Biochimica et Biophysica Acta 862 (1986) 285–293
Elsevier

BBA 73295

Localization of cyanine dye binding to brush-border membranes by quenching of *n*-(9-anthroyloxy) fatty acid probes

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(Received 10 April 1986)
(Revised manuscript received 3 July 1986)

Key words: Cyanine dye; Fluorescence quenching; Stopped-flow; Brush-border membrane; Anthroyloxystearic acid

The location and orientation of 3,3′-dipropylthiodicarbocyanine (diS-C_3-(5)) binding sites in renal brush-border membrane vesicles was examined from the quenching of *n*-(9-anthroyloxy) fatty acid (*n*-AS) fluorescence. Based on previous kinetic studies (Cabrini, G. and Verkman, A.S. (1986) J. Membrane Biol. 90, 163–175) monomeric aqueous diS-C_3-(5) binds to brush-border membrane vesicles (BBMV) by an initial 6 ms association to form bound monomer, a 30–40 ms equilibrium between bound monomer (M) and bound dimer (D), and a 1–1.3 s translocation of D from the outer to inner membrane leaflet. Based on Stern-Volmer and lifetime analyses, M and D quench the fluorescence of the *n*-AS probes by a collisional mechanism. At low [diS-C_3-(5)]/[BBMV] (R), where M predominates, the *n*-AS quenching efficiencies (Q) are similar ($n = 2–16$); at high R, where D predominates, Q increases with n ($16 > 12 \gg 6 > 2$), suggesting that M is oriented parallel, and D perpendicular, to the phospholipid chains deep within the membrane. Mixture of diS-C_3-(5) with brush-border membrane vesicles containing *n*-AS in a stopped-flow apparatus gave a biexponential fluorescence decrease (excitation 390 nm, emission above 450 nm) with time constants 30–40 ms and 1–1.5 s; there was no 6 ms quenching process. These findings are incorporated into a model in which diS-C_3-(5) adheres loosely to the outer membrane surface in 6 ms, binds parallel to the membrane phospholipid in 30–40 ms, dimerizes and rotates by 90° in much less than 30 ms, and translocates to the opposite half of the bilayer in 1–1.5 s.

Introduction

The potential-sensitive carbocyanine dye diS-C_3-(5) has been used extensively to study static and time-dependent membrane potentials in artificial liposomes [1], cells [2], mitochondria [3], and membrane vesicles isolated from sarcoplasmic reticulum [4] and renal proximal tubule [5–7]. DiS-C_3-(5) is particularly useful to study ion transport in membrane vesicles because of its relatively rapid response to changes in membrane potential (less than 1 s) and its large signal intensity of up to 50% for a 100 mV change in membrane potential. The location and orientation of diS-C_3-(5) binding sites within biological membranes, and the precise mechanism by which diS-C_3-(5) responds to changes in membrane potential in vesicle systems have not been established.

In the renal brush-border membrane vesicle, we have previously shown that aqueous diS-C_3-(5) is

Abbreviations: diS-C_3-(5), 3,3′-dipropylthiocarbocyanine iodide; *n*-AS, *n*-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; $(Me)_2$POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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in equilibrium with membrane-bound monomeric and dimeric diS-C_3-(5) [8]. Stopped-flow fluorescence studies revealed that binding of diS-C_3-(5) to brush-border membrane vesicles consists of at least three distinct processes: an initial binding (6 ms), followed by reorientation-dimerization (30–40 ms), and translocation to a site on the opposite leaflet of the bilayer (1–1.3 s). At a 622 nm excitation wavelength, aqueous diS-C_3-(5) has an emission peak at 670 nm which red shifts to 695 nm for bound monomer; bound dimer is non-fluorescent. These measurements defined a minimal kinetic mechanism for diS-C_3-(5) binding to brush-border membrane vesicles; however, it was not possible to determine the location of the binding sites for the monomer and dimer at each stage of binding.

In order to localize the diS-C_3-(5) binding sites and to define further the sequence of diS-C_3-(5)/membrane binding processes, we observed that diS-C_3-(5) strongly quenches the fluorescence of the *n*-(9-anthroyloxy) fatty acids (*n*-AS). The *n*-AS probes contain an anthroyloxy fluorophore located at graded depths within the transverse plane of the bilayer [9–11]. Because the degree of *n*-AS quenching is determined by diS-C_3-(5) location, static fluorescence intensity and stopped-flow kinetic measurements could be used to examine the spatial orientation and kinetics of interconversion of diS-C_3-(5) binding sites.

