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Mechanism of Interaction of the Cyanine Dye DiS-C_3-(5) with Renal Brush-Border Vesicles

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Summary. The equilibrium binding mechanism and kinetics of binding of diS-C_3-(5) (3,3′-dipropylthiodicarbocyanine iodide) to rabbit renal brush-border membrane vesicles (BBMV) were examined using steady-state and time-resolved fluorescence, and fluorescence stopped-flow methods. In aqueous solution, diS-C_3-(5) exists as a monomer at concentrations < 5 μM with fluorescence emission peak at 670 nm (excitation 622 nm), anisotropy $r = 0.102$, and lifetime $\tau = 1.2$ nsec (23°C). Upon addition of increasing BBMV (voltage clamped to 0 mV using K^+/valinomycin), the 670 nm emission peak decreases, corresponding to formation of a nonfluorescent membrane dimer, and subsequently a new emission peak at 695 nm increases, corresponding to membrane monomer. Dynamic depolarization studies show that aqueous diS-C_3-(5) rotation is unhindered with a rotational rate $R = 0.57$ nsec^{-1} while membrane monomer is hindered with steady-state anisotropy $r = 0.190$, lifetime $\tau = 2.1$ nsec, $R = 0.58$ nsec^{-1} and limiting anisotropy $r_\infty = 0.11$. Based on equilibrium fluorescence titrations, the membrane monomer-dimer (*M-D*) dissociation constant, $K_d = [M]^2/[D][\text{BBMV}]$, is 0.0013, where BBMV is expressed as membrane phospholipid concentration. Three distinct kinetic processes are identified by stopped-flow experiments in which BBMV are mixed with diS-C_3-(5). There is rapid binding of diS-C_3-(5) to the membrane to form bound monomer with a 6-msec exponential time constant. The membrane monomer at the membrane outer surface then aggregates to form bound dimer at the outer surface with a concentration independent time constant of 30 msec. The overall dimerization reaction probably consists of a rate-limiting reorientation process (30 msec) followed by a rapid dimerization which occurs on a nanosecond time scale. Finally, there is a 0.8 to 1 sec translocation of membrane dimer between symmetric sites at the inner and outer membrane surfaces. The translocation reaction is the step which is probably sensitive to changes in transmembrane electrical potential.

Key Words cyanine dye · membrane potential · stopped-flow · brush-border membrane · fluorescence lifetimes

Introduction

Potential-sensitive optical dyes have been used increasingly to measure membrane potential in vesicles, viable cells and intracellular organelles, and intact tissues such as conducting nerves and contracting muscle. The carbocyanine dye diS-C_3-(5) (3,3′-dipropylthiodicarbocyanine iodide) is one of the most useful probes because of its relatively large change in fluorescence intensity with variations in membrane potential. DiS-C_3-(5) has been used to monitor membrane potential changes in erythrocytes (Sims et al., 1974; Tsien & Hladky, 1978), mitochondria (Kinnally et al., 1978), liposomes (Guillet & Kimmich, 1981), sarcoplasmic reticulum vesicles (Ivkova et al., 1983), and, most recently, to study Na^+-coupled transport of sugars, amino acids and tricarboxylic acids in renal brush-border membrane vesicles (BBMV) (Beck & Sactor, 1978; Burckhardt et al., 1980; Wright et al., 1981; Kragh-Hansen et al., 1982; Wright, 1984).

The mechanism by which diS-C_3-(5) responds to changes in membrane potential was studied first by Sims et al. (1974), and subsequently in a number of other laboratories (Waggoner et al., 1977; Smith et al., 1980; Ivkova et al., 1983). DiS-C_3-(5) partitions strongly into lipid bilayer membranes where it undergoes membrane potential-dependent changes in fluorescence intensity. Membrane-bound dye is probably present in at least two forms: a fluorescent monomer and a nonfluorescent dimer. The mechanism and kinetics of interconversion among solution and bound dye forms, as well as the location and physical environment of the bound dye forms have not been determined.

In order to define the interaction mechanism of diS-C_3-(5) with a biological membrane, we studied the fluorescence response of diS-C_3-(5) in renal brush-border membrane vesicles isolated from rabbit renal cortex. The BBMV is a model vesicle system which is comprised of the plasma apical membrane of the proximal tubule cell which faces the tubule lumen (Booth & Kenny, 1974). The BBMV preparation is quite homogeneous and has been ex-

tensively characterized with respect to its enzymatic and membrane transport properties using tracer uptake methods, light scattering (Verkman et al., 1985), pH-dependent fluorescent probes and diS-C_3-(5) (Wright, 1984).

We used steady-state fluorescence, and fluorescence lifetime and dynamic depolarization methods to characterize the affinities and environments of free and bound diS-C_3-(5). Fluorescence stopped-flow was used to define the sequence and kinetics of interconversions among free and bound dye forms. Based on this approach, we proposed a model in which solution diS-C_3-(5) monomer binds to the outer BBMV membrane surface rapidly (~6 msec) and undergoes a red shift in fluorescence emission and a decrease in rotational freedom. The membrane-bound monomer is rapidly interconverted to a nonfluorescent dimer (~30 msec) which can slowly permeate the membrane (~1.2 sec) to bind to a symmetric site on the inner membrane surface.

Materials and Methods

Materials

All chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, Mo.). DiS-C_3-(5) was obtained from Molecular Probes Inc. (Seattle, Wash.) and was stored as a 5-mM stock solution in ethanol at 0°C in the dark. 10 × 10 × 48 mm acrylic cuvettes (Sarstedt, W. Germany) were used to eliminate dye binding which was observed using glass and quartz cuvettes. Most experiments were performed using 'standard buffer' consisting of 250 mM sucrose, 10 mM MES/Tris, 150 mM KCl at pH 7.4.

Vesicle Preparation

Brush-border membrane vesicles (BBMV) were prepared from 1 to 2 kg female New Zealand white rabbits by a Mg aggregation and differential centrifugation procedure described previously (Booth & Kenny, 1974; Aronson, 1978). BBMV were prepared in standard buffer containing valinomycin (25 μg/mg membrane protein) and stored at −70°C as a concentrated pellet of ~30 mg protein/ml. BBMV concentrations are expressed as [membrane phospholipid] using the conversion factor 0.4 μmol phospholipid/mg protein (Hise et al., 1984). Protein concentration was determined by the method of Lowry et al. (1951).

Fluorescence Measurements

Steady-state fluorescence intensity and anisotropy spectra were measured on an SLM 8225 fluorimeter (Urbana, Ill.) with a double-excitation monochromator and single-emission monochromator which was interfaced to an IBM-XT computer. Steady-state anisotropy spectra were measured in the L-format with subtraction of background.

Fluorescence lifetime and polarization experiments were performed on an SLM 4800 nanosecond fluorimeter with a thermostatted sample compartment. The fluorescence of solutions containing diS-C_3-(5) was excited at 622 nm (0.5 mm slit) and measured using Corion 650 or 697 nm high-pass filters. Less than 1% of the measured fluorescence intensity was due to scattered light.

Fluorescence lifetime measurements were performed with unpolarized light at modulation frequencies of 18 and 30 MHz using a glycogen scattering reference solution. As pointed out by Lakowicz et al. (1981), the modulation lifetimes obtained for short lifetimes (<2 nsec) using a glycogen scattering reference solution have considerable uncertainty and are frequently negative. We found this to be true for measurements using diS-C_3-(5). Phase lifetimes (τ) were calculated from the relation,

$$\tau = \omega^{-1} \tan(\phi_s - \phi_r) \tag{1}$$

where ϕ_s and ϕ_r are sample and reference phase angles, and ω is the modulation frequency expressed in radians/sec.

