quences can occur in sequences such as those in the arms of the junction.

It is important to point out that the NMR analysis of this junction is consistent with previous physical studies on the complex. Circular dichroism studies have indicated that the presence of the junction does not perturb the structure of the DNA distal to it (Seeman et al., 1984); the CD spectrum is characteristic of B DNA. The close correspondence seen between the spectra observed for the pairwise arm duplexes and the spectra of the complete intact junction is consistent with this observation. Thus, only the local neighborhood of the junction is likely to display unusual structural features.

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Spectra, Membrane Binding, and Potentiometric Reponses of New Charge Shift Probes†

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ABSTRACT: The properties of a series of new potentiometric membrane probes have been explored. The probes all contain an (aminostyryl)pyridinium chromophore or a more highly conjugated analogue. The spectral properties of the dyes are discussed in terms of the excitation-induced charge shift from the pyridine to the aniline; this charge shift also provides the basis for the voltage dependence of the spectra according to an electrochromic mechanism. The spectral responses to a membrane potential on a hemispherical bilayer have been obtained and, grossly, are quite similar for all probes tested. The more subtle variations from dye to dye can be partially rationalized by consideration of binding parameters, the depth within the membrane, and structural factors. The most potential sensitive dye in this collection has been designated di-4-ANEPPS and has a 6-amino-2-naphthyl group in place of the *p*-anilino on the parent chromophore. Both the relative fluorescence emission and excitation responses have maxima of 8% per 100 mV, and these two spectra display a striking symmetry.

Optical potentiometric probes have become important tools in electrophysiology. These organic molecules display spectroscopic responses to membrane potential and have been used for the study and characterization of model membranes, nerve and muscle tissues, organelles, microorganisms, and red blood cells (Waggoner, 1979; Cohen & Salzberg, 1978; Freedman & Laris, 1981). They can often be used in place of conventional microelectrodes and lend themselves to many systems not accessible to microelectrodes. Our laboratory has concentrated on the development of probes that respond via an electrochromic mechanism (Loew et al., 1978; Loew, 1982).

In an electrochromic mechanism, molecules undergo an electronic redistribution upon excitation, and if the direction of the charge shift is parallel to an external field (membrane

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potential), the energy of the electronic transition is altered (Chance & Balteschеffsky, 1975). Since this response involves only movement of electrons upon excitation, assuring a virtually instantaneous coupling between the external field and electronic states of the chromophore, the probe response should be fast enough to follow any physiological process. In addition, this mechanism might be expected to impart a relatively uniform sensitivity to the probe irrespective of its specific environment. This expectation has been examined and found to be of limited practical value because of the occasional and somewhat unpredictable intrusion of secondary, nonelectrochromic response mechanisms (Loew et al., 1985). On the other hand, this paper demonstrates that the concept of electrochromism does provide a successful theoretical framework for the design of fast potentiometric dyes with high sensitivities.

We report the spectroscopic and membrane binding properties of a series of probes recently synthesized in our laboratory (Hassner et al., 1984). These probes are all analogues of the (aminostyryl)pyridinium (ASP)[1] dyes described in earlier papers (Loew & Simpson, 1981; Loew et al., 1979). The absorption, fluorescence excitation, and emission responses to a transmembrane potential across a hemispherical lipid bilayer have been dissected and are presented for the most effective dyes. Evidence for an electrochromic mechanism will be discussed as well as an analysis of the way in which structural variations may effect probe response and binding. Some of the factors that may allow complication from secondary motion-dependent response mechanisms are also traced to specific structural features of the dyes.

Experimental Procedures

The dyes were all synthesized by the aldol condensation or Pd-catalyzed coupling procedures as detailed by Hassner et al. (1984). Egg phosphatidylcholine was obtained from Sigma Chemical Co. (type XI-E); decane was the high-purity grade ("Gold Label") available from Aldrich Chemical Co.; deuterated water (99.8%) was also obtained from Aldrich; oxidized cholesterol was prepared according to the procedure described by Tien (1974). Lipid vesicles were prepared by adding 1 mL of double-distilled water to 10 mg of egg phosphatidylcholine, which had been deposited as a thin film on the flat bottom of a 10-mL vial, and sonicating to clarity with a Branson W-185 probe-type sonicator at 0 °C.