Materials and Methods

Materials. All chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). DiS-C_3-(5), 2-AS, 6-AS, 12-AS and 16-AP were obtained from Molecular Probes (Junction City, OR). DiS-C_3-(5) was stored as a 5 mM stock solution in ethanol at 0°C in the dark. The *n*-AS probes were stored as 2 mM solutions in ethanol at −70°C. $10 \times 10 \times 48$ mm acrylic cuvettes (Sarstedt, F.R.G.) 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 (pH 7.4).

Vesicle preparation. Brush-border membrane vesicles were prepared from 1–2 kg female New Zealand white rabbits by an Mg aggregation and differential centrifugation procedure described previously [12,13]. Brush-border membrane vesicles were prepared in standard buffer containing valinomycin (25 μg/mg membrane protein) and stored at −70°C as a concentrated pellet of about 30 mg protein/ml. Brush-border membrane vesicle concentrations are expressed as [membrane phospholipid] using the conversion factor 0.4 μmol phospholipid/mg protein [14]. Protein concentration was determined by the method of Lowry et al. [15].

Fluorescence measurements. Steady-state fluorescence intensity and spectra were measured on an SLM 8225 fluorimeter (Urbana, IL) with a double excitation monochromator and single emission monochromator which was interfaced to an IBM-XT computer. Excitation and emission wavelengths were (in nm): 369/436 (2-AS), 368/435 (6-AS), 351/440 (12-AS) and 366/440 (16-AS) with 4 nm bandpass. For steady-state quenching measurements, brush-border membrane vesicles (100 μM membrane phospholipid) were incubated with 2 μM *n*-AS at 4°C for 12 h, at which time *n*-AS fluorescence was stable. Less than 1% of measured fluorescence arose from aqueous *n*-AS. μl quantities of the stock diS-C_3-(5) solution were added to a stirred cuvette containing brush-border membrane vesicles and *n*-AS. The maximum concentration of ethanol used (2% v/v) had no effect on *n*-AS fluorescence in brush-border membrane vesicles. Fluorescence intensities were corrected for inner filter effects and brush-border membrane vesicle scattering. For [diS-C_3-(5)] < 10 μM the correction was less than 5%; at 20 μM diS-C_3-(5), the maximum correction was 30%. Based on dielectric measurements in lipid bilayers, *n*-AS compounds have been reported to perturb the lipid environment at the dye: lipid ratios required in the present experiments for good signal-to-noise ratios [16]. However, control experiments performed using 0.2 μM 12-AS in place of 2 μM 12-AS showed that these effects are not important in the present measurements.

Fluorescence lifetime measurements were performed on an SLM 4800 nanosecond fluorimeter with 390 nm excitation wavelength (0.5 nm bandpass) and 420 ± 10 nm emission using a Corion 420 interference filter. Studies were performed with unpolarized light at modulation frequencies

of 18 and 30 MHz using a $(Me)_2$POPOP reference solution with 1.45 ns lifetime which was matched in intensity to the sample solution. Phase and modulation lifetimes were calculated as described elsewhere [17]. The *n*-AS probes are known to undergo an excited-state reaction in liposomes [18] with emission wavelength-dependent steady-state anisotropies (r) and lifetimes. In brush-border membrane vesicles, r decreased monotonically by 10–30% over the emission wavelength range 410–520 nm for each of the *n*-AS probes, indicating that an *n*-AS excited-state reaction also occurs in the brush-border membrane vesicle. The blue end of the emission spectrum (420 nm) was chosen for lifetime measurements in order to minimize effects of *n*-AS excited state reaction kinetics on measured lifetimes [19].

Stopped-flow experiments. Stopped-flow measurements were performed on a Dionex-130 stopped-flow apparatus (Sunnyvale, CA) which has 99% mixing efficiency and a 2 ms dead time. 0.15 ml of solutions containing brush-border membrane vesicles and *n*-AS were mixed with an equal volume of solutions containing diS-C_3-(5) and the time-course of fluorescence was recorded by a MINC/23 computer (Digital Equipment Corp., Maynard, MA) for subsequent analysis. Monochromatic excitation light (390 nm, 4 nm bandpass) was obtained using a Zeiss MM12 double monochromator (F.R.G.). Emitted light was filtered by a 450 nm long-pass filter. The maximum rate of data acquisition is 512 data points (16 bits/data point) in 40 ms. The electronic response time of the instrument is less than 1 ms. The time-course of fluorescence intensity, $F(t)$, was fitted to mono- or biexponential functions by the non-linear Newton's method.

