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Two Classes of Binding Site for Hydrophobic Molecules on Bacterioopsin†

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ABSTRACT: Using environmentally sensitive fluorescent probes, we demonstrate the presence of two classes of binding site for hydrophobic compounds on the apoprotein of bacteriorhodopsin derived from the purple membrane of *Halobacterium halobium*. Dansyl fatty acids bind at two to four sites per monomer, and we suggest that these sites are located at protein–protein interfaces (nonannular sites). Dansylpropranolol, an amine, binds at six to eight sites, probably at the lipid–protein interface (annular sites). We find that binding of the fluorescent probes to the lipid component of the membrane, essentially all of which is closely associated with protein, is restricted relative to probe binding to simple lipid bilayers.

Purple membrane, a specialized region of the cytoplasmic membrane of *Halobacterium halobium*, contains a single type of protein, bacteriorhodopsin (Stoeckenius & Kunau, 1968; Oesterhelt & Stoeckenius, 1971), arranged in a regular pseudocrystalline lattice (Blaurock & Stoeckenius, 1971). The extensive two-dimensional order within the purple membrane has enabled the broad outline of the structure of the protein to be determined by a combination of electron diffraction and imaging (Henderson & Unwin, 1975; Hayward & Stroud, 1981; Agard & Stroud, 1982; Henderson et al., 1986). Until recently (Diesenhofer et al., 1985), bacteriorhodopsin was the only example of an intrinsic membrane protein to be so characterized and has therefore served as an archetype for many others (Stoeckenius & Bogomolni, 1982; Henderson, 1979). The observed cluster of α-helical segments traversing the membrane may be a common feature of all transmembrane proteins (Burres & Dunker, 1980; Engleman & Steitz, 1981); available sequence data for many membrane proteins contain stretches of polypeptide chain that could be folded into α-helices of the required length and arranged so as to present a hydrophobic face to the bilayer lipid (Engelman & Zaccai, 1980; Argos et al., 1982; Eisenberg, 1984; MacLennan et al., 1985).

Purple membrane assembles spontaneously in vivo and in vitro to form a rigid array of protein trimers, each surrounded by a single shell of phospholipid (Henderson & Unwin, 1975). This stable arrangement, which may or may not be required for physiological function, is evidently the result of a balance of lipid–protein, lipid–lipid, and protein–protein interactions. The forces between the membrane components, predominantly hydrophobic in nature, will be modulated by electrostatic and geometrical constraints, which may be particularly influential in determining the nature and degree of protein–protein associations. To gain an insight into the nature of these interactions, we have studied the binding of a number of environmentally sensitive fluorescent probes to purple membrane modified by extraction of the retinaldehyde chromophore. Bleaching of the purple membrane in this way serves a 3-fold

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purpose: it removes the need for large corrections for reabsorption of fluorescence at wavelengths around 570 nm, it eliminates quenching of bound dansyl[1] groups by retinal, and it relieves the quenching of protein tryptophan residues, thus allowing fluorescence energy transfer to probe molecules. Removal of the prosthetic group does, however, result in significant structural differences between bacteriorhodopsin and the apoprotein bacterioopsin although it seems likely that the gross structural features of the membrane are preserved, as discussed later.

In previous publications we have shown that the fluorescence emission spectra of probes containing the dansyl group are sufficiently different when they are bound to lipid and protein sites within a membrane to allow the fluorescence emission from probes in these sites to be resolved (Lee et al., 1982, 1983; Rooney & Lee, 1986). It is then possible to use such spectra to quantitate binding to the membrane. Since, in general, significant fractions of the probes will be bound to both lipid and protein sites within the membrane, it is necessary to account for binding to both classes of site. For charged probe molecules binding to lipid sites, charge effects are important, praticularly when, as in the purple membrane (Kushawa et al., 1976; Kates et al., 1982), a large fraction of the phospholipids are themselves charged. It has been shown that such charge effects can be interpreted in the framework of Gouy–Chapman theory (Rooney et al., 1983). In the purple membrane, the lipid to protein ratio is low, and thus a large fraction of the lipid will be at the lipid–protein interface (Glaeser et al., 1985). It is therefore necessary to consider the possibility that binding of probe to lipid at the lipid–protein interface (annular lipid) will be different from binding to a simple lipid bilayer. Finally, we need to consider binding of hydrophobic probe molecules to the protein both at the lipid–protein interface and at protein–protein interfaces within the trimer.

Materials and Methods

H. halobium (strain R_1) was grown in batch culture and purple membrane isolated from freshly harvested cells according to published procedures (Oesterhelt & Stoeckenius, 1974). The purified membranes gave a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis corresponding to a molecular weight of about 26 000. The apoprotein, bacterioopsin, was produced by extraction of the retinal moiety with 1 M hydroxylamine in the presence of diethyl ether, largely as described by Mullen et al. (1981). The bleached membranes were washed twice more in 1 M hydroxylamine followed by three washes in distilled water.