Absorption spectra of the dyes in solutions of ethanol, chloroform, and water, as well as bound to egg phosphatidylcholine vesicles, were obtained on a Beckman spectrophotometer (Model 25, Beckman Instruments, Inc., Fullterton, CA 92634). Corrected emission and excitation spectra of the same solutions were obtained with a Perkin-Elmer Model MPF 44B fluorescence spectrometer. Fluorescence quantum yields were obtained relative to a quinine sulfate standard by integration of the normalized corrected emission spectra.

Binding of the dyes was assayed by monitoring the large fluorescence enhancement for vesicle-bound vs. free dye. Aliquots of vesicles, prepared as described earlier, from 10 or (for high-affinity dyes) 1 mg/mL lipid vesicle preparations were used to titrate stirred 1 μM aqueous solutions of the dyes. The fluorescence was determined at wavelengths corresponding to maximal excitation and emission of the bound form, corrected for dilution (never more than 10%), and fit to a one-site saturable binding model via nonlinear least-squares analysis:

$$F_C = \frac{(F_\infty - F_0)C}{1 + K_d/n} + F_0$$

where F_C is the fluorescence of the lipid concentration C, K_d is the single-site binding constant, and n is the number of sites. These latter parameters cannot be determined independently by this simple procedure (Bashford et al., 1979). The parameter K_d/n is reported in units of mg/mL and serves as a quantitative measure of the relative membrane affinities of the dyes.

The apparatus and methodology used for measuring the potential-dependent spectroscopic responses of the probes when bound to a hemispherical lipid bilayer of oxidized cholesterol have been described earlier (Loew & Simpson, 1981; Loew, 1982). The only change in the apparatus has been the incorporation of a voltage clamp device (Model 8500, Dagan Corp., Minneapolis, MN), in order to more accurately control and monitor the membrane voltage, current, and capacitance.

Transmittance response spectra were obtained by varying the wavelength of the incident light and monitoring the potential-dependent changes in the transmitted light. Pure emission response spectra were obtained by using an excitation wavelength corresponding to the null point in the transmittance response spectrum for each probe and by varying the measured emission wavelength point by point via a set of narrow-band interference filters. This method eliminates any contributions from the shifting excitation spectrum. Similarly, pure excitation spectra, void of any emission contributions, were obtained by varying the incident radiation and monitoring emission at a wavelength corresponding to the emission response crossover (Loew, 1982). The data obtained in this manner were processed by a Hewlett Packard 9825T computer to obtain the ratio of the differential to the total light signals. This method provided the relative transmittance and fluorescence responses, $\Delta T/T$, $\Delta F/F_{em}$, and $\Delta F/F_{ex}$, as a function of wavelength.

Results

Spectroscopic Characteristics. The spectroscopic properties of a collection of the new charge-shift dyes are summarized in Table I. Included are the absorption and emission maxima of the probes in solutions of ethanol, chloroform, water, and a suspension of lipid vesicles. Also included are extinction coefficients and quantum yields. Some of the entries are left blank because of poor solubility or undetectable fluorescence; also, in some cases the emission maxima were beyond the upper wavelength limit of our correction calibration.

In an earlier paper (Loew et al., 1979) on the first in this series of potentiometric probes, di-5-ASP, we pointed out the unusual trend in the solvent-induced shifts in absorption maximum; specifically, nonpolar organic solvents led to bathochromic shifts relative to water while the lipid vesicles, normally assumed to provide a nonpolar membrane environment to bound probes, led to a blue-shifted spectrum. Both observations could be reconciled by considering the displacement of a semilocalized charge down the long axis of the chromophore upon excitation. The inability of solvent molecules to reorganize around the charge on the time scale of the absorption process means that the excitation energy varies primarily according to stabilization of the ground state; nonpolar solvents raise the energy of the ground state, decreasing the excitation energy. In a membrane, the chromophore orientation is such that the charge in the ground state is exposed to the aqeuous interface while the excited state is destabilized by the localization of its charge in the hydrocarbon region; thus, the energy of the excitation is increased relative

[1] Abbreviations: ASP, (aminostyryl)pyridinium; ABP, (anilinobutadienyl)pyridinium; APP, (anilinopentadienyl)pyridinium.

to water and a blue shift is observed.

Table I reveals that this behavior is found for all but one of the new probes and is reflected in the emission maxima as well. This is consistent with the idea that the charge shifts from the hydrophilic pyridine end to the hydrophobic aniline end upon excitation. The exception, di-6-ASQPS, contains a large perturbation to the rod shape needed for the appropriate orientation in the membrane. This picture of the probe binding is also consistent with the fluorescence quantum yields, which are uniformly low in water and quite large in the vesicles; in the solvent-relaxed excited state, strong polar interactions with solvent can provide thermal pathways for decay to the ground state; for the membrane-bound probe, the excited-state charge is shielded from the solvent, making emission a more preferred decay pathway.

