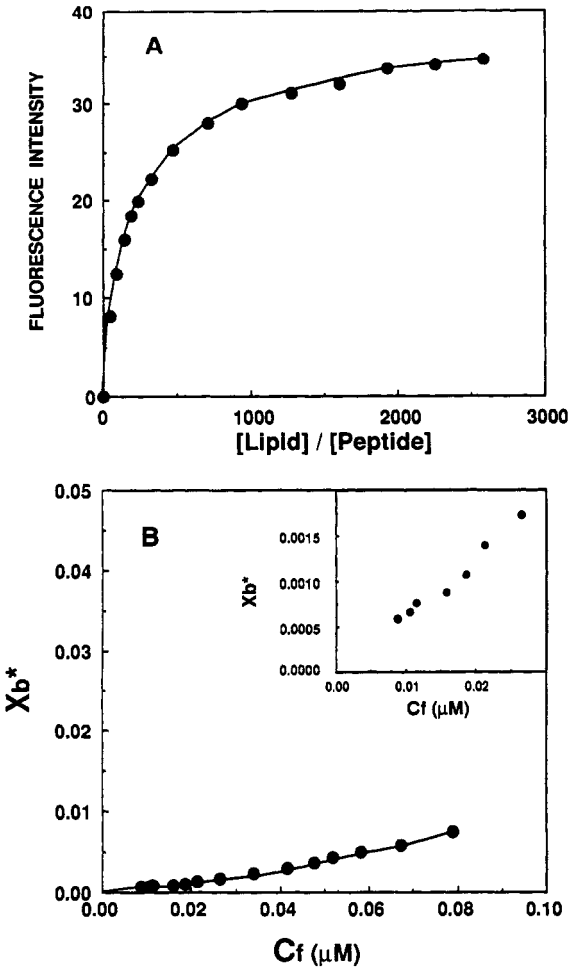


FIGURE 2: (A) Increase in the fluorescence of Ac-S4-C-NBD upon titration with PC vesicles. Peptide (0.1 $\mu$M total concentration) was titrated with PC vesicles with excitation monitored at 468 nm and emission recorded at 530 nm. The experiment was performed at 24 °C in 50 mM $Na_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8. (B) Binding isotherm derived from panel A by plotting $X_b$* (molar ratio of bound peptide per total lipid) versus $C_f$ (equilibrium concentration of free peptide in the solution). The inset shows the low concentration range of the main figure.

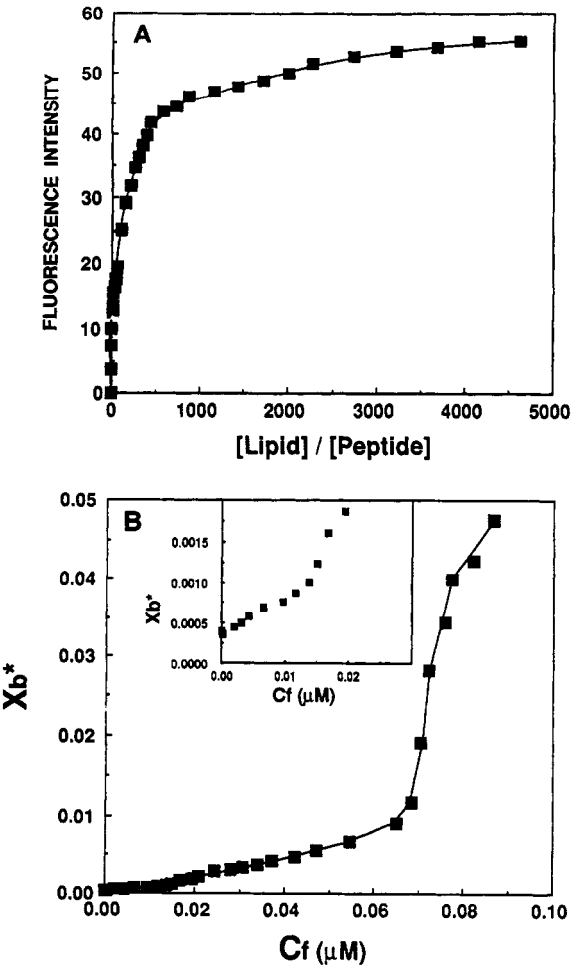


FIGURE 3: (A) Increase in the fluorescence of Ac-S4-C-NBD upon titration with PC/PS vesicles. Peptide (0.1 $\mu$M total concentration) was titrated with PC/PS vesicles with excitation monitored at 468 nm and emission recorded at 530 nm. The experiment was performed at 24 °C in 50 mM $Na_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8. (B) Binding isotherm derived from panel A as described for Figure 2B.