Total lipids were extracted from bleached membranes as described by Kates et al. (1982). Polar lipids were separated from the total lipid extract (which included residual retinal and retinal oxime) by acetone precipitation (Tornabene et al., 1969). The acetone-insoluble fraction was taken up in chloroform, and the lipid concentration was determined by drying and weighing, assuming an average molecular weight of 991 (Kates et al., 1982).

Protein concentrations were determined by total protein hydrolysis (0.2% phenol, 6 M HCl at 106 °C in vacuo for 24 h) followed by amino acid analysis. Protein concentrations for purple membrane calculated in this way agreed well with those estimated by using an extinction coefficient of 63 000 M^{-1} cm^{-1} at 568 nm for the purple complex (Oesterhelt & Hess, 1973), while for bleached membranes, the procedure circumvents the pitfalls of the more usual Lowry analysis (Rehorek & Heyn, 1979).

11-Dansylundecanoic acid was obtained from Molecular Probes and dansylpropranolol [1-[(2-dansylaminoethyl)-amino]-3-(1-naphthalenyloxy)-2-propanol] from Calbiochem. 12-Dansyldodecanoic acid was prepared by reaction of 12-aminolauric acid (Aldrich) with dansyl chloride (Sigma) in aqueous acetone (50% v/v) saturated with $NaHCO_3$. The product gave the anticipated ^1H NMR spectrum and ran as a single spot on thin-layer chromatography. Extinction coefficients of 3800 M^{-1} cm^{-1} for the dansyl fatty acids and 4100 M^{-1} cm^{-1} for dansylpropranolol were determined at 345 nm, in buffer at pH 7.5.

Fluorescence spectra were recorded by using a Spex Fluorolog fluorometer interfaced with a Z80-based microcomputer (Cromemco System 3). One hundred twenty-five points were collected at 2-nm intervals between 400 and 650 nm, integrating for 2 s at each wavelength. Spectra were corrected for the wavelength-dependent response of the emission monochromator and detector system as described elsewhere (Rooney & Lee, 1986). A band-pass of 10 nm was used for both excitation and emission, and a glass filter was placed between the sample and the emission monochromator to eliminate the second-order diffraction artifact.

We have shown elsewhere (Lee et al., 1982) that fluorescence emission spectra for dansyl probes in a variety of environments can be described as skewed Gaussians:

$$A = A_0 \exp\{-\ln 2[\ln [1 + 2b(\lambda - \lambda_0)/w_\lambda]/b]^2\}$$
$$= 0 \text{ for } [2b(\lambda - \lambda_0)/w_\lambda] \leq -1 \quad (1)$$

where A represents the fluorescence intensity at wavelength λ, A_0 is the maximum intensity (at λ_0), w_λ is the half-width, and b is the skew factor. Multicomponent fluorescence spectra were therefore treated as the sum of a number of skewed Gaussian bands due to probe in different types of environment and were resolved by curve fitting as described by Rooney and Lee (1986).

Fluorescence titrations were performed by serial addition of fluorescence probes in concentrated methanol solution to membrane samples, with excitation and emission wavelengths of 345 and 460 nm, respectively. Buffer was 40 mM HEPES, pH 7.5 at 25 °C, containing 100 mM NaCl and 0.1 mM EDTA. In no case did the volume of methanol added exceed 1% of the total volume.

Electrophoretic mobilities of bleached membrane fragments and of liposomes of extracted polar lipids were measured in 40 mM HEPES, 100 mM NaCl, and 0.1 mM EDTA, at pH 7.5 and 25 °C, by using a Rank Brothers Mark I microelectrophoresis apparatus with a cylindrical sample cell. Care was taken to focus at the stationary layer.

Analysis of Binding to Membranes. Under conditions where charge effects can be ignored (zwitterionic lipids and high ionic strength), binding of the charged fluorescence probes to lipid bilayers can be described in terms of an effective dissociation constant K_d^l (Lee et al., 1983). Assuming simple mass-action binding of the probes to n independent and identical binding sites on the protein with dissociation constant K_d^p, the concentrations of probe bound to protein $[D]_b^p$ and to lipid $[D]_b^l$ are given in terms of total concentrations of probe [D], protein [P], and lipid [L] by

$$[D]_b^p = \{(n[P] - [D]_b^p)([D] - [D]_b^p - [D]_b^l)\}/K_d^p \quad (2)$$

$$[D]_b^l = \{([L] - [D]_b^l)([D] - [D]_b^p - [D]_b^l)\}/K_d^l \quad (3)$$

[1] Abbreviations: BSA, bovine serum albumin; dansyl, 1-(dimethylamino)naphthalene-5-sulfonyl; dansylpropranolol, 1-[(2-dansylaminoethyl)amino]-3-(1-naphthalenyloxy)-2-propanol; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