Results

The *n*-(9-anthroyloxy) fatty acid compounds are known to probe specified depths within artificial liposomes [9,20] and biological membranes [21,22]. Using energy transfer and fluorescence quenching methods, these dyes have been used to assess membrane fluidity gradients [9] and to determine the locations of intrinsic membrane proteins and exogenous ligands within the membrane [22–24,25,26]. We find that the membrane-potential-sensitive dye diS-C_3-(5) quenches the fluorescence of the *n*-AS compounds in a dose-dependent manner.

The Stern-Volmer plots for diS-C_3-(5) quenching of 2, 6 and 12-AS and 16-AP are given in Fig. 1. At low [diS-C_3-(5)] (under μM), quenching of the *n*-AS compounds by diS-C_3-(5) is comparable as shown by the similar slopes of the Stern-Volmer plot given in the figure legend. At high [diS-C_3-(5)] (above 10 μM), the anthroyloxy probes located deepest within the membrane (12-AS and 16-AP) are quenched most. In addition, there is a discontinuity in the slope of the Stern-Volmer relation at about 6 μM diS-C_3-(5) for 12-AS and 16-AP.

We have shown previously that diS-C_3-(5) is present in brush-border membrane vesicles in the form of monomers and dimers [8]. Based on fluorescence binding titrations, we showed that bound dimer is primarily present when the [diS-C_3-(5)]/[membrane phospholipid] ratio is above 0.06 whereas bound monomer is primarily present when the ratio is below 0.03. Since 100 μM membrane phospholipid was used for the titrations in Fig. 1, the predominant form of bound diS-C_3-(5) is monomer for [diS-C_3-(5)] below 3 μM and dimer for [diS-C_3-(5)] above 6 μM. The results of the

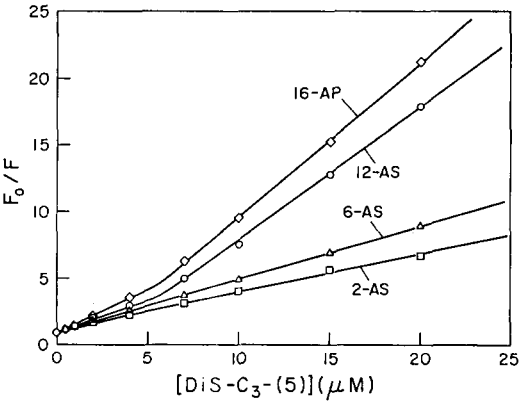

Fig. 1. Stern-Volmer plot for quenching of anthroyloxy probes by diS-C_3-(5) in brush-border membranes. DiS-C_3-(5) was added to brush-border membrane vesicles (100 μM membrane phospholipid) containing 2 μM *n*-AS at 23°C in one experiment typical of two. F_0/F represents the ratio of corrected *n*-AS fluorescence before and after diS-C_3-(5) addition. Slopes fitted to data for [diS-C_3-(5)] < 2 μM were (in μM^{-1}): 0.36 ± 0.01 (2-AS), 0.37 ± 0.01 (6-AS), 0.43 ± 0.01 (12-AS) and 0.56 ± 0.01 (16-AP). Slopes fitted to data for [diS-C_3-(5)] > 10 μM were (in μM^{-1}): 0.26 ± 0.03 (2-AS), 0.40 ± 0.01 (6-AS), 1.04 ± 0.01 (12-AS) and 1.17 ± 0.02 (16-AP).

quenching studies suggest, therefore, that bound monomer quenches the anthroyloxy group with similar efficiencies at all membrane depths, whereas the dimer primarily quenches those compounds located nearest the center of the bilayer. This is possible if the monomer is oriented parellel to the plane of the bilayer and if the dimer is oriented perpendicular to the plane of the bilayer, deep within the membrane. A number of control experiments were performed to test the validity of this conclusion.