as well as those of bound peptide, $F$, the fraction of membrane bound peptide, $f_b$, could be calculated using

$$f_b = (F - F_0)/(F_\infty - F_0) \quad (5)$$

Having calculated the value of $f_b$, it is then possible to calculate $C_f$, as well as the extent of peptide binding, $X_b$. In practice, it was assumed that the peptides were initially partitioned only to the outer leaflet of the SUV (60% of the total lipid), as had been previously suggested (Beschiaschvili & Seelig, 1990). Therefore, values of $X_b$ were corrected as such:

$$X_b^* = X_b/0.6 \quad (6)$$

and eq 4 becomes

$$X_b^* = K_p^* C_f \quad (7)$$

The curve resulting from plotting $X_b$* versus free peptide, $C_f$, is referred to as the conventional binding isotherm. The experimental binding isotherms obtained from the interactions of the Ac-S4-C-NBD peptide with either PC or PC/PS SUV, at pH 6.8, are presented in traces B of Figures 2 and 3, respectively. The binding isotherm of N-NBD-S4 greatly resembled that of Ac-S4-C-NBD and as such is not included. Since sufficient data points of $C_f$ could be collected at very low concentrations, the partition coefficients were estimated from the initial slopes of the curves extrapolated to zero. The surface partition coefficients, $K_p$*, of the Ac-S4-C-NBD peptide were calculated (with an uncertainty of 20%) to be 6.2 $\times 10^4$ $M^{-1}$ in the case of PC vesicles and 9.0 $\times 10^4$ $M^{-1}$ using PC/PS vesicles. These values are within the range obtained for bioactive peptides such as melittin and its derivatives in zwitterionic vesicles ($10^4$–$10^5$ $M^{-1}$) (Stankowski & Schwarz, 1990), the *Staphylococcus* $\delta$-toxin (Thiaudière et al., 1991), and pardaxin analogues (Rapaport & Shai, 1991).

The shape of binding isotherms can provide information about the organization of peptides within a membrane. The isotherms obtained from PC/PS vesicles display an initial "lag"; i.e., the curves are initially flat but eventually rise sharply upon the crossing of a threshold concentration ($C_f$*). On the other hand, isotherms obtained with PC vesicles rise only slightly once a threshold concentration has been reached. The shape of the PC/PS isotherms argues in favor of a process whereby peptides first incorporate into the membrane and then aggregate (Schwarz et al., 1986, 1987). Similar behavior was observed for alamethicin (Rizzo et al., 1987) and NBD-labeled pardaxin and some of its derivatives (Rapaport & Shai, 1991), peptides postulated to form aggregation-derived pores in lipid bilayers.

*Fluorescence Energy-Transfer Studies.* In order to study intermolecular energy transfer, modified peptides, labeled with

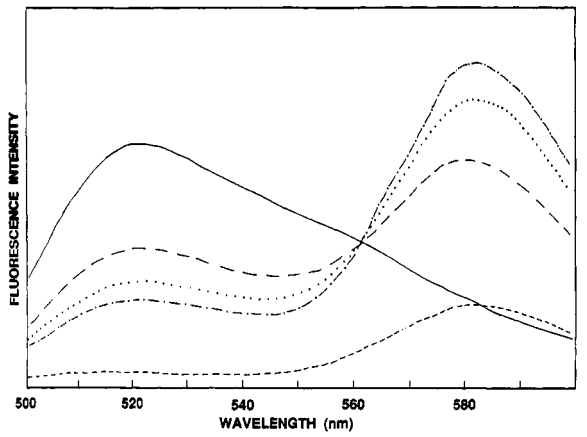


FIGURE 4: Fluorescence energy transfer dependence on peptide acceptor concentration. The spectrum of donor–peptide, Flu-S4, was determined in the presence or absence of various concentrations of acceptor–peptide, Rh-S4. Each spectrum was recorded in the presence of 70 $\mu$M PC/PS vesicles in 50 mM $Na_2SO_4$, 25 mM HEPES–$SO_4^{2-}$, pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. Symbols: (—) 0.1 $\mu$M Flu-S4; (- - -) spectrum of 0.1 $\mu$M Rh-S4 taken as control; (– –) a mixture of 0.1 $\mu$M Flu-S4 and 0.1 $\mu$M Rh-S4; (···) a mixture of 0.1 $\mu$M Flu-S4 and 0.2 $\mu$M Rh-S4; (– · –) a mixture of 0.1 $\mu$M Flu-S4 and 0.3 $\mu$M Rh-S4.