which are solved numerically for given values of n, K_d^p, and K_d^l (Lee et al., 1983). Binding of the fatty acid probes to the lipid component of bleached membranes was found to be negligible under our experimental conditions (see below) and was ignored in the subsequent analysis. In this case eq 2 reduces to a simple quadratic. In the analysis of the binding of the positively charged probe dansylpropranolol to the membrane lipids, charge effects become important, because the membrane lipids are negatively charged. The necessary equations (Rooney & Lee, 1983) are too complex to allow least-squares fitting to the experimental data. An approximate method was therefore adopted. The binding of dansylpropranolol to the membrane lipids was described by an effective binding constant $K_d^{l,eff}$ given by

$$K_d^{l,eff} = ([L] - [D]_b^l)([D] - [D]_b^l)/[D]_b^l \quad (4)$$

where $[D]_b^l$ is the concentration of dansylpropranolol bound to the lipid component of the membrane and [D] and [L] are the total concentrations of dansylpropranolol and lipid, respectively. This effective binding constant will vary with the concentrations of probe and lipid since binding will depend on membrane charge which, in turn, will depend on the extent of binding of dansylpropranolol to the membrane. In a previous paper we have presented the equations for analyzing binding to charged lipid bilayers and have determined dissociation constants for binding of the neutral and protonated species of dansylpropranolol to dioleoylphosphatidylcholine (Lee et al., 1983). Assuming that the initial charge density on the lipid portion of the membrane is equal to that measured by microelectrophoresis of liposomes of extracted lipid under the same ionic conditions and assuming that the intrinsic binding constants of dansylpropranolol are largely independent of lipid structure, the equations presented by Rooney and Lee (1983) can be used to calculate the concentration of dansylpropranolol bound to lipid, $[D]_b^l$, at any given concentrations of lipid, [L], and dansylpropranolol, [D], and thus the effective lipid binding constant, $K_d^{l,eff}$, for that set of conditions. Under the experimental conditions used here, $K_d^{l,eff}$ was calculated to vary between 10 and 50 μM, assuming that the probe bound to only 30% of the membrane lipid (see below). Under the same conditions, a value of 80–100 μM would be calculated for binding to a bilayer of a zwitterionic lipid such as dioleoylphosphatidylcholine. The effective lipid binding constant was then used in eq 2 and 3 to calculate probe binding to lipid and protein sites in the membrane.

The fluorescence intensity F_{obsd} for the dansyl fluorophor in the presence of the membrane arises from both bound and free probe and is attenuated by absorption of the exciting light. Thus

$$F_{obsd} = (\alpha[D]_f + \beta[D]_b^p + \gamma[D]_b^l + F_{membr}) \times 10^{-x} \quad (5)$$

where the subscript f denotes free probe, the coefficients α, β, and γ relate the concentrations of free probe and probe bound to protein and lipid sites, respectively, to their respective fluorescence intensities, F_{membr} is the fluorescence of the membrane sample in the absence of probe (mainly due to the presence of residual amounts of retinal oxime, which varies between preparations), and x is a parameter describing the inner filter correction. For optically clear samples the inner filter correction can be expressed as

$$x = \epsilon c l \quad (6)$$

where c is the concentration of the absorbing species, ϵ is its extinction coefficient at the excitation wavelength, and l is the relevant path length; with a standard square 1-cm cuvette in a fluorometer with right-angle optics, l should be about 0.5 cm, the precise value depending on the difference in acceptance angle between the fluorometer and the spectrophotometer used to determine ϵ. For the particular geometry of the Spex Fluorolog, the effective pathlength, l, was determined to be 0.494 by measuring the fluorescence intensity of dansylundecanoic acid in methanol as a function of concentration and fitting (Dixon & Brown, 1979) the resulting curve to

$$F_{obsd} = k[D] \times 10^{-\epsilon[D]l} \quad (7)$$

where k is an intensity coefficient (arbitrary units). This correction factor was used to account for absorption by the dansyl group. The membrane samples used also showed significant optical density at the excitation wavelength (345 nm). However, for turbid samples, measured optical density includes contributions from both absorption and light scattering and therefore overestimates the true absorbance of the sample. The correction for inner filter effects arising from absorption of light by the turbid membrane suspensions was therefore estimated empirically by using the intrinsic fluorescence of residual retinal oxime as an internal standard; fluorescence emission intensities and optical densities at 345 nm (without any correction for scatter) were measured for a series of dilutions of a concentrated suspension of bleached membrane. The data were fitted (Dixon & Brown, 1979) to an equation of the same form as eq 7:

$$F_{obsd} = kA_p \times 10^{-A_p\delta} \quad (8)$$

where k is again an intensity coefficient, in this case relating the fluorescence of membrane-associated retinal oxime to the optical density of the sample A_p. The coefficient δ incorporates both the effective path length of the sample and a correction for the contribution of light scatter to the measured optical density. Excellent fits to eq 8 were obtained up to an optical density of 0.8, the highest observed in the course of these experiments, with best fit values for δ varying between preparations, in the range 0.2–0.3. Thus the total correction for the inner filter effect was expressed as

$$x = 0.494A_D + \delta A_p \quad (9)$$

where A_D is the optical density of the dansyl probe.