The first seven entries to Table I reveal how variations in the hydrophilic side chain affect the spectral properties of the probes. Specifically, it was of interest to determine how the proximity of a fixed negative charge can influence the excitation energy. Unfortunately, no strong trends are apparent in the absorption maxima. It is probable that the flexibility of the carbon chains holding the negative charges does not allow a simple correlation of chain length with physical distance; even in the case of di-4-ASP, one cannot assume that the iodide ion is completely dissociated, with significant ion pairing likely in the nonpolar solvents. Furthermore, the steric bulk of the side chains introduces variations in the degree of ground-state solvation of the chromophore within this series. Still, it is possible to discern a weak trend to shorter wavelength absorption with a decreasing number of intervening carbons between the chromophore and the negative charge (entries 1–5) within the chloroform spectra where solvation is least important; di-4-ASPBS does not fit even this limited trend, presumably because the flexibility of the butyl chain allows the sulfonate to wrap around toward the positively charged chromophore. The only other noteworthy observation within this series is the uniformly red-shifted spectra of di-4-ASPEA—probably traceable to hindrance to solvation by the bulky ethylacetyl group.

Several other entries to Table I are worthy of individual comment. Many of the new dyes have extended conjugation compared to the original ASP chromophore (entries 11, 13, and 15–17 in Table I). By and large, only small bathochromic shifts are observed in the absorption spectra, but large shifts are evident in the emission. Such large Stokes shifts are usually associated with large differences in geometry or solvation between the relaxed ground and excited states, the latter having already been associated with the solvent dependence. Any change in excited-state geometry would be largely governed by torsional motions. Insertion of a methyl group increases the energy of the completely planar trans conformation, making deactivating torsional modes accessible to the excited state. Thus, entry 12 in Table I has a significantly reduced fluorescence quantum yield. On the other hand, the tied-back alkyl groups in JULEPPS (entry 20) should enforce a more planar trigonal geometry about the aniline nitrogen; this compound does indeed display strongly red-shifted spectra indicative of enhanced conjugation. Unfortunately, this dye does not bind to membranes (vide infra). Finally, entries 18 and 19 show probes that have cross-conjugated aniline nitrogens, resulting in significant blue shifts.

Membrane Binding. The binding parameters K_d/n for the probes are also given in Table I. As mentioned earlier, this parameter provides a quantitative measure of the relative affinities of the probes to the membrane and actually represents the concentration of lipid needed to bind 50% of the dye present in solution. From these binding constants and structural variations in probe design, it is clear that two factors greatly affect probe binding. First, and most obvious, are the effects that various hydrophobic appendages to the chromophore have on binding. In general, it appears that increases in the length of the hydrophobic side chains on the aniline nitrogen (compare entries 2, 3, and 6 with 8–10, respectively) result in more efficient binding. On the other hand, if the hydrocarbon chains are tied back or otherwise restricted, as in probes 18–20, poor binding is observed. A second parameter that appears to effect probe binding to the membrane is the overall length of the probe chromophore. This is quite apparent when one considers the strong binding of entries 13–18 in Table I, each of which has an extended rod shape compared to the ASP chromophore.

To further gauge the dependence of binding on the structural features of the probe molecules, we employed a method described by Radda (1971) to determine how well shielded the membrane-bound probes are from the water. In this method, the size of the D_2O-induced fluorescence enhancement in the presence or absence of lipid vesicles is used to assess the degree of exposure of the bound probe. Table II shows the ratio of fluorescence intensity in water to that in D_2O for a selection of the probes in the pure solvents as well as in vesicle dispersions; the intensities are the means of at least three determinations with standard deviations of no more than 5%. The last column of the table gives a parameter defined by the expression

$$\text{fraction exposure} = \frac{1 - (F_{H_2O}/F_{D_2O})_{ves}}{1 - F_{H_2O}/F_{D_2O}}$$

This parameter can be used to provide a rough feeling for the depth of the probe chromophores within the membrane. The similarity of the chromophores in this series imparts an additional measure of confidence in our application of such an empirical approach. Two general trends emerge: the depth of the chromophore increases with the number of carbons between it and covalently linked counterion; the depth of the chromophore increases with the length of the hydrocarbon tails. It also appears that the dienyl probe di-4-ABPPS is more exposed than is the equivalent structure with only one double bond, di-4-ASPPS.