fluorescein (Flu-S4), serving as an energy donor, or with rhodamine, serving as a fluorescence acceptor (Rh-S4), were used. The $R_0$ value for the Flu/Rh donor/acceptor pair was calculated to be 45 Å (Rapaport & Shai, 1992). The extent of energy transfer from Flu-S4 to Rh-S4 within membranes is presented in Figure 4: Line F represents the emission spectrum of Flu-S4 alone, at the fluorescein excitation wavelength (470 nm), while line R represents the Rh-S4 emission spectrum at the same excitation wavelength. When Rh-S4, at a final concentration of 0.1, 0.2, or 0.3 $\mu$M, was added to a mixture of Flu-S4 (0.1 $\mu$M) and PC/PS lipid vesicles (70 $\mu$M), dose-dependent quenching of the donor emission was observed, consistent with energy transfer (Figure 4, lines A, B, and C, respectively). To reduce energy transfer between unassociated peptide monomers, the surface density of the donors and acceptors was maintained at relatively low levels (lipid:peptide ratio of 700:1). In control experiments, when the acceptor–S4 was replaced by an equal amount of the unlabeled peptide Ac-S4-Et, little or no change in the emission spectrum of fluorescein was observed (data not shown). Furthermore, no decrease in emission at 520 nm was observed when the acceptor probe was attached to ethanolamine (which does not interact with the donor-labeled peptide), confirming that the decrease in donor emission at 520 nm was caused by a specific association of donor- and acceptor-labeled peptides and was not an artifact due to direct absorption of light by the acceptor.

In the case of PC/PS vesicles, the transfer efficiency observed was higher than that calculated if a surface density of random distribution was assumed (Fung & Stryer, 1978). Thus, the donor and acceptor were not randomly distributed throughout the membrane but were instead associated. In the case of PC vesicles (data not shown), the recorded transfer efficiencies are not higher than theoretically generated values for equal amounts of randomly distributed acceptor molecules. These results support the concept of self-association of fluorescently labeled S4 peptides within PC/PS vesicles. Unfortunately, these experiments do not allow for a determination of the nature of this nonrandom distribution. In PC bilayers, the peptide exists in a more randomly distributed state.

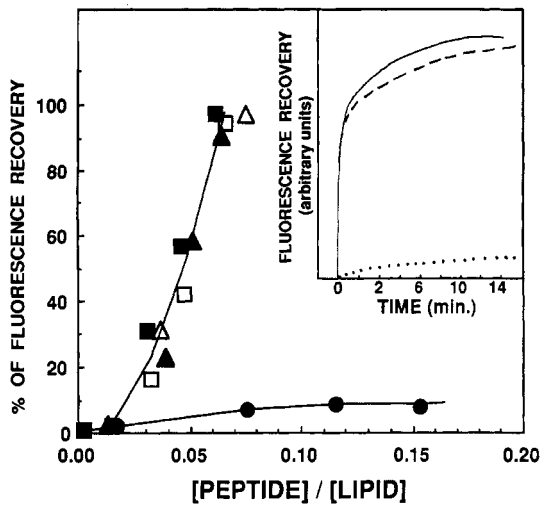


FIGURE 5: Dissipation of diffusion potentials by several S4 analogues in PC or PC/PS vesicles. Peptides were added to 1 mL of buffer containing a constant concentration of vesicles (38 $\mu$M), preequilibrated with the fluorescent dye diS-$C_2$-5 and valinomycin. Fluorescence recovery was measured after 10–20 min and is plotted as a function of the peptide:lipid molar ratio. Symbols: (■) Ac-S4-EtOH in PC/PS; (▲) S4 in PC/PS; (□) Ac-S4-C-NBD in PC/PS; (△) Rh-S4 in PC/PS; (●) Ac-S4-EtOH in PC vesicles. Flu-S4 gave similar results and as such is not given. (Inset) The dissipation rate of a diffusion potential as a function of time: (—) 1.3 $\mu$M Ac-S4-EtOH in PC/PS; (- - -) 1.3 $\mu$M Ac-S4-EtOH in PC/PS; (···) Ac-S4-EtOH in PC vesicles.

In order to investigate the influence of negative diffusion potential on the self-aggregation process, the experiment was also performed using SUV prepared in $K^+$ buffer and suspended in $K^+$-free buffer. Quenching was observed when acceptor–peptide was added to a solution of vesicles preincubated with donor–peptide. Addition of valinomycin had no effect on the degree of quenching, demonstrating that the negative diffusion potential formed in the presence of valinomycin did not cause detectable changes in the probes' intermolecular distance (data not shown).