Anisotropy decay experiments were performed by differential phase fluorimetry using vertically and horizontally polarized excitation light modulated at 30 MHz (Lakowicz & Prendergast, 1978; Lakowicz et al., 1979). The differential phase angle Δ is defined by,

$$\Delta = (\phi_V - \phi_H)_V - (\phi_V - \phi_H)_H \tag{2}$$

where $(\phi_V - \phi_H)_V$ and $(\phi_V - \phi_H)_H$ are phase angle differences between vertically and horizontally polarized emission light with excitation light polarized in the vertical and horizontal directions, respectively.

Based on the measured r, Δ and τ values it was possible to calculated the rate of diS-C_3-(5) rotation and the freedom with which diS-C_3-(5) can rotate as a function of temperature. For isotropic rotations of a hindered fluorophore,

$$\tan \Delta = \frac{(r_0 - r_\infty)(2R\tau)}{[(1 + 2r_0)(1 - r_0)(1 + \omega^2\tau^2)/9 + (1 + 2r_\infty)(1 - r_\infty)(2R\tau)^2 + (2 + r_0 + r_\infty(1 - 4r_0))(2R\tau)/3]} \tag{3}$$

where R is the inverse rotational correlation time in $nsec^{-1}$, r_0 is the fluorophore anisotropy in the absence of depolarizing rotations and r_∞ is the limiting anisotropy at times long compared to τ. $r_0 = 0.36$ was estimated from the measured anisotropy of a 3-μM diS-C_3-(5) solution in glycerol at 0°C. 0.36 was the highest anisotropy measured for a series of solvents at −70 and 0°C. The steady-state equation relating R and r_∞ to r, r_0 and τ is

$$r = r_\infty + (r_0 - r_\infty)/(1 + 6R\tau). \tag{4}$$

R and r were calculated using Eqs. (3) and (4) at each temperature using $r_0 = 0.36$ and measured r, Δ and τ. The maximum value for tan Δ (tan Δ_{max}) was calculated from r_0, ω and τ as given elsewhere (Lakowicz et al., 1979). The rotational cone angle θ was estimated from the relation,

$$\cos \theta = \sqrt{0.25 + 2(r_\infty/r_0)^{1/2}} - 0.5. \tag{5}$$

Stopped-Flow Experiments

Stopped-flow measurements were performed on a Dionex-130 stopped-flow apparatus (Sunnyvale, Calif.) which has 99% mixing efficiency within a 2-msec dead time and temperature control between 5 and 60°C. 0.15 ml of solutions containing BBMV were mixed with equal volumes of solutions containing diS-C_3-(5) and the time course of fluorescence was recorded by a MINC/23 computer (Digital Equipment Corp., Maynard, Mass.) for subsequent analysis. A 100-watt tungsten-halogen lamp powered by a 12V deep-cycle battery was used for maximal stability of the light source. Monochromatic excitation light (622 nm, 4 nm bandpass) was obtained using a Zeiss MM12 double monochromator (W. Germany). Emitted light was filtered by 650 or 697 nm high-pass filters (Corion Corp., Holliston, Mass.). The ratio of fluorescence intensity to random electronic noise was generally >100 : 1. The maximum rate of data acquisition is 512 data points (16 bits/data point) in 300 msec. The electronic response time of the instrument is <1 msec.

The time course of fluorescence intensity $F(t)$ was fitted to mono- or biexponential functions by the nonlinear Newton's method,

$$F(t) = A \exp(-t/\tau) + B \quad (6)$$

$$F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + B \quad (7)$$

where the A's are amplitudes, the τ's are exponential time constants and B is instrument offset.

Results

Data will be presented in the following order: Fluorescence spectra and titrations will be used to establish that diS-C_3-(5) can exist in three forms: free dye, bound monomer and bound dimer; and to estimate the equilibrium constants which describe the reactions. The environment and characteristics of bound dye will then be studied using fluorescence polarization, lifetime and dynamic depolarization methods. The detailed sequence of dye interactions with the membrane and the kinetic parameters will be determined from stopped-flow fluorescence experiments. Based on a proposed reaction mechanism, the mechanism of potential sensitivity of diS-C_3-(5) will be examined to determine the particular reaction step which is potential sensitive.

Equilibrium Partitioning of diS-C_3-(5) into Brush-Border Membranes

Figure 1 shows equilibrium fluorescence spectra for diS-C_3-(5) binding to BBMV. The wavelength of the excitation peak (622 nm) is independent of diS-C_3-(5) and BBMV concentrations. The emission spectrum reveals to distinct fluorescent species. Aqueous diS-C_3-(5) has an emission peak at 670 nm. As

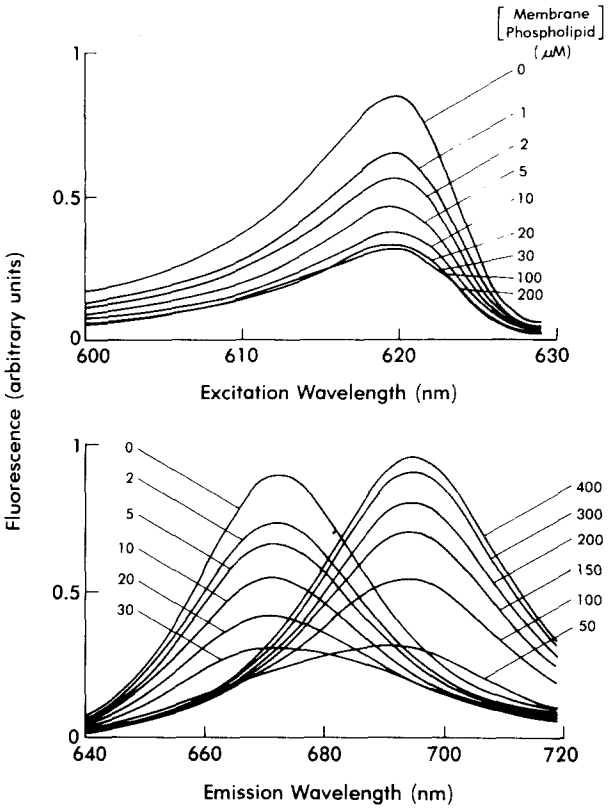

Fig. 1. Fluorescence spectra of diS-C_3-(5) in renal brush-border vesicles. Solutions consist of 3 μM diS-C_3-(5) in standard buffer (23°C) containing specified quantities of BBMV expressed as concentration of membrane phospholipids in μM. Top: Corrected excitation spectra shown using a rhodamine B quantum counter, 670-nm emission wavelength and 4-nm excitation and emission bandpass. Spectra for [membrane phospholipid] = 20 to 200 μM were virtually overlapping. Bottom: Uncorrected emission spectra at 622 nm excitation wavelength and 4 nm bandpass

[membrane phospholipid] is increased from 0 to 30 μM with addition of BBMV, the fluorescence intensity at 670 falls without appearance of a second peak, suggesting equilibration between aqueous diS-C_3-(5) and a membrane-bound, nonfluorescent diS-C_3-(5) species. Since the nonfluorescent species exists at relatively high [diS-C_3-(5)] to [membrane phospholipid] ratios, it is probably a dye aggregate, predominantly dye dimer. Several studies based on absorbance measurements have shown that cyanine and merocyanine dyes can exist in the membrane as a dimeric species (Dragsten & Webb, 1978; Tsien & Hladky, 1978; Ivkova et al., 1983; Verkman & Frosch, 1985).