Response Spectra from Stained Hemispherical Lipid Bilayer. The relative transmittance, emission, and excitation response spectra for a hemispherical lipid bilayer stained with di-4-ANEPPS are shown in Figures 1–3, respectively. These spectra are in response to a 100-mV transmembrane potential, while illuminating only the approximately planar bottom region of the bilayer. The relative signal changes are determined by dividing the change in the transmitted or emitted light intensity by the resting intensity, at each wavelength. All three response spectra have a characteristic biphasic shape and cross zero near the maxima of the respective resting spectra. These spectral characteristics are consistant with those expected for an electrochromic mechanism (Loew, 1982) and are displayed by all our dyes. Notice in addition that the absolute magnitudes of the fluorescence emission and excitation responses are very nearly equal (8% maximum) and that these spectra (compare Figures 2 and 3) have nearly perfect inverse symmetry. This behavior is evidence that a purely electrochromic mechanism is operating in both emission and excitation with equal effectiveness for di-4-ANEPPS.

This need not be the case and is not for some of the other probes. For example, if the excitation response was electro-

Table I: Spectroscopic and Binding Properties of Charge-Shift Probes

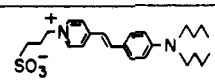
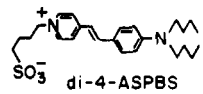
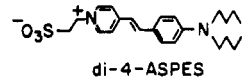
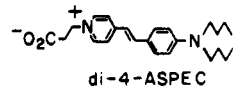
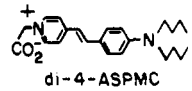
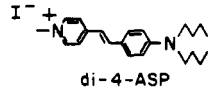
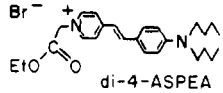
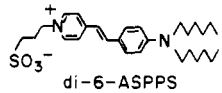
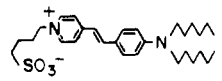
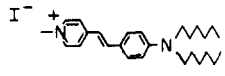
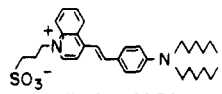
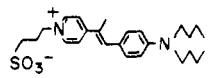
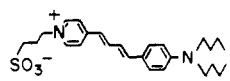
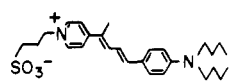
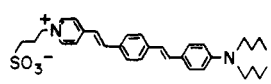
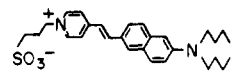
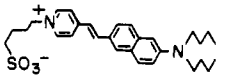
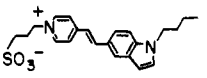
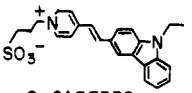
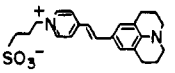
entry	structure	λ_{max}^{abs} (nm); log ϵ	λ_{max}^{em} (nm); ϕ	K_d/n (mg/mL)
1	 di-4-ASPPS	492; 4.7[a] 525; 4.8[b] 482; 4.6[c] 467; 4.6[d]	625; 0.04[a] 595; 0.3[b] 635; 0.003[c] 595; 0.3[d]	0.10
2	 di-4-ASPBS	492; 4.7[a] 517; 4.7[b] 478; 4.5[c] 465; 4.5[d]	615; 0.04[a] 600; 0.3[b] 635; 0.005[c] 597; 0.3[d]	0.13
3	 di-4-ASPES	490; 4.7[a] 519; 4.7[b] 487; 4.6[c] 465; 4.5[d]	620; 0.03[a] 600; 0.4[b] 602; 0.2[d]	0.09
4	 di-4-ASPEC	487; 4.4[a] 511; 4.3[b] 480; 4.3[c] 468; 4.2[d]	612; 0.05[a] 578; 0.15[b] 630; 0.003[c] 602; 0.2[d]	0.20
5	 di-4-ASPMC	486; 4.6[a] 509; 4.5[b] 484; 4.4[c] 467; 4.4[d]	614; 0.06[a] 580; 0.5[b] 630; 0.004[c] 602; 0.3[d]	0.13
6	 di-4-ASP	490; 4.6[a] 518; 4.7[b] 476; 4.5[c] 467; 4.4[d]	615; 0.5[a] 600; 0.02[b] 625; 0.002[c] 595; 0.3[d]	0.09
7	 di-4-ASPEA	507; 4.7[a] 523; 4.8[b] 498; 4.6[c] 482; 4.6[d]	630; 0.01[a] 605; 0.07[b] 640; 0.001[c] 607; 0.1[d]	0.07
8	 di-6-ASPPS	496; 4.8[a] 526; 4.9[b] 468; 4.6[c] 467; 4.5[d]	624; 0.04[a] 600; 0.3[b] 635; 0.002[c] 595; 0.3[d]	0.015
9	 di-6-ASPBS	495; 4.8[a] 519; 4.8[b] 469; 4.5[c] 465; 4.5[d]	622; 0.05[a] 600; 0.5[b] 630; 0.003[c] 595; 0.3[d]	0.010
10	 di-6-ASP	490; 4.7[a] 518; 4.9[b] 476; 4.6[c] 462; 4.4[d]	615; 0.03[a] 605; 0.06[b] 630; 0.003[c] 595; 0.2[d]	0.012
11	 di-6-ASQPS	565; 4.7[a] 587; 4.8[b] 500; 4.4[c] 533; 4.5[d]	692[a] 665; 0.2[b] 665[d]	0.06
12	 di-4-AMSPPS	475; 4.5[a] 507; 4.5[b] 460; 4.3[c] 450; 4.1[d]	610; 0.003[a] 605; 0.003[b] 584; 0.04[d]	0.3
13	 di-4-ABPPS	498; 4.7[a] 524; 4.7[b] 485; 4.5[c] 471; 4.4[d]	625; 0.05[a] 545; 0.5[b] 640; 0.004[c] 605; 0.3[d]	0.08
14	 di-4-APPPS	485; 4.6[a] 521; 4.5[b] 467; 4.4[c] 463; 4.2[d]	715[a] 690[d]	0.04
15	 di-4-ASTEPPS	482; 4.6[a] 532; 4.6[b] 461; 4.4[d]	~750[b] 670[d]	0.02
16	 di-4-ANEPPS	493; 4.5[a] 542; 4.4[b] 470; 4.1[c] 468; 4.3[d]	745[a] 675[b] 640[d]	0.03