### *Membrane Permeability Studies*

*Valinomycin-Mediated Diffusion Potential Assay.* In order to examine the membrane-permeating behavior of S4 and its N- or C-terminal-labeled analogues, the peptides were tested for their ability to dissipate a diffusion potential in SUV. Peptides, at increasing concentrations, were mixed with SUV composed of either PC or PC/PS, pretreated with the fluorescent dye diS-$C_2$-5 and valinomycin. Recovery of fluorescence was monitored with time, using peptide:lipid molar ratios ranging from 0.01:1 to 0.2:1. Each experiment was repeated three times, each time with fresly prepared SUV. The ability of the peptides to permeate the membrane was elucidated by monitoring the fluorescence recovery until a plateau was observed (usually after 10–20 min). This level was taken as the maximal activity attainable at each concentration of the tested analogues (see Figure 5). The time course of fluorescence recovery provides information about the rate of membrane permeation. Figure 5 (inset) shows typical profiles of fluorescence recovery as a function of time for Ac-S4-Et using PC or PC/PS vesicles, at similar lipid:peptide molar ratios. Similar profiles were obtained using the other S4 analogues, although with slightly different kinetics. The results reflect the high perturbing activity of S4 and its N- or C-terminal-modified analogues on PC/PS vesicles, in marked contrast to the low level observed with PC vesicles.

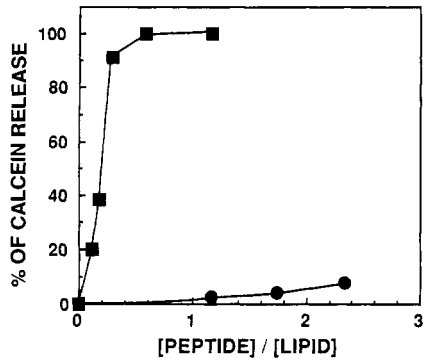


FIGURE 6: Calcein release induced by Ac-S4-Et. Increasing amounts of peptide were added to 2.5 $\mu$M of either PC or PC/PS vesicles containing entrapped calcein at a self-quenching concentration in 1 mL of buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). Fluorescence recovery was measured after 5 min and is plotted as a function of the peptide:lipid molar ratio. Symbols: (■) PC/PS vesicles; (●) PC vesicles.

*Calcein Leakage from Vesicles.* The Ac-S4-Et peptide was tested for its potency in evoking calcein releasing from liposomes composed of either PC or PS/PC (1:1), at pH 7.4. Increasing amounts of peptide were added to a vesicle suspension, and the level of calcein release reached after 5 min, as a function of the [peptide]/[lipid] ratio, was recorded (Figure 6). Note that significant levels of calcein release could only be induced from vesicles composed of acidic PC/PS membranes, while using high molar ratios of peptide:lipid.

## DISCUSSION

An alternative approach to structure–function studies of ion channels is based on the use of small synthetic peptides to reconstitute channel function (Lear et al., 1988). The major thrust of synthetic peptide experiments has been to reconstruct an ion-selective pore in artificial bilayers whose properties match as closely as possible those of the native channel (Stühmer, 1991). Synthetic peptides corresponding to the transmembrane segments S3 of the brain sodium channel (Oiki et al., 1988a) and the S4 repeat IV of the *E. electrophorus* sodium channel (Tosteson et al., 1989) have been shown to form conductive pores in lipid bilayers. Similarly, synthetic peptides corresponding to the transmembrane M2-$\delta$ (Oiki et al., 1988b) and MA-$\beta$ (Ghosh & Stroud, 1991) of the acetylcholine receptor, as well as a peptide similar to the M-2 and M-4 segments of the glycine receptor (Langosh et al., 1991), are also able to form channel pores in lipid bilayers. In line with this approach, we decided to perform a detailed study on the interaction of the putative voltage sensor, the S4 segment of the sodium channel, with membranes by employing a variety of biophysical techniques.

A peptide corresponding to the S4 repeat I segment of the *E. electrophorus* sodium channel was synthesized, modified, and labeled with the fluorescent probes NBD, Flu, or Rh. Fluorescence measurements were then employed to investigate three aspects of membrane–peptide interaction: (1) the orientation of peptide bound to SUV membranes; (2) the organization of peptide monomers within the membrane; and (3) the calculation of the surface partition coefficient of the peptides in lipid vesicles. Furthermore, the influence of a valinomycin-mediated diffusion potential, as well as the correlation between the state of aggregation within the membrane and the peptide's membrane perturbing activity, were also studied.