As more BBMV are added to diS-C_3-(5) ([membrane phospholipid] = 50 to 400 μM), an emission

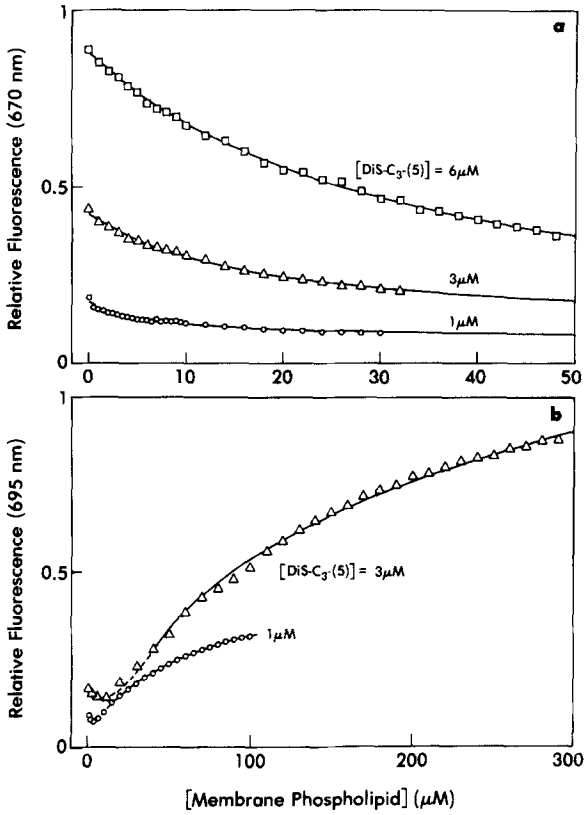

Fig. 2. Equilibrium binding of diS-C_3-(5) to brush-border membranes. Solutions consist of diS-C_3-(5) and BBMV in standard buffer (23°C). Each fluorescence intensity along a titration curve was measured after complete dye/membrane equilibration has occurred (15 to 30 sec); curves shown represent one experiment typical of two. (*a*) Fluorescence titration (622 excitation, 670 nm emission, 4 nm bandpass) for [diS-C_3-(5)]/[membrane phospholipid] > 0.1 showing disappearance of solution dye to form nonfluorescent membrane-bound dimer with increasing [membrane phospholipid] at constant [diS-C_3-(5)]. Curves fitted to a single-site binding model (Eq. 8) with K_{app} = 33 ± 2 μM (6 μM diS-C_3-(5)), 17 ± 2 μM (3 μM diS-C_3-(5)) and 6 ± 1 μM (1 μM diS-C_3-(5)). (*b*) Fluorescence titration (622 excitation, 695 emission, 4 nm bandpass) for [diS-C_3-(5)]/[membrane phospholipid] < 0.1 showing conversion of bound dye dimer to fluorescent dye monomer with increasing [membrane phospholipid] at constant [diS-C_3-(5)]. Fluorescence intensities were corrected for absorbance of excitation and emission light by the BBMV. Scattered light was negligible at even the highest [membrane phospholipid]. Curves were obtained from a fit to Eq. (A5) in the Appendix with K_d = 0.0013 ± 0.0001 (3 μM diS-C_3-(5)) and 0.0013 ± 0.0002 (1 μM diS-C_3-(5))

peak at 695 nm appears. As [diS-C_3-(5)] to [membrane phospholipid] ratio decreases, the fluorescence intensity at 695 nm increases without a change in spectral shape, suggesting the appearance of a fluorescent, membrane-bound monomer. Similar series of spectra were obtained with 1 and 6 μM diS-C_3-(5).

In order to determine whether the presence of proteins in the brush-border membrane modifies these spectral changes, similar experiments were performed in unilamellar phosphatidylcholine (PC) vesicles prepared by the method of Huang and Thompson (1974). With an excitation wavelength of 622 nm, a series of emission spectra were obtained which were almost identical to those obtained using the renal BBMV. There was a decrease in fluorescence at 670 nm (0 to 40 μM PC) followed by an increase in fluorescence at 692 nm (>40 μM PC). Therefore the presence of transmembrane proteins does not affect significantly the equilibrium binding properties of diS-C_3-(5) to membrane phospholipid.

It is known that cyanine dyes may exist as both monomers and higher aggregates in aqueous solution (West & Pearce, 1965). In order to examine whether dimers were present in the buffer used for these experiments at the highest diS-C_3-(5) concentrations used, it was shown that the shape of the emission peak at 670 nm and the diS-C_3-(5) fluorescence lifetime (1.1 to 1.2 nsec) was independent of concentration in the range 0.5 to 6 μM. In addition, the fluorescence intensity of diS-C_3-(5) at 670 nm, corrected for inner filter effects, was linear with concentration (0.5 to 6 μM). These results show that if a diS-C_3-(5) aggregate was present in aqueous solution, its fluorescence properties must be identical to those of the aqueous monomer. Since cyanine and merocyanine aggregates are generally nonfluorescent, aggregates are probably not present in aqueous solution, consistent with the dimer dissociation constant of 16 μM reported by West and Pearce (1965) for diS-C_3-(5) in water.

The equilibrium relations among aqueous diS-C_3-(5) and the bound monomer and dimer can be estimated from the dependence of fluorescence intensity on BBMV concentration (Fig. 2). Panel *a* shows the decrease in fluorescence at 670 nm resulting from conversion of aqueous diS-C_3-(5) to membrane dimer. Data were fitted to a single-site binding model,

$$F = F_0 - \frac{F_\infty[\text{membrane phospholipid}]}{[\text{membrane phospholipid}] + K_{app}} \qquad (8)$$

where F_0 and F_∞ are the fluorescence intensities (F) in the absence and presence of maximum BBMV, and K_{app} is the concentration of membrane phospholipid which results in 50% membrane binding of diS-C_3-(5). Figure 2 indicates that K_{app} increases with increasing [diS-C_3-(5)]. If binding of diS-C_3-(5) to BBMV occurred by simple partitioning, K_{app} would be independent of [diS-C_3-(5)]; the observed dependence suggests saturable binding, in agreement with the analysis of Ivkova et al. (1983) for diS-C_3-(5) binding to azolectin liposomes and to sar-

Table 1. Fluorescence properties of diS-C_3-(5) in different solvents and in the brush-border membrane[a]

Solvent	Peak (nm)	F	ε	η (cp)	τ_{30} (nsec)	τ_{18} (nsec)	tan A	r	r_∞	R (nsec^{-1})
Buffer	670	1.0	80	0.9	1.21 ±0.01	1.14 ±0.04	0.035 ±0.009	0.1021 ±0.001	0.030 ±0.020	0.57 ±0.14
Ethanol	680	3.8	24	1.2	1.64 ±0.01	1.65 ±0.03	0.040 ±0.001	0.0504 ±0.0005	0.001 ±0.002	0.67 ±0.03
Ethylene glycol	685	4.0	38	20	2.01 ±0.02	2.04 ±0.01	0.074 ±0.003	0.2335 ±0.002	0.090 ±0.01	0.09 ±0.01
BBMV	695	4–5			2.13 ±0.02	2.11 ±0.02	0.035 ±0.004	0.1920 ±0.0003	0.110 ±0.004	0.54 ±0.06