Table I (Continued)

entry	structure	λ_{max}^{abs} (nm); log ϵ	λ_{max}^{em} (nm); ϕ	K_d/n (mg/mL)
17	 di-4-ANEPBS	495; 4.6[a] 542; 4.6[b] 467; 4.5[d]	745[a] 675[b] 640[d]	0.04
18	 4-INEPPS	427; 4.5[a] 423; 4.1[b] 410; 4.4[c]	600; 0.002[a] 587; 0.04[b]	1.9
19	 2-CAREPBS	440; 4.5[a] 462; 4.5[b] 422; 4.5[c] 421; 4.3[d]	588; 0.3[a] 560; 0.1[b] 599; 0.02[c] 566; 0.3[d]	0.3
20	 JULEPPS	523; 4.5[a] 553; 4.7[b] 496; 4.4[c]	640; 0.03[a] 620; 0.5[b] 655; 0.003[c]	no binding

[a] Ethanol. [b] Chloroform. [c] Water. [d] Lipid vesicles.

Table II: Solvent Isotope Effects on Probe Fluorescence

dye	F_{H_2O}/F_{D_2O}	$(F_{H_2O}/F_{D_2O})_{ves}$	fractional exposure
di-4-ASPBS	0.71	0.96	0.17
di-4-ASPPS	0.68	0.89	0.34
di-4-ASPES	0.76	0.85	0.60
di-4-ASPEC	0.67	0.83	0.52
di-4-ASPMC	0.70	0.84	0.53
di-4-ASP	0.71	0.88	0.41
di-6-ASPBS	0.69	0.96	0.13
di-6-ASPPS	0.70	0.94	0.20
di-6-ASP	0.82	0.95	0.28
di-4-ABPPS	0.71	0.86	0.48

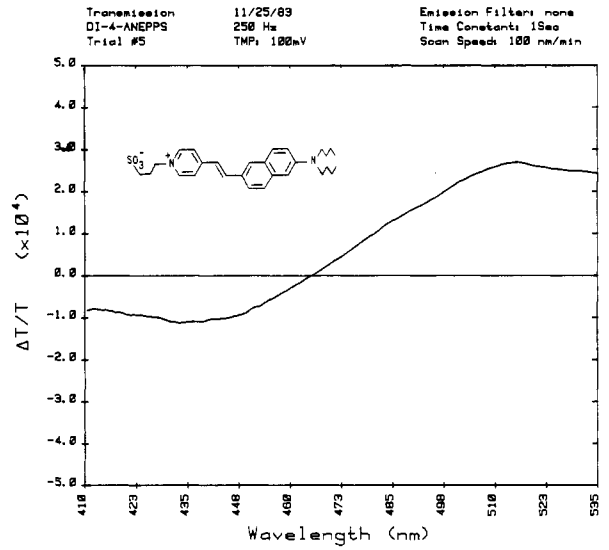


FIGURE 1: Relative transmittance response spectrum for a hemispherical bilayer stained with di-4-ANEPPS. The spectrum is the result of a 100-mV, 250-Hz transmembrane potential.