It is necessary to show that the *n*-AS compounds have equilibrated completely with brush-border membrane vesicles before addition of diS-C_3-(5), and that *n*-AS fluorescence quenching is not due to removal of *n*-AS from the bilayer by formation of a non-fluorescent complex with diS-C_3-(5) or by a diS-C_3-(5)-induced change in the *n*-AS partition coefficient. Based on the time-course of fluorescence increase when *n*-AS (n = 2–16) was added to dipalmitoyl phosphatidylcholine vesicles, Rockley and Najjar [27] showed that the *n*-AS compounds fully partitioned into the membrane in less than 15 min. Using the same technique with addition of 2 μM *n*-AS to brush-border membrane vesicles (100 μM phospholipid), the exponential time constants for binding of 2-AS, 6-AS, 12-AS and 16-AP to brush-border membrane vesicle were 3.9, 3.7, 3.6 and 3.9 min, respectively, at 23°C. There was no further change in *n*-AS fluorescence after 20 min. A single activation energy of 16 $\pm$ 1 kcal/mol was measured for 12-AS in the temperature range 10–55°C. All experiments were performed well after full equilibrium between *n*-AS and brush-border membrane vesicles occurred.

To demonstrate that quenching of *n*-AS fluorescence by diS-C_3-(5) is not due to formation of a static complex, spectral and lifetime measurements were performed. Excitation and emission spectra were obtained for brush-border membrane vesicles containing each *n*-AS compound with diS-C_3-(5) in the range 0–50 μM. A typical experiment is shown in Fig. 2 for the 16-AP excitation spectrum. Addition of diS-C_3-(5) decreases fluorescence without causing significant changes in the 16-AP spectral shape. Similar findings were obtained for excitation and emission spectra of each *n*-AS probe in brush-border membrane vesicles.

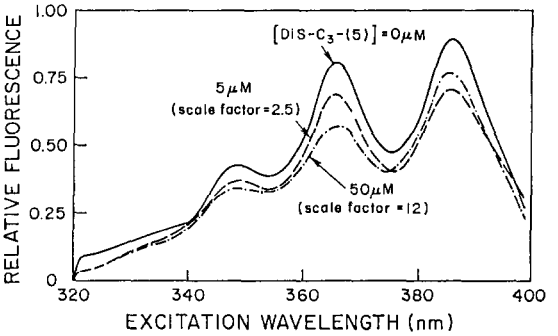

Fig. 2. Effect of diS-C_3-(5) on excitation spectra of 16-AP. Corrected excitation spectra (bandpass 4 nm) were obtained for 2 μM 16-AP in brush-border membrane vesicles (100 μM membrane phospholipid) at 440 nm emission wavelength, 23°C. Spectra obtained in the presence of 5 and 50 μM diS-C_3-(5) were scaled upward to demonstrate the lack of dependence of spectral shape on diS-C_3-(5) quenching.

The fluorescence lifetime of *n*-AS in brush-border membrane vesicles is predicted to decrease with increasing [diS-C_3-(5)] if quenching occurs by a collisional mechanism, whereas the lifetime would not change if quenching occurs by static complex or by partitioning of *n*-AS from membrane to aqueous solution. Table I indicates that the phase and modulation lifetimes measured for 12-AS and 16-AP decrease with increasing diS-C_3-(5). The decrease occurs both when bound monomer is primarily present (1 μM diS-C_3-(5)) and when bound dimer is primarily present (over 10 μM diS-C_3-(5)). The fractional decrease in lifetimes roughly correlates with the decrease in fluorescence intensities given in Fig. 1. A quantitative comparison is not possible, however, because a homogeneous lifetime was not measured due to the presence of an *n*-AS excited state reaction.

A lifetime titration is shown in Fig. 3 in which the *n*-AS phase lifetime at 18 MHz was measured as a function of [diS-C_3-(5)] for all four *n*-AS probes. The diS-C_3-(5) concentrations at which τ_p decreases by 50% of its maximal decrease ($K_{1/2}$), estimated from a fit to a single site dissociation model, are 2.5, 3.8, 4.1 and 1.8 μM for n = 2, 6, 12 and 16, respectively. Using the data in Fig. 1, a fit of F/F_0 vs. [diS-C_3-(5)] data to a single-site dissociation model gives $K_{1/2}$ = 3.2, 3.4, 4.3 and 2.0 μM for n = 2, 6, 12 and 16, respectively. The decrease in *n*-AS lifetimes with increasing [diS-C_3-

TABLE I

EFFECT OF diS-C_3-(5) ON 12-AS AND 16-AP LIFETIMES

Solutions consisted of brush-border membrane vesicles (100 μM membrane phospholipid) containing 2 μM 12-AS or 16-AP at 23°C. Phase and modulation lifetimes were measured in triplicate as described in the 'Methods' section; errors represent 1 S.D.