[a] DiS-C_3-(5) (3 μM) was dissolved in the indicated solvent at 23°C. The BBMV solution consisted of diS-C_3-(5) and 350 μM membrane phospholipid is standard buffer. The excitation wavelength was 622 nm. F is the relative intensity as the indicated emission peak, ε is dielectric constant and η is bulk solution viscosity. ε and η values at 20°C were taken from the Handbook of Chemistry and Physics, CRC Press, Cleveland, Ohio (1979).

coplasmic reticulum vesicles. As pointed out by Bashford et al. (1979), K_{app} depends both on binding affinity and on total binding stoichiometry; independent determination of binding stoichiometry cannot easily be accomplished from fluorescence titrations alone.[1]

Panel *b* in Fig. 2 shows increasing fluorescence intensity at 695 nm with increasing BBMV. For low [membrane phospholipid], fluorescence decreases because of conversion of aqueous diS-C_3-(5) to bound dimer prior to formation of significant quantities of bound monomer. As [membrane phospholipid] increases further, the predominant reaction is conversion of bound dimer to bound monomer. Using the fitting procedure given in the Appendix, the fluorescence titration yields a dimer dissociation constant of 0.0013. For [diS-C_3-(5)]/[membrane phospholipid] < 0.03, it is predicted that less than 2% of total diS-C_3-(5) is present as aqueous dye.

Environment of Bound Dye

There is considerable evidence that diS-C_3-(5) binds to lipid bilayers somewhere near the aqueous interface rather than in the hydrophobic center of the bilayer (Waggoner, 1976). DiS-C_3-(5) does not partition into hydrophobic solvents such as hexanol and we find that the mineral oil : water partition coefficient for diS-C_3-(5) is less than 10^{-3}. In order to further define the characteristics of the diS-C_3-(5) binding site, the fluorescence properties of the bound monomer were compared to those of a series of pure solvents of known viscosities and dielectric constants (Table 1).

As solvent dielectric constant decreases from 80 (water) to 25–50 (ethanol and ethylene glycol) the peak emission wavelength increases from 670 to 680–685 nm. The peak is further red shifted with BBMV. These results are the opposite of what is predicted by the Lippert equation for general solvent relaxation effects; a specific solvent-fluorophore interaction must occur. The relative quantum yield for diS-C_3-(5) parallels the red shift in emission peak. These findings suggest that the environment of the bound monomer is less polar than ethanol (dielectric constant 24.3), though higher than the center of the lipid bilayer (dielectric constant 2).

For all solvents tested with the exception of water, there is a homogeneous fluorescence lifetime as judged from the agreement between phase lifetimes obtained at two modulation frequencies (18 and 30 MHz). There is no clear relationship between fluorescence lifetime and the solvent dielectric constant or peak emission wavelength. The steady-state anisotropy (r) is roughly correlated with solvent viscosity. r is dependent upon fluorescence lifetime (τ), fluorophore rotational rate (R) and the degree to which rotation is hindered (limiting anisotropy, r_∞). DiS-C_3-(5) rotation is unhindered in water, ethanol and ethylene glycol ($r_\infty \sim 0$), with R decreasing from ~0.6 to 0.09 nsec^{-1} as sol-

[1] We attempted to determine diS-C_3-(5) binding stoichiometry to BBMV directly using a centrifugation technique. Because diS-C_3-(5) bound avidly to all materials except acrylic, mixtures of BBMV and diS-C_3-(5) were centrifuged at 20,000 × g for 15 min in acrylic cuvettes cemented into plastic centrifuge tubes using epoxy. Under these conditions there was less than 2% binding of 3 μM diS-C_3-(5) to the cuvette. The concentration of bound diS-C_3-(5) was inferred from total diS-C_3-(5) and supernatant diS-C_3-(5) measured by fluorescence at 670 nm. Unfortunately, at any BBMV concentration, the amount of diS-C_3-(5) removed from the solution by BBMV was two- to threefold less than that inferred from the fluorescence titrations in Fig. 2, top. We concluded that the BBMV pellet bound much less dye than would be bound by an equivalent number of isolated vesicles and were therefore unable to measure total site stoichiometry.

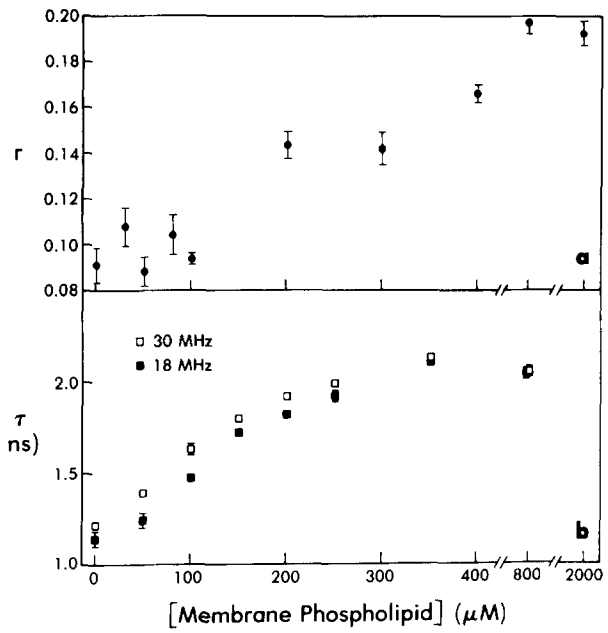

Fig. 3. Fluorescence lifetimes and steady-state polarization of diS-C_3-(5). Solution consists of 3 μM diS-C_3-(5) in standard buffer (23°C) with varying [membrane phospholipid]. (*a*) Steady-state anisotropy (*r*) measured at 622 nm excitation and 695 nm emission wavelengths (4 nm bandpass) in the L-format with correction for scattered light. Each measurement performed in triplicate; errors represent 1 SD. (*b*) Phase lifetimes were measured in quadruplicate at 18 and 30 MHz modulation frequencies using a glycogen reference solution. Excitation wavelength 622 nm and emission >695 nm using a Corion 695 nm high-pass filter

vent bulk viscosity increases 20-fold. The rate of diS-C_3-(5) rotation in the BBMV membrane is similar to that in aqueous solution.

The fluorescence titrations shown in Fig. 2 give only limited information about the diS-C_3-(5) reaction mechanism. Since only two distinct fluorescence emission peaks were identified, it was necessary to assume that only two fluorescent species were present. Furthermore, direct information about the location and characteristics of the bound dimer was unavailable because the dimer is nonfluorescent. Steady-state fluorescence polarization and fluorescence lifetime titrations yield complementary information to steady-state intensity titrations (Fig. 3). A measured *r* value is equal to the average of *r* values for the individual reaction species weighted by their relative fluorescence intensities. The analysis of lifetime heterogeneity allows for a direct measure of the relative fluorescence intensities and lifetimes of the individual fluorescent species.

The *r* values increases from 0.09 (aqueous solution) to 0.19 (bound monomer) with a half-point for transition between the two values at [membrane phospholipid] = 200 to 300 μM for 3 μM diS-C_3-(5). The titration was performed at an emission wavelength of 695 nm, where the fluorescence of the membrane monomer is expected to dominate the signal. It is quite surprising that *r* increases relatively little under conditions where most of the fluorescence at 695 arises from the bound monomer ([membrane phospholipid] = 100 to 300 μM). In order to examine this observation further, complete emission polarization spectra were obtained (660 to 710 nm) for 3 μM diS-C_3-(5) and varying BBMV (*data not shown*). For a single [membrane phospholipid], *r* did not depend on emission wavelength; the average *r* value agreed with the data in panel *a*. These unexpected results suggest the presence of monomer-monomer or dimer-monomer interactions in the membrane on a nanosecond time scale. If bound monomer-monomer interactions are very rapid (<10 nsec), then it is possible that an excited state complex could form and decay rapidly with different characteristics from those of the isolated monomer; for example, the angle between excitation and emission dipoles may increase resulting in apparent depolarization of the excitation light. It is also possible that there is significant membrane perturbation by the dimer or by a high monomer density which could alter the rotational characteristics of the monomer or the angle between monomer excitation and emission dipoles. Energy transfer could also decrease *r*; however, there is no measurable overlap between the 622 excitation and 695 emission peaks of bound monomeric diS-C_3-(5).