Examining the environment encountered by an NBD group located at either the N or C terminus of the peptide, we noted that the fluorescence probe was sequestered to a region of higher hydrophobicity (emission maxima of 522 nm and 528 nm, respectively) than that detected with an NBD probe located on the surface of the membrane (emission maximum 533 nm; Chattopadhyay & London, 1987). We thus conclude that the S4 segment incorporates into the lipid bilayer and that both the N- and C-terminals of the segment are located within the acyl-chain region of the membrane. A difference of 6 nm in the blue shift of the NBD group when attached to the N terminus as compared to the C-terminus is significant, and as such it argues in favor of S4 existing in an organized manner within the lipid bilayer, rather than a random distribution. Moreover, we believe that this result reflects the preferential organization of S4 monomers within the aggregate in a parallel manner, as proposed for the shark repellent neurotoxin pardaxin within the membrane on the basis of similar observations (Rapaport & Shai, 1992). However, such experiments cannot determine whether both peptide terminals lie in the cis side of the membrane or whether the peptide fully traverses the lipid bilayer, and as such further investigation is required. It is important to note that the formation of negative diffusion potential had no detectable effects on the location of the peptide's N or C terminus within the lipid bilayer. These results can be explained by the notion that none of the proposed models for the involvement of S4 in the gating mechanism require movement of more than a few angstroms within the membrane. Thus, our observation that the formation of a diffusion potential across the membrane did not cause any detectable changes in the location of the probes within the membrane could be explained by a lack of sensitivity of our method to such minor changes. Alternatively, as suggested by Stühmer (1991), charge movement is more likely to arise from slight rearrangements not only of charges and dipoles within S4 but also of other charged structures, in particular the countercharges of S4. Therefore, in the absence of other structures, as is the case in our system, only minor, if any, charge movement occurs. Moreover, our results can be explained by the propagating helix model, which proposes the helical portion of the segment to be the moving element, while the ends of the segment remain almost fixed relative to the membrane surface.

Apart from investigating the localization of the S4 peptide within the membrane, we studied the affinity of the segment to lipid bilayers. The partition coefficients obtained from binding isotherms with zwitterionic vesicles (formed from PC) or with the acidic vesicles (formed from PC/PS) did not differ markedly. This suggests that electrostatic interactions do not play a major role in the partitioning of the peptide into lipid vesicles. However, such interactions are crucial for the organization of the peptides within the membrane. As inferred from the shape of the binding isotherm and from FET experiments, a self-association process of peptide monomers was observed, in acidic but not in zwitterionic SUV. Due to the higher sensitivity of this method, energy transfer could be observed even at lower values of the peptide:lipid molar ratio, corresponding to the critical concentration of free peptide, $C_f^*$ (reflected in Figure 3B). This difference could be explained by the fact that negative and positive charges counteract each other in a PC/PS-S4 system. The negatively-charged headgroups of the acidic vesicles are able to compensate for the electrostatic repulsion formed between the positively-charged arginine and lysine side chains, a situation arising when several peptide monomers are brought into close proximity. According to the propagating helix model, positive charges of S4, in the open state, should interact well with

negative charges found near the putative extracellular ends of other transmembrane segments. In our system the negatively-charged headgroups of the lipid molecules might play a similar role. The lack of influence of a negative diffusion potential on intermolecular distances as reflected in FET measurements can be explained as follows: The fluorescent probes were attached to the N terminus of the segment. It is possible that other parts of the peptide change their location with changes in the membrane potential, maintaining the N terminus at a fixed position relative to the N termini of adjacent peptide molecules. Moreover, the propagating helix model postulates a relatively small spatial distance between the S4 segments in the deactivated state, when a bundle of such segments is hypothesized to occlude the pore.

Good correlation between the formation of peptide aggregates in acidic lipid vesicles and the ability of the peptide to permeate these membranes is obtained. Hence, we can conclude that in our system membrane permeability is an aggregation-dependent process. Our experiments on membrane permeabilization potential, as reflected in the release of entrapped molecules, give support to the hypothesis that S4 segments form part of the channel lining, also as suggested by the propagating helix model. Further studies, with additional putative transmembrane segments, are currently being performed, in order to obtain detailed information on the mutual interactions of these segments within lipid bilayers.

## ACKNOWLEDGMENT

We thank Dr. Y. Marikovsky for his help in electron microscopy. Aurelia Finkels for her technical assistance, and Jerry Eichler for editing the manuscript.

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