[DiS-C_3-(5)] (μM)	18 MHz		30 MHz	
	τ_p	τ_m	τ_p	τ_m
12-AS				
0	8.3±0.1	11.7±0.5	6.5±0.1	9.9±0.1
1	7.2±0.1	9.8±0.2	5.1±0.1	8.1±0.1
7	3.8±0.1	7.5±0.1	2.9±0.1	5.1±0.1
20	2.6±0.1	8.8±0.2	0.8±0.3	1.2±0.1
16-AP				
0	9.1±0.1	11.9±0.2	7.6±0.3	11.5±0.2
1	6.7±0.1	9.9±0.1	6.0±0.2	9.4±0.1
7	3.5±0.1	7.4±0.2	2.9±0.2	6.4±0.2
15	2.8±0.1	5.5±0.1	2.4±0.2	3.1±0.7

(5)] and the close agreement between $K_{1/2}$ for intensity and lifetime measurements indicate that diS-C_3-(5) quenches *n*-AS fluorescence primarily by a collisional mechanism.

Another approach which is frequently useful to distinguish between static and collisional quenching is the dependence of F_0/F on temperature at constant quencher concentration. In collisional quenching, F_0/F may increase with temperature because of increased quencher diffusion, whereas in static quenching, F_0/F would decrease because of tighter fluorophore-quencher binding. F_0/F increases with temperature for quenching of 16-AP by bound diS-C_3-(5) dimer as expected for a collisional quenching mechanism (Table II). Interestingly, however, there is little effect of temperature on 2-AS or 16-AP quenching by diS-C_3-(5) monomer. The explanation for this finding may involve the increased fluidity near the center of the bilayer known for pure lipid membranes [9,28]. In brush-border membrane vesicles a similar fluidity gradient appears to exist [29]. The collisional rate of diS-C_3-(5) dimers with 16-AP may be strongly temperature-dependent because of the fluid environment, whereas temperature may have little effect on the mobility of the diS-C_3-(5) monomer. This possibility is supported by our previous dynamic depolarization measurements showing that the nanosecond rotational rate for bound diS-C_3-(5) monomer (0.55–0.6 ns^{-1}) was nearly independent of temperature [32]. The rotational rate for bound dimer could not be measured because the dimer is non-fluorescent. The lack of temperature effect of 2-AS quenching by bound diS-C_3-(5) dimer supports the interpretation of Fig. 1 that 2-AS fluorescence is quenching minimally by bound dimer.

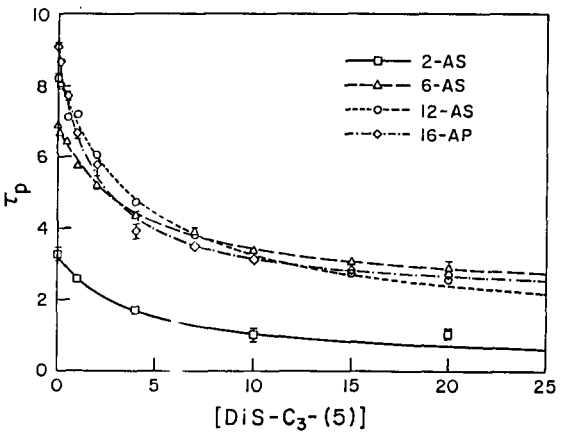

Fig. 3. Dependence of anthroyloxy probe lifetimes on diS-C_3-(5) concentration in brush-border membrane vesicles. Solutions consist of 2 μM *n*-AS in brush-border membrane vesicles (100 μM membrane phospholipid) with varying [diS-C_3-(5)] at 23°C. Phase lifetimes (τ_p) at 18 MHz modulation frequency were measured in triplicate; errors are 1 S.D. Data were fitted to a saturable single-site binding model with $[\text{diS-C}_3\text{-(5)}]_{1/2}$ (in μM) = 2.5±0.8 (2-AS), 3.8±0.6 (6-AS), 4.1±0.8 (12-AS) and 1.8±0.4 (16-AP).

TABLE II

TEMPERATURE DEPENDENCE OF *n*-AS QUENCHING BY diS-C_3-(5)

Solutions consisted of brush-border membrane vesicles (50 μM membrane phospholipid) containing 2 μM 2-AS or 16-AP and 3 or 20 μM diS-C_3-(5). Indicated values represent the quenching ratio (F_0/F) normalized to its value at 10°C.