Lifetime measurements were performed to help distinguish among these possibilities (Fig. 3, panel *b*). Bound monomer has a homogeneous lifetime of 2 to 2.1 nsec. Aqueous diS-C_3-(5), unlike the less polar solvents examined in Table 1, does not have a single lifetime as shown by the difference between phase lifetimes measured at 18 and 30 MHz modulation frequencies. Since τ measured at 18 MHz is less than that measured at 30 MHz, ground state heterogeneity cannot account for the difference; there is probably a nonexponential decay process which may result from an excited state reaction (Lakowicz & Balter, 1982). Since the phase lifetime at 18 MHz is less than that at 30 MHz for [membrane phospholipid] < 300 μM, a two-component ground state heterogeneity analysis cannot be performed; the lifetime results will therefore be used only in a semi-qualitative manner. Recognizing this limitation, it is observed that at 695 nm the 'average lifetime' undergoes half transition from 1.2 nsec (aqueous) to 2.1 nsec (bound monomer) in the range [membrane phospholipid] = 100 to 200 μM, well above the membrane phospholipid concentration where the fluorescence signal arises mainly from

the bound monomer. These results would support the suggestion that a rapid dye monomer-monomer interaction occurs; at a low monomer : dimer ratio the lifetime decreases due to a 'collisional quenching' mechanism where the monomer-monomer interaction results in an enhanced rate of decay of the excited state.

The rotational mobilities of diS-C_3-(5) in aqueous solution and in bound monomer forms are examined in Fig. 4 assuming isotropic rotation of diS-C_3-(5) in the membrane. The fluorescence lifetimes are weakly dependent on temperature (panel *c*). The measured tan Δ is a complex function of r, r_0, r_∞, τ and R (*see* Materials and Methods). The calculated value for tan Δ_{max}, which represents the maximum value for tan Δ at any temperature for an unhindered isotropic rotator (Lakowicz et al., 1979) is 0.042 for aqueous diS-C_3-(5) and 0.073 for membrane monomer. The tangent defect, which is the difference between the theoretical tan Δ_{max} and the maximum measured tan Δ at any temperature, is nearly zero for aqueous diS-C_3-(5) and 0.038 for bound monomer (panel *c*), suggesting that rotation of bound monomeric dye is significantly hindered. Since tan Δ does not exhibit a maximum value at any temperature, the estimated tangent defect represents an upper limit. Based on measured τ, tan Δ and r values, R and r_∞ were calculated at each temperature. It is quite interesting that the diS-C_3-(5) rotational rate (R) is virtually the same in aqueous solution and in the membrane, while the extent of rotation is unhindered in aqueous solution and significantly hindered in the membrane. These findings suggest that the diS-C_3-(5) monomer rests in a pocket in the membrane which allows for rapid rotation within a restricted angle. At 20°C, the angle of restriction, estimated from r_0 and r_∞ (Eq. 5), is 48°. 90° represents unhindered rotation and 0° represents an immobile fluorophore.

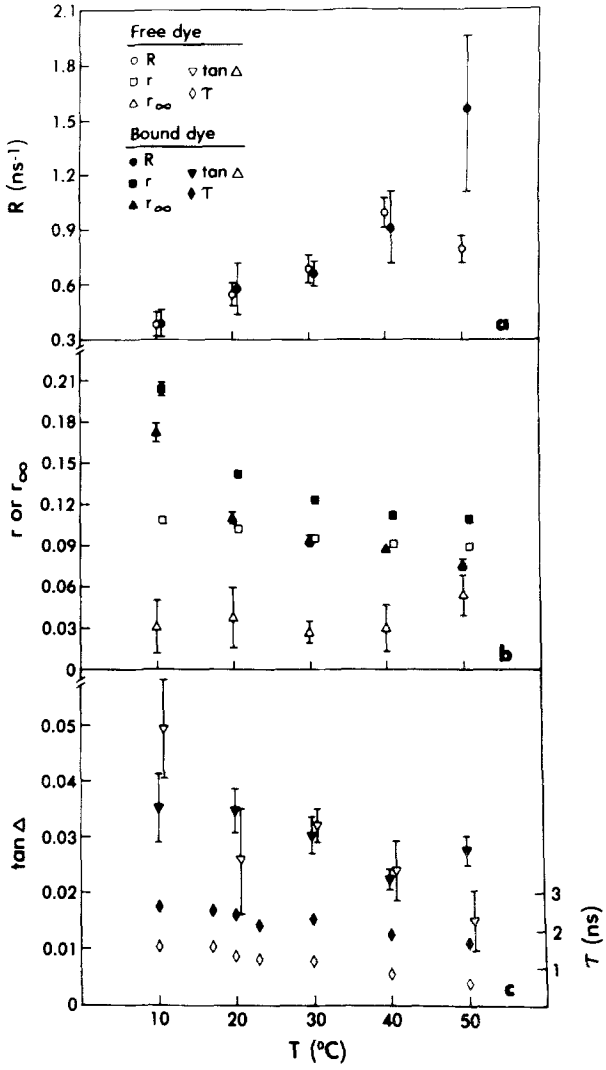

Fig. 4. Fluorescence lifetime and dynamic depolarization studies of diS-C_3-(5) in brush-border vesicles. Solutions consist of 3 μM diS-C_3-(5) in standard buffer in the absence and presence of BBMV (350 μM membrane phospholipid). Steady-state anisotropy (r), tan Δ and lifetime (τ) values were measured three or more times as described in Materials and Methods; errors represent 1 SD. R and r_∞ were calculated from measured r, tan Δ, τ and r_0 = 0.352; indicated errors in R and r_∞ were obtained by propagating errors in the measured quantities

Stopped-Flow Experiments

Fluorescence stopped-flow experiments were performed to define the sequence of interactions between diS-C_3-(5) and the BBMV membrane. The time course of fluorescence intensity (>670 and >697 nm emission wavelengths) following mixture of 3 μM diS-C_3-(5) and BBMV ([membrane phospholipid] = 30 μM) is shown in Fig. 5. For an emission wavelength > 670 nm, there is a biexponential decrease in fluorescence. The same experiment as measured with an emission wavelength > 695 nm showed a small initial fluorescence decrease followed by a monoexponential increase in fluorescence. Similar time courses were measured for a series of diS-C_3-(5) concentrations (0.5 to 6 μM) and BBMV concentrations ([membrane phospholipid] = 10 to 100 μM). As discussed below, the fast exponential process observed at >670 nm represents conversion of bound monomer to bound dimer, and the slow exponential observed using either emission wavelength represents transmembrane movement of bound dimer.