chromic but a reorientation took place during the lifetime of the excited state, the shifting charge would no longer be parallel to the transmembrane field, and the emission response would be much smaller than the excitation response. The characteristics of a selection of the dyes tested on the hemispherical bilayer are summarized in Table III. This table provides the magnitude and spectral position $[(\Delta x/x)_{max}; \lambda]$ of the relative response maximum in each of the transmittance, emission, and excitation response spectra for a given probe.

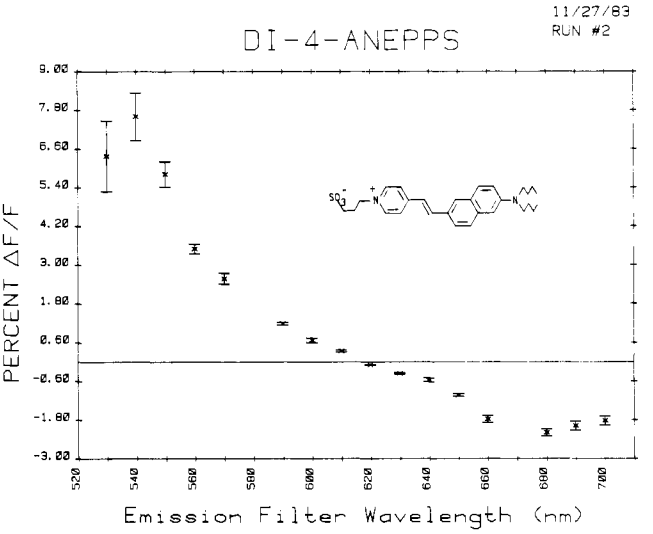


FIGURE 2: Relative fluorescence emission response of hemispherical bilayer stained with di-4-ANEPPS. The spectrum is obtained by exciting at the transmittance spectrum crossover and varying the emission wavelength with a set of narrow-band interference filters.

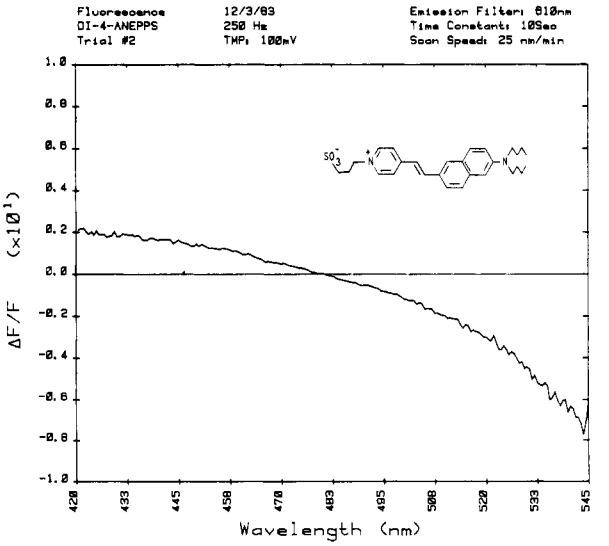


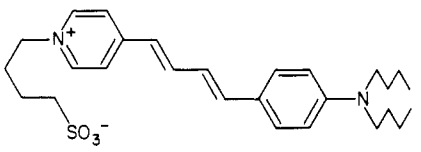
FIGURE 3: Relative fluorescence excitation spectrum of di-4-ANEPPS-stained bilayer. An interference filter at the emission spectrum crossover wavelength is used, and excitation is varied with a motor-driven monochromator.