Temperature		2-AS		16-AP	
(°C)	[diS-C_3-(5)]:	20 μM	3 μM	20 μM	3 μM
10		1.00	1.00	1.00	1.00
20		1.07	0.97	1.02	0.97
30		1.07	0.89	1.13	0.98
40		1.16	0.98	1.40	1.00
50		1.06	0.95	1.61	1.00

The equilibrium Stern-Volmer plot in Fig. 1 indicates that bound diS-C_3-(5) monomer lies parallel to the phospholipid chains within the brush-border membrane vesicle membrane. Previous kinetic studies suggested that at least two species of bound monomer exist, one type which is formed within 6 ms after diS-C_3-(5) addition to brush-border membrane vesicles, and a second type formed later (30–40 ms) which may be more stable and lie deeper in the membrane. To examine the locations of the bound diS-C_3-(5) monomeric species, stopped-flow experiments were performed in which brush-border membrane vesicles containing *n*-AS were mixed rapidly with diS-C_3-(5). As shown in Fig. 4 and Table III, there is a biphasic decrease in fluorescence with exponential time constants of 34–41 ms and 1.1–1.4 s. There was no component of decreasing fluorescence occurring in less than 34 ms, indicating that the first type of bound monomer does not quench *n*-AS fluorescence. Thus the first type of bound monomer is probably loosely associated to the brush-border membrane vesicle membrane (see Discussion) and is unable to quench 6-AS, 12-AS and 16-AP fluorescence. Because of the low quantum yield of 2-AS, stopped-flow kinetic measurements could not be performed with this compound.

The fast (34–41 ms) and slow (1.1–1.4 s) phases of *n*-AS fluorescence quenching by diS-C_3-(5) probably represent the overall processes of binding to the outer leaflet of the bilayer (reorientation and dimerization, see Discussion) and translocation across the center of the bilayer, respectively. The ratio of the amplitude of the fast phase of *n*-AS quenching to the total amplitude ($A_1/(A_1 + A_2)$, Table III) would then represent the fraction of total *n*-AS present in the outer leaflet of the bilayer weighted by the quantum yields and quenching efficiencies of *n*-AS present in the outer and inner bilayer leaflets. As expected, $A_1/A_1 + A_2$) is relatively constant (0.72–0.85), except for quenching of 16-AP by diS-C_3-(5) dimer, where $A_1/(A_1 + A_2) = 0.55 \pm 0.04$. This value does not represent a difference in 16-AP partitioning because $A_1/(A_1 + A_2) = 0.75$ for quenching of 16-AP by 5 μM diS-C_3-(5), but may represent a slight

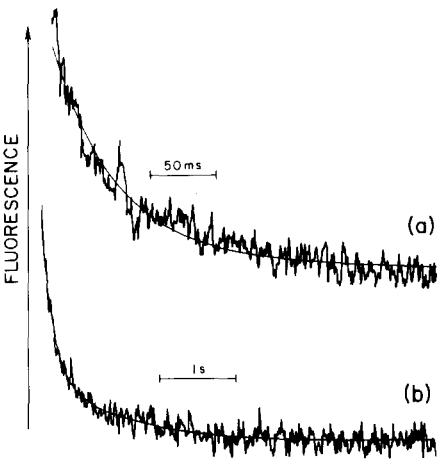

Fig. 4. Time-course of 16-AP fluorescence quenching by diS-C_3-(5). Brush-border membrane vesicles containing 16-AP were mixed with diS-C_3-(5) in a stopped-flow apparatus to given final concentrations of 100 μM membrane phospholipid and 5 μM diS-C_3-(5) (23°C). Two time-scales are shown. (a) Single exponential fit with time constant 43 ns and amplitude 415 fluorescence units. (b) Double exponential fit with slower time constant 1.15 s with amplitude 129 fluorescence units.

TABLE III

STOPPED-FLOW MESUREMENT OF *n*-AS QUENCHING BY diS-C_3-(5)

Final solutions consisted of brush-border membrane vesicles (100 μM membrane phospholipid) containing 2 μM *n*-AS and indicated [diS-C_3-(5)]. τ_1 and τ_2 refer to the faster and slower time constants; $A_1/(A_1 + A_2)$ is the fractional amplitude of the faster process. Measurements were performed in triplicate and errors represent 1 S.D.