Table 2 gives the concentration dependence of the fast and slow exponential processes. The time

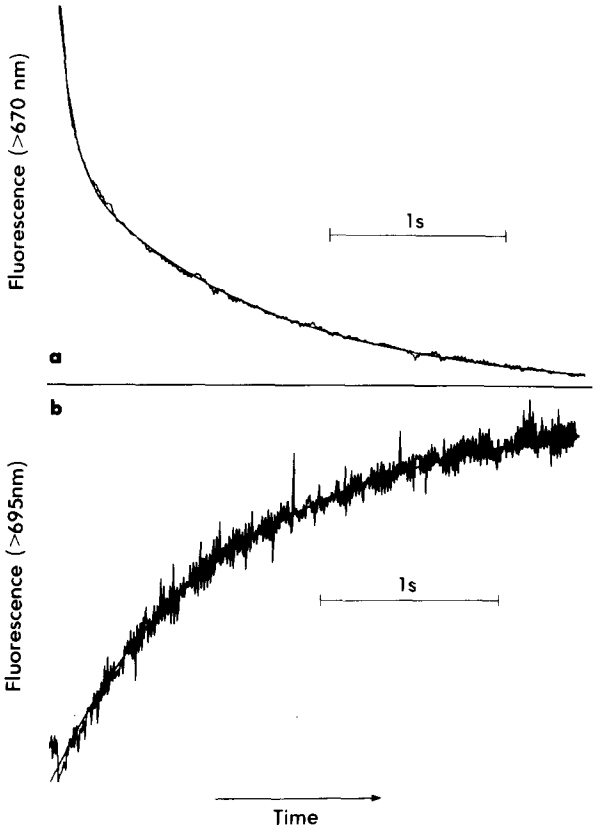

Fig. 5. Time course of diS-C_3-(5) binding to brush-border membranes. 0.1 ml of 3 μM diS-C_3-(5) was mixed with an equal volume of BBMV (30 μM membrane phospholipid) in standard buffer (23°C) in a stopped-flow apparatus. Excitation wavelength was 622 nm with 8 nm bandwidth. (*a*) Emission measured at >650 nm using a high-pass filter showing a biexponential fluorescence decrease (Eq. 7) with τ_1 = 81 msec, τ_2 = 1.1 sec and A_1/A_2 = 0.82 (*b*) Emission measured at >697 nm using a high-pass filter showing a small decrease in fluorescence followed by a single exponential increase with τ = 1.5 sec

Table 2. Concentration dependence of diS-C_3-(5) binding to brush-border vesicles[a]

[Membrane phospholipid] = 10 μM	30 μM	50 μM
Fast process	Time constant (msec)	
[DiS-C_3-(5)] (μM)		
0.5	18 ± 1	29 ± 4
1	33 ± 5	26 ± 2
2	30 ± 2	28 ± 1
3	34 ± 4	31 ± 1
5	37 ± 1	
Slow process	Time constant (sec)	
[DiS-C_3-(5)] (μM)		
0.5	0.8 ± 0.1	0.9 ± 0.1	1.4 ± 0.1
1	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
3	0.9 ± 0.1		0.6 ± 0.1
5	1.3 ± 0.1		0.9 ± 0.1

[a] Specified concentrations of BBMV and diS-C_3-(5) in standard buffer were mixed in a stopped-flow apparatus. The excitation wavelength was 622 nm. The emission wavelength was >650 nm for 10 μM membrane phospholipid experiments and for the fast process at 30 μM membrane phospholipid; the remaining experiments were carried out at emission wavelengths >697 nm. The exponential time constants, obtained by a single (50 μM membrane phospholipid) or a double (all other experiments) exponential fit were measured in three or more experiments; errors represent 1 SD.

constants for both the fast and slow processes are relatively concentration independent (~30 msec for the fast process and ~1 sec for the slow process). If the slow process represents dye movements from binding sites at the external membrane interface to sites at the internal membrane interface, then the rate for dye movement in the opposite direction should be similar since diS-C_3-(5) should bind to voltage-clamped membranes, symmetrically. Figure 6*b* shows an unbinding experiment in which BBMV preincubated with diS-C_3-(5) are subjected to a 1 : 1 dilution to induce unbinding of dye and thus movement of dye from internal to external binding sites. The time constant in three experiments is 1.1 ± 0.2 sec, similar to 1 sec for dye movement from external to internal binding sites.

If the fast process represents a simple dimerization reaction, then its rate should increase markedly with increasing [diS-C_3-(5)] to [membrane phospholipid] ratio. There was no significant increase in the measured rate of the fast process over a fivefold change in dye-to-lipid ratio. For merocyanine 540, Verkman and Frosch (1985) showed that the overall dimerization reaction consists of a rate-limiting dye reorientation (~1000 sec^{-1}) followed by a rapid dimerization process (>250,000 sec^{-1}). A very rapid dimerization (1 to 100 nsec) was predicted theoretically from the membrane dye density and an assumed lateral diffusion coefficient for dye in membrane. Because of the structural similarity of the cyanine and merocyanine dyes, diS-C_3-(5) may also undergo a rate-limiting reorientation and rapid dimerization. If the 30-msec time constant for the fast process represented the reorientation, then it should be relatively concentration independent, as observed. In addition, the steady-state anisotropy and fluorescence lifetime titrations (Fig. 3) are consistent with a very rapid dimerization step (<10 nsec).

At higher BBMV concentrations ([membrane phospholipid] = 200 to 400 μM), there is a very rapid fluorescence increase at >695 nm which precedes the monoexponential increase in fluorescence (Fig. 6*a*). The time course has an exponential time

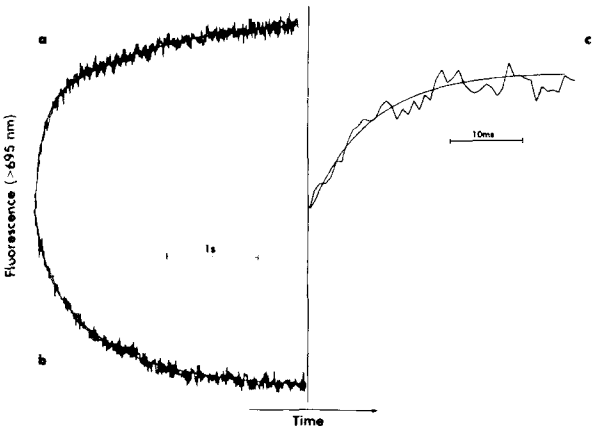

Fig. 6. Time course of diS-C_3-(5) binding to brush-border membranes. Fluorescence measured at a 622-nm excitation wavelength (8 nm bandpass) and >697 emission wavelength. Left: Binding and unbinding of diS-C_3-(5) from BBMV. (*a*) 1 μM diS-C_3-(5) in standard buffer (23°C) mixed with BBMV (200 μM membrane phospholipid). The biexponential fit is shown with τ_1 = 99 msec, τ_2 = 4.1 sec and A_1/A_2 = 1.9. Due to an electronic filtering time constant of 50 msec, the 99 msec time constant is artificially prolonged. (*b*) 3 μM diS-C_3-(5) + BBMV (50 μM membrane phospholipid) mixed with an equal volume of standard buffer to induce release of dye from the membrane. The parameters of the fitted biexponential function were τ_1 = 115 msec, τ_2 = 0.8 sec and A_1/A_2 = 0.54. Right: Initial, rapid binding of diS-C_3-(5) to BBMV. 5 μM diS-C_3-(5) were mixed with an equal volume of solution containing BBMV (200 μM membrane phospholipid). Experiment performed in standard buffer (23°C) with single fitted exponential τ = 6 msec