Table III: Spectral Responses to 100-mV Potential Changes on the Hemispherical Bilayer

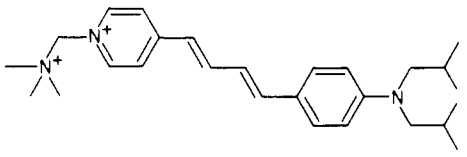
dye	$(\Delta T/T)_{max}$; λ (nm)	λ_t^0 (nm)	$(\Delta F/F)_{max}^{em}$; λ (nm)	λ_{em}^0 (nm)	$(\Delta F/F)_{max}^{ex}$; λ (nm)	λ_{ex}^0 (nm)
di-4-ASPPS	0.5×10^4; 505	476	3×10^{-2}; 510	550	-4×10^{-2}; 520	470
di-6-ASQPS	2.1×10^4; 572	522	5.2×10^{-2}; 560	615	-3.5×10^{-2}; 585	524
di-4-APPPS	0.95×10^4; 520	469	6.1×10^{-2}; 570	620	-4×10^{-2}; 535	467
di-4-ANEPPS	2.6×10^4; 515	462	7.64×10^{-2}; 540	620	-8×10^{-2}; 545	469
di-4-ANEPBS	1.3×10^4; 508	463	8.3×10^{-2}; 530	600	-6.3×10^{-2}; 550	460
di-4-ABPPS	2.5×10^4; 540	477	9×10^{-2}; 560	630	-4×10^{-2}; 540	484
di-6-AMSPPS	5×10^5; 497	458	5.8×10^{-2}; 500	535	-1.2×10^{-2}; 495	467
di-4-ASTEPPS	-3×10^4; 420	471	6×10^{-2}; 590	675	-5×10^{-2}; 540	474
RH-421	0.8×10^4; 536	478	2.92×10^{-2}; 540	625	-5×10^{-2}; 550	478
RH-460	0.75×10^4; 555	498	4.5×10^{-2}; 550	615	-3.5×10^{-2}; 575	505

In addition, the table provides the position of the zero crossover for each spectrum (λ^0). The results for a series of substituted ASP probes were reported earlier (Loew & Simpson, 1981), but di-4-ASPPS is repeated here for comparison. The fractional change in transmittance depends on the optical density of the bound dye and is not, therefore, as good a measure of intrinsic sensitivity as either of the relative fluorescence responses. These dyes as well as those in the earlier report, all possess similar spectral characteristics to those of di-4-ANEPPS. In general, the zero-crossover points are close to the maxima of the resting spectra (cf. Table I), indicating that a simple spectral shift is operative. It is important to note, however, that a few of the dyes display considerable differences between the magnitudes of their emission and exitation responses with apparent changes in quantum yield superimposed on the shift. This could be the result of additional contributions from nonelectrochormic mechanisms as previously mentioned.

Also included in the table are two dyes prepared by Amiran Grinvald and Rena Hildesheim (Grinvald et al., 1982, 1983), RH-421 and RH-460, each of which contains an ABP chromophore. RH-421 provides the largest response ($\Delta F/F$ = 21%) of any dye measured on the neuroblastoma cell preparation employed by Grinvald to screen probes (Grinvald et al., 1983). On the hemispherical bilayer it has a sensitivity similar to that of di-4-ABPPS. The double positive charge on RH-460 was of special interest; any molecular motion dependent components of the response mechanism should be highlighted in a comparison between RH-460 and its zwitterionic relatives. That RH-460 displays no strikingly aberrant behavior is good evidence against the importance of potential-dependent motions for these probes on the hemispherical bilayer [cf. Loew & Simpson (1981)].



RH-421



RH-460

Discussion

The most sensitive and least complex response on the hemispherical bilayer is that obtained with the new charge-shift probe di-4-ANEPPS. The chromophore of this probe is lengthened compared to the ASP series by the substitution of a naphthalene ring system in the place of a phenyl ring. This has the effect of lengthening the path for the excitation-induced charge shift (increasing the electrochromic effect) without introducing any additional flexibility to the chromophore (which could allow the intrusion of nonelectrochromic components to the response).

Evidence from coupling patterns in the NMR spectra of the ASP probes (Hassner et al., 1984) indicates that they are in the completely extended all-trans configuration. We have obtained 360-MHz NMR spectra of the dienyl ABP and APP probes and find them much more difficult to rationalize in terms of a single configuration or conformation. It is possible that potential-dependent changes in the structures of these more flexible probes could contribute to the signals and either reinforce or detract from the electrochromic response at a given set of wavelengths. The extent of such interference may also depend on the specific membrane environment and could completely dominate the potentiometric response. Indeed, interactions with proteins or charged lipids could so alter the conformation or orientation of a probe as to completely suppress the electrochromic response. Thus, despite the fact that any nonelectrochromic components appear to be minor from the hemispherical bilayer results summarized in Table III, a thorough analysis is necessary to completely define the mechanisms underlying an observed response in a different membrane preparation. The impressive sensitivity of RH-421 in neuroblastoma cells (Grinvald et al., 1984), for example, is clearly too large to be attributable to electrochromism.

Tables I and II show that the probe binding loosely follows some trends based on the structural features of the molecules. The binding constant and the depth within the bilayer both increase with increasing chain length of the hydrocarbon tails on the aniline nitrogen. Depth also increases with increasing separation between the chromophore and a covalently linked counter charge; binding seems less sensitive to this factor. In addition to the ASP probes listed in Tables I–III, a number of additional longer chain and/or positively charged probes containing this chromophore were examined earlier (Loew & Simpson, 1981). All of these probes have similar sensitivities and display the characteristic biphasic response spectra exemplified in Figures 1–3. Thus, despite a wide spectrum of binding sites and strengths, the characteristics of the potentiometric response of the ASP probes on the hemispherical bilayer are largely invariant. This argues strongly against any mechanism for the response involving a potential-dependent change in the degree or site of binding. Electrochromism is the only mechanism that fits all the data comfortably.

In general, the largest absorbance and excitation responses occur on the red wing of the absorption bands (di-4-ASTEPPS is an exception) while the emission response is largest on the blue side of the emission maximum. In an experimental application of the probes, wavelengths could be chosen so that the emission and excitation responses reinforce for maximal sensitivity. For example, from Figures 2 and 3, it can be seen that excitation of di-4-ANEPPS at 540 and monitoring emission at 680 should provide a sensitivity of ~10%/100 mV.

In addition to potential sensitivity, many of the dyes have properties that may make them useful as reporters of other membrane properties or processes. They are strongly fluorescent when bound and practically nonfluorescent in water. Their large Stokes shifts make it possible to use broad bandwidths for excitation and emission. They have lipid-like structures, which should not cause severe perturbations to the membrane structure; this is especially true for the zwitterionic probes. They are chemically and photochemically quite stable and are insensitive to pH. Finally, they bind with their transition moments perpendicular to the membrane surface, making them attractive complements to the class of long-chain cyanine dyes that have been widely used in membrane studies.

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Binding Stoichiometry and Structural Mapping of the ϵ Polypeptide of Chloroplast Coupling Factor 1†

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ABSTRACT: Fluorescent probes were attached to the single sulfhydryl residue on the isolated ϵ polypeptide of chloroplast coupling factor 1 (CF_1), and the modified polypeptide was reconstituted with the ϵ-deficient enzyme. A binding stoichiometry of one ϵ polypeptide per CF_1 was obtained. This stoichiometry corresponded to a maximum inhibition of the Ca^{2+}-dependent ATPase activity of the enzyme induced by ϵ removal. Resonance energy transfer between the modified ϵ polypeptide and fluorescent probes attached to various other sites on the enzyme allowed distance measurements between these sites and the ϵ polypeptide. The ϵ-sulfhydryl is nearly equidistant from both the disulfide (23 Å) and the dark-accessible sulfhydryl (26 Å) of the γ subunit. Measurement of the distance between ϵ and the light-accessible γ-sulfhydryl was not possible due to an apparent exclusion of modified ϵ from ϵ-deficient enzyme after modification of the light-accessible site. The distances measured between ϵ and the nucleotide binding sites on the enzyme were 62, 66, and 49 Å for sites 1, 2, and 3, respectively. These measurements place the ϵ subunit in close physical proximity to the sulfhydryl-containing domains of the γ subunit and approximately 40 Å from the membrane surface. Enzyme activity measurements also indicated a close association between the ϵ and γ subunits: ϵ removal caused a marked increase in accessibility of the γ-disulfide bond to thiol reagents and exposed a trypsin-sensitive site on the γ subunit. Either disulfide bond reduction or trypsin cleavage of γ significantly enhanced the Ca^{2+}-ATPase activity of the ϵ-deficient enzyme. Thus, the ϵ and γ polypeptides of coupling factor 1 are closely linked, both physically and functionally.

Chloroplast coupling factor 1 (CF_1)[1] catalyzes ATP synthesis by utilizing energy derived from a transmembrane proton gradient. The enzyme is also a latent ATPase which may be activated by a number of different treatments both on and off the membrane [cf. McCarty & Moroney (1985)]. CF_1 is composed of five different polypeptides designated α–ϵ in order

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; $CF_1(-\epsilon)$, CF_1 lacking the ϵ polypeptide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine 5′-triphosphate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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