[diS-C_3-(5)] (μM)	τ_1 (ms)	τ_2 (s)	$A_1/(A_1 + A_2)$
6-AS			
5	49 ± 8	1.2 ± 0.2	0.85 ± 0.11
20	39 ± 6	1.4 ± 0.3	0.78 ± 0.06
12-AS			
5	41 ± 7	1.1 ± 0.2	0.76 ± 0.08
20	37 ± 4	1.1 ± 0.1	0.72 ± 0.07
16-AP			
5	34 ± 6	1.4 ± 0.5	0.75 ± 0.08
20	37 ± 2	1.3 ± 0.1	0.55 ± 0.04

difference in the locations of bound dimer in the two membrane leaflets.

Discussion

A number of assumptions are required for the use of *n*-AS fluorescence to localize ligand-binding sites. These assumptions were implicit in studies of the lcoation of local anesthetics in lipid vesicles [26], of ubiquinones in mitochondrial membranes [22], and of band 3 tryptophans in the red-cell membrane [25]. It is first assumed that the ligand does not perturb the location of the *n*-AS probes so that binding information is reported accurately from known depths within the membrane. If a complex is formed between the ligand and *n*-AS which causes displacement of the anthroyloxy group or expulsion of the anthroyloxy group from the membrane, then all localization information is lost. In the present studies of diS-C_3-(5) quenching of *n*-AS, the demonstration of a collisional quenching mechanism makes this unlikely. It is also assumed that the quenching efficiency of anthroyloxy groups by a ligand is fairly independent of the position of the anthroyloxy group, so that F_0/F is proportional to the local quencher concentration. This assumption appears to be justified based on studies of *n*-AS quenching by ligands with known locations [10,21]. Finally, it is assumed that *n*-AS does not itself alter the location of the ligand being studied. Measurements of *n*-AS quenching by diS-C_3-(5) using 0.2 μM *n*-AS instead of 2 μM *n*-AS support this assumption; however, it is never possible to rigorously exclude undesired probe-ligand interactions because measured fluorescence information necessarily originates from the probe molecule.

Bearing in mind these assumptions, the data presented suggest that the diS-C_3-(5) monomer is oriented parallel to the phospholipids and quenches *n*-AS fluorescence at all levels ($n = 2$–16) similarly. The spacing between the anthroyloxy groups in 2-AS and 16-AP (about 15 Å) is similar to the length of diS-C_3-(5) (about 16 Å [4]), so that the diS-C_3-(5) monomer spans most of one leaflet of the lipid bilayer. Quenching of *n*-AS by the bound dimer occurs primarily for 12-AS and 16-AP, suggesting that the dimer is oriented perpendicular to the phospholipids and deep within the bilayer. The deep location of the dimer may be related to charge shielding effects which make the partitioning of diS-C_3-(5) dimers into the nonpolar environment more favorable.

The finding of parallel monomers and perpendicular dimers is similar to results obtained for the potential-sensitive dye, merocyanine 540. Using fluorescence anisotropy mesurements in hemispherical lipid bilayers, Dragsten and Webb [30] showed that parallel merocyanine 540 monomers were in equilibrium with perpendicular dimers and that the monomer-dimer equilibrium was sensitive to membrane potential. The absolute location of the merocyanine 540 monomers and dimers could not be determined from their studies.

Based on the present results and our previous kinetic studies of diS-C_3-(5) binding to brush-border membrane vesicles [8], a mechanism for the sequence of interactions which occur following exposure of brush-border membrne vesicle to diS-C_3-(5) is shown in Fig. 5. There is initial binding of aqueous diS-C_3-(5) (labelled '1' in Fig. 5) to form bound dye adhering to the membrane surface (2). This binding process was observed as a 6-ms increase in diS-C_3-(5) fluorescence (excitation 622 nm, emission 695 nm) but was not measured in

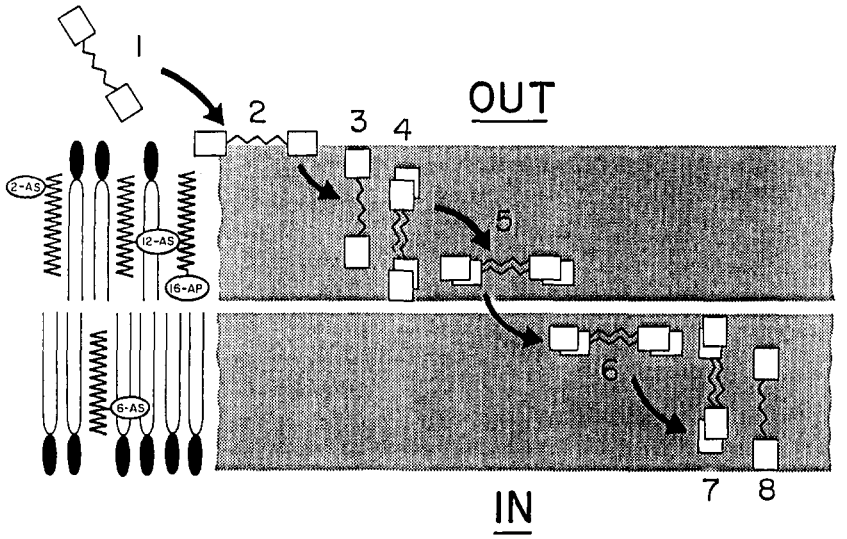

Fig. 5. Model for diS-C_3-(5) transport across a phospholipid membrane. The phospholipid bilayer containing anthroyloxy fatty acid probes is depicted schematically. Steps for diS-C_3-(5) binding include (1–2) surface binding, (2–3) monomer adsorption, (3–4) dimerization, (4–5) reorientation, (5–6) translocation, (6–7) reorientation and (7–8) dimer dissociation.

stopped-flow *n*-AS quenching experiments ($n = 6$, 12 and 16), suggesting that initial diS-C_3-(5) binding occurs to a site nearer the membrane surface than the 6-AS position.

Following initial binding (2), there is a reorientation to form a more stable bound monomer (3). This process was observed best as a concentration-independent, 30–40 ms decrease in fluorescence of aqueous diS-C_3-(5) (excitation 622 nm, emission 670 nm). In the present studies, this process is observed as a 36–41 ms decrease in *n*-AS fluorescence following mixture of diS-C_3-(5) with brush-border membrane vesicles containing *n*-AS. Bound monomer (3) is oriented parallel to the phospholipids. Previous lifetime and dynamic depolarization measurements indicated that bound monomer (combination of (2) and (3)), weighted by intensities) resides in a relatively non-polar environment (dielectric constant < 10), suggesting that the equilibrium between (2) and (3) favors (3).

Bound dimer (5) lies perpendicular to the phospholipids near the center of the bilayer. Because the 30–40 ns time-course represents conversion of (2) to (3), the dimerization and rotation processes ((3)–(4) and (4)–(5)) occur on a faster time scale (much less than 30 ms) and were not measured directly. Using the temperature-jump technique, the time-course of the rotation process for merocyanine 540 in phosphatidylcholine vesicles was measured directly and occurs in 0.5–1 ms, whereas the dimerization process occurs in under 6 μs [31]. Based on calculations of the frequency of monomer collisions expected at measurable dye : lipid ratios, it was concluded that the dimerization process occurs in 1–100 ns.

The translocation process ((5)–(6)) was measured as a 1–1.3 s decrease in *n*-AS fluorescence, or previously, by a decrease in aqueous diS-C_3-(5) fluorescence (emission 670) over the same time scale. The process was symmetric in rates and slower at very low [diS-C_3-(5)]/[membrane phospholipid], suggesting that dimer translocation occurs much faster than monomer translocation. The dimer present at the opposite leaflet of the bilayer [6] then undergoes rapid rotation and dimer dissociation ((6)–(7) and (7)–(8)) which was measured as a 1–1.3 s increase in diS-C_3-(5) monomer fluorescence (emission 695 nm).

In studies of the potential-sensitive response mechanism of diS-C_3-(5), Cabrini and Verkman [32] showed that diS-C_3-(5) translocation was the reaction step driven by changes in transmembrane potential. The fluorescence response of diS-C_3-(5) to changes in membrane potential was shown to result from the coupling of translocation to the

other reaction steps (binding, reorientation, dimerization). The results reported in this paper provide information about the location of diS-C_3-(5) monomers and dimers, and additional details containing individual steps in the overall process for binding of diS-C_3-(5) to brush-border membrane vesicles.

Acknowledgments

Supported by N.I.H. grant AM35124. G.C. was supported by Associazione Veneta Fibrosi Cistica and USL 25 Regione Veneto of Italy.

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