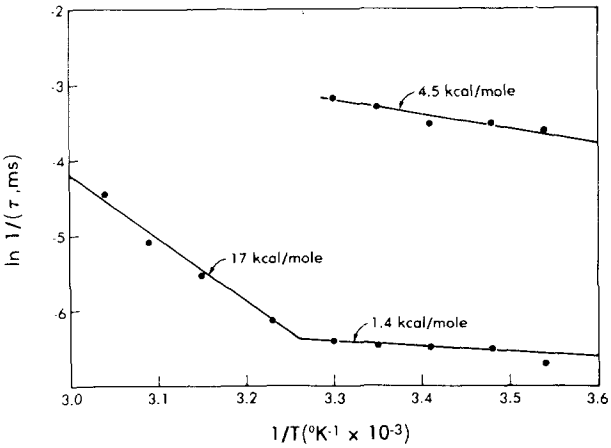

Fig. 7. Temperature dependence of diS-C_3-(5) binding to brush-border vesicles. BBMV (30 μM membrane phospholipid) were mixed with 3 μM diS-C_3-(5) in standard buffer in a stopped-flow apparatus. The time constants for binding were calculated from a biexponential fit to the time course of fluorescence decrease at 622 nm excitation and >650 nm emission wavelengths. Data points on the Arrhenius plot are the average of three or more determinations. Fitted reaction enthalpies (ΔH) for the fast and slow exponential processes are shown

constant of 6 ± 1 msec (3 experiments, Fig. 6*c*). The very fast process represents the initial binding of aqueous diS-C_3-(5) monomer to the membrane to form bound monomer. The process is best observed at very high BBMV when the equilibrium concentration of bound monomer is highest and the bound monomer-dimer equilibrium strongly favors the monomer.

The reaction enthalpies (ΔH) for the fast (30 msec) and slow (1 sec) processes were estimated from the temperature dependence of the fast and slow reaction rates at a single diS-C_3-(5) and membrane phospholipid concentration (Fig. 7). Data for the fast process were obtained only up to 30°C (ΔH = 4.5 kcal/mole) because the fluorescence signal deteriorates due to an increase in the rate of the fast reaction and a decrease in the diS-C_3-(5) quantum efficiency. There is a discontinuity in the slope of the Arrhenius plot for the slow process with transition from ΔH = 1.4 kcal/mole to 17 kcal/mole at ~33°C. Verkman et al. (1985) also found marked discontinuities in the Arrhenius plots for BBMV osmotic water and urea transport in the temperature range 30 to 33°C. It is possible that a membrane phase transition occurring at 30 to 33°C could cause the slope discontinuities in the Arrhenium plots; however, steady-state and dynamic fluorescence studies of diphenylhexatriene in BBMV have failed to reveal significant thermotropic phase transitions (Ives & Verkman, 1985; Verkman & Ives, 1986). Another possible explanation for the discontinuity is the presence of two parallel pathways for diS-C_3-(5) movement; at low temperature the 1.4 kcal/mole pathway would dominate whereas at high temperature the 17 kcal/mole pathway would become faster and dominate.

Discussion

A proposed mechanism for diS-C_3-(5) binding to brush-border membranes is given in Fig. 8. Aqueous monomer in the external solution (M_f^i) has a fluorescence emission peak at 670 nm. Aqueous monomer binds at the outer leaflet of the membrane bilayer rapidly (~6 msec) to form a bound monomer (M_1^o) with a red shifted emission peak at 695 nm (Fig. 6, curve *c*). Equilibrium binding studies suggest that the fluorescent-bound monomer is in equilibrium with nonfluorescent-bound dimer (D_o). The 30-msec time course, observed best at >650 nm and at relatively low [diS-C_3-(5)] to [membrane phospholipid] ratios, represents overall conversion of bound monomer to bound dimer (Fig. 5, curve *a*). As bound dimer forms, there is depletion of the

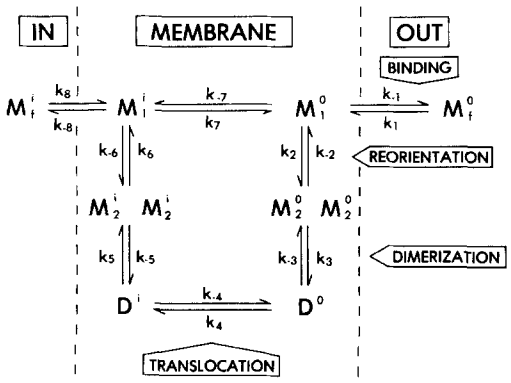

Fig. 8. Proposed mechanism for diS-C_3-(5) binding to brush-border membranes. M_f: aqueous monomer, M_1 and M_2: bound monomers, D: bound dimer. The superscripts i and o refer to diS-C_3-(5) binding sites in the inner and outer membrane leaflets. The rate constants (k_i's) refer to the indicated reaction

solution aqueous monomer and a resultant decrease in the fluorescence at 670 nm.

Two lines of evidence suggest that the overall dimerization process consists of at least two individual reactions, a unimolecular 'reorientation' and a bimolecular dimerization. Since the measured rate of the overall dimerization reaction is not strongly concentration dependent, the reorientation process is rate limiting with a 30-msec time course. As discussed in the Results section, the dimerization reaction probably occurs on a nanosecond time scale. It was observed that the fluorescence lifetimes and steady-state anisotropies of diS-C_3-(5)/BBMV solutions, measured under conditions when the fluorescence signal was arising almost exclusively from the bound monomer, were very different from those obtained under conditions when only bound monomer was present (Figs. 2 and 3). Therefore there must be a monomer-monomer interaction which occurs on the time scale of the diS-C_3-(5) fluorescence lifetime in the membrane. Since the interaction would be a collisional process, the dimerization reaction could have a comparable rate.

The slow reaction process is symmetric and has a time course of ~1 sec (Fig. 6, curve *b* and Table 2) under conditions when the dimerization reaction favors bound dimer. Under these conditions, the reaction is observed as a time course of decreasing fluorescence at >650 nm and of increasing fluorescence at >695 nm. As the dimer moves across the membrane there is depletion of aqueous monomer, and thus a decrease in fluorescence at >650 nm (since the signal from 670 nm is greater than that from 695 nm when bound dimer predominates over bound monomer). The increase in fluorescence intensity at 695 nm results from formation of bound monomer (M_1^i and M_2^i) due to rapid dimer dissociation at the inner membrane leaflet. This increase in fluorescence intensity is consistent with the assumption that D^i undergoes further dissociation after translocation has occurred.

In order to examine whether bound monomer has a significant rate of translocation, stopped-flow experiments were performed using 200 μM membrane phospholipid and 1 to 3 μM diS-C_3-(5). Under these conditions the dimerization reaction favors the bound monomer. In seven experiments the time constant for the slow process was 4.5 ± 0.5 sec, much greater than the time constant (1 sec) obtained for higher [diS-C_3-(5)] to [membrane phospholipid] ratios. These measurements are difficult to perform because most of the fluorescence increase is due to the initial binding reaction rather than to the translocation process (Fig. 6, curve *a*). The increase in time constants from 1 to 4.5 sec with a fourfold increase in [membrane phospholipid] suggests that monomer permeation is much slower than dimer permeation, consistent with the notion that lipid : water partition coefficient of a bulky charged dimer is greater than that of a charged monomer.