The coefficient α relating fluorescence intensity to the concentration of free probe was obtained by measuring the fluorescence of the probe in buffer alone. The coefficient γ for lipid-bound dansylpropranolol was obtained by fluorescence titration of extracted lipid, by using effective binding constants calculated as described above.

Finally, the fluorescence intensities measured as a function of protein and probe concentrations were fitted to eq 5 by using the nonlinear least-squares program described by Dixon and Brown (1979) to obtain best fit values for β, n, and K_d^p.

Results

Microelectrophoresis of bleached purple membranes in 0.1 M NaCl at pH 7.5 revealed a net negative surface charge density σ^- of 1.4×10^{-3} charges/Å^2 (Table I). This is 2–3 times greater than previous reports of $(4–5) \times 10^{-4}$ (Packer et al., 1984) and $(6–7) \times 10^{-4}$ charges/Å^2 (Ehrenberg & Meiri, 1983), although Ehrenberg and Meiri found that σ^- varied somewhat between membrane preparations. Most of the surface charge is due to the presence of negatively charged lipid head groups, as is shown by microelectrophoresis of liposomes of extracted membrane lipids, where σ^- increases to 3.7×10^{-3} charges/Å^2, corresponding to a surface potential of −63 mV. If allowance is made for binding of Na^+ to the

Table I: Surface Charge Properties of Purple Membranes and Extracted Lipids[a]

	purple membranes	extracted lipids
electrophoretic mobility[b] ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)	−1.79 ± 0.24	−3.81 ± 0.25
ζ potential (mV)	−23 ± 3	−48 ± 3
surface potential[c] (mV)	−29 ± 3	−63 ± 4
surface charge density (charges/Å^2)	−1.4 (±0.3) × 10^{-3}	−3.7 (±0.3) × 10^{-3}

[a] Purple membrane concentration was 2.5 mg of protein/mL and lipid concentration was 0.22 mg/mL. [b] Values given as mean ± SD of measurements on 15 individual particles. [c] Calculated assuming that the plane of shear (at which ζ potential is measured) is 2 Å from the plane of the membrane surface charges (Rooney et al., 1983).

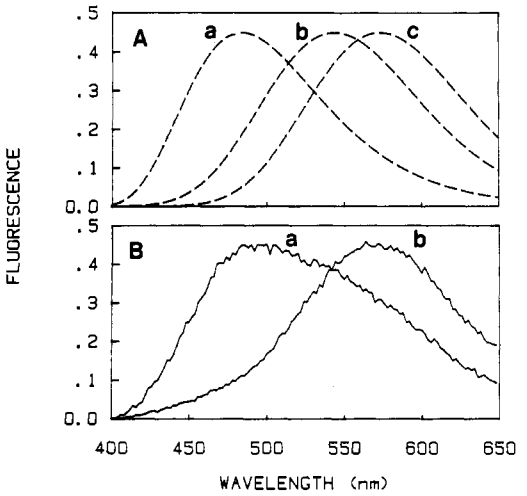

FIGURE 1: (A) Fluorescence emission spectra for dansylundecanoic acid bound to (a) BSA and (b) liposomes of DOPC and (c) free in solution at pH 7.5. (B) Fluorescence emission spectra of 10.6 μM dansylundecanoic acid in the presence of bleached membranes (6 μM protein) at pH 7.5, excited at 284 (a) or at 345 nm (b). All spectra have been normalized to the same maximum intensity.

lipid head groups in the same way as to phosphatidylserine, with an intrinsic association constant of 0.6 M^{-1} (Eisenberg et al., 1979), the surface charge density due to lipid is ca. 7 × 10^{-3} charges/Å^2. This value agrees well with the average of the estimates of Renthal et al. (1984) for the surface charge densities due to lipid on the cytoplasmic and external faces of purple membranes, under low-salt conditions.

Absorption spectra of membranes before and after bleaching show that the absorption band due to the retinylidene protein at 570 nm is eliminated, to be replaced by a band at 366 nm due to residual retinal oxime and weak absorption around 470 nm.

In previous studies, we have shown that the environmental sensitivity of the dansyl group is sufficient to