An alternate explanation for the physical meaning of the 1-sec reaction must be examined. The 1-sec reaction may represent a further aggregation or reorientation of D^o to form a reactant at the outer membrane leaflet, rather than the proposed process of transmembrane dye movement. Waggoner et al. (1977) found that the time constant for transmembrane diS-C_3-(5) movement across glycerylmonooleate-cholesterol planar bilayers was <0.8 to 25 sec, depending upon the cholesterol content. In BBMV, after completion of the 1-sec time course, there was no further decrease in fluorescence at 670 nm for at least 15 min. If transmembrane movement of diS-C_3-(5) occurred during this time, an additional time course of decreasing fluorescence, with approximately equal amplitude to the initial drop in fluorescence, would have been observed. The similarity in rates for the forward and reverse slow processes supports the existence of a 1-sec translocation. In addition, the discontinuity in the Arrhenius plot for the slow process is similar to temperature dependences measured for other BBMV transmembrane transport processes (Verkman et al., 1985). The reaction enthalpy above the transition temperature (17 kcal/mole) is similar to that reported for lipid-mediated transport processes (Fettiplace & Haydon, 1980).

It has been suggested that movement of diS-C_3-(5) across an unstirred layer adjacent to the aqueous-membrane interface may be responsible for potential-dependent changes in diS-C_3-(5) absorbance. We find that the initial binding of 5 μM diS-C_3-(5) to

BBMV occurs with a 6-msec time constant. In order to examine whether this process represents a bimolecular binding event limited by diS-C_3-(5) diffusion in aqueous solution, the time constant for the initial binding of 1 μM diS-C_3-(5) to BBMV (200 μM membrane phospholipid) was measured and found to be identical (7 ± 2 msec, 3 experiments) to the time constant obtained using 5 μM diS-C_3-(5). If the binding reaction were a simple bimolecular association, then a fivefold change in [diS-C_3-(5)] would generally accelerate the measured reaction time constant, except when the unbinding rate greatly exceeds the binding rate. Since no change was observed, the results are most consistent with a rate-limiting barrier at the membrane aqueous interface for diS-C_3-(5) access to its binding site.

It is possible to estimate the predicted time constant for binding of 5 μM diS-C_3-(5) to BBMV if no unstirred layer is present to examine whether the observed time course (6 msec) is slower than the maximum rate of bimolecular binding. It is difficult to use classical diffusion theory (Chandrasakhar, 1943) to estimate the binding rate because there is considerable uncertainty in the interaction radius and in the nature of the diS-C_3-(5) binding site on a membrane which does not have distinct, immobile receptors. Using the temperature-jump technique, Verkman and Solomon (1980) have measured directly the rate for diffusion-limited binding of a molecule of similar size, phloretin, to phosphatidylcholine membrane vesicles. At 50 μM phloretin the binding time constant was ~100 μsec. Assuming that phloretin and diS-C_3-(5) have similar binding characteristics because of similar Stoke's radii, the time constant for binding of 5 μM diS-C_3-(5) would be 1 msec, slightly faster than the observed time constant of 6 msec.

The measurements reported here establish a kinetic mechanism for diS-C_3-(5) binding to brush-border membranes in the absence of induced membrane potentials. They do not address the questions of where diS-C_3-(5) binds within the membrane and of the mechanism by which diS-C_3-(5) responds to changes in transmembrane electrical potentials. The location of diS-C_3-(5) binding sites has recently been studied from the quenching of *n*-(9-anthroyloxy) fatty acid (*n*-AS) fluorescence (Cabrini & Verkman, 1986). These lipophilic fluorescent probes contain a fluorescent moiety located at specified depths within the membrane which is strongly quenched by diS-C_3-(5) by a collisional mechanism. Based on efficiencies of quenching of *n*-AS fluorescence by bound diS-C_3-(5) monomer and dimer, it was suggested that bound monomer is oriented parallel to the membrane phospholipids and that bound dimer is oriented perpendicular to the membrane phospholipids deep within the membrane.

Based on the mechanism for diS-C_3-(5) binding to BBMV given in Fig. 8, there are several possible reaction steps which could be sensitive to changes in membrane potential. An external electric field could modify solution-membrane partitioning of diS-C_3-(5). Waggoner et al. (1977) suggested this mechanism based on the measured optical response of diS-C_3-(5) in glycerylmonooleate lipid bilayers to a series of square-wave electrical pulses. They concluded, however, that a potential-sensitive intramembrane dimerization reaction could not be rigorously excluded. A second mechanism for potential sensitivity of diS-C_3-(5) is a direct effect of membrane potential on the reorientation/dimerization reactions as has been demonstrated for merocyanine 540 (Dragsten & Webb, 1978). A third mechanism is translocation in which diS-C_3-(5) redistributes across the membrane in response to an external electric field. Preliminary evidence favors a potential-sensitive translocation mechanism for diS-C_3-(5) in BBMV (Cabrini & Verkman, *unpublished results*). The response time for changes in diS-C_3-(5) fluorescence to induced transmembrane potentials is 0.5 to 1.5 sec, similar to the time constant for the translocation process. At 3 μM diS-C_3-(5) and 30 μM membrane phospholipid, changes in fluorescence at 670 and 695 nm are in the same direction in response to induced transmembrane potentials, making a solution-membrane partitioning mechanism unlikely. In addition, diS-C_3-(5) fluorescence responds well to membrane potential changes over a tenfold change in [diS-C_3-(5)]/[membrane phospholipid] ratio. A reorientation-dimerization mechanism would predict potential sensitivity over a relatively narrow range of dye-to-lipid ratios, where the monomer-dimer equilibrium is near its midpoint. Further experiments are required to define the kinetics of diS-C_3-(5) binding to BBMV in the presence of transmembrane electrical potentials.

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Appendix

Under conditions when [membrane phospholipid] > 30 [diS-C_3-(5)] (Fig. 2, top), there are predominantly two diS-C_3-(5) species present, a bound monomer M and a bound dimer D. For purposes of an equilibrium binding calculation M represents the sum of M_1^i, M_2^i, M_1^o and M_2^o, and D is the sum of D^i and D^o as given in Fig. 8. The dimerization reaction can be written,

$$M/\text{BBMV} + M/\text{BBMV} \longleftrightarrow D/\text{BBMV} \qquad \text{(A1)}$$

where each reactant concentration is expressed as the reactant concentration (moles/liter of solution) divided by the membrane concentration (moles membrane phospholipid/liter of solution) (Verkman & Frosch, 1985). The dimerization dissociation constant K_d is dimensionless and is defined by the relation,

$$K_d = [M]^2/[D][\mathrm{MP}] \quad \text{(A2)}$$

where [MP] is the membrane phospholipid concentration. The condition for conservation of total dye, $[\text{diS-C}_3\text{-(5)}]_t$, can be written,

$$[\text{diS-C}_3\text{-(5)}]_t = [M] + 2[D]. \quad \text{(A3)}$$

It is assumed that the fluorescence intensity at 695 nm, F_{695} is linearly related to $[M]$,

$$F_{695} = a[M] \quad \text{(A4)}$$

where a is expressed in fluorescence units/μM.

Combining Eqs. (A2)–(A4),

$$F_{695} = a\left[\sqrt{\frac{K_d^2[\mathrm{MP}]^2}{16} + \frac{K_d[\mathrm{MP}][\text{diS-C}_3\text{-(5)}]_t}{2}} - \frac{K_d[\mathrm{MP}]}{4}\right]. \quad \text{(A5)}$$

Data in Fig. 2 (bottom) for F_{695} as a function of [MP] at constant $[\text{diS-C}_3\text{-(5)}]$ was fitted to Eq. (A5) with parameters K_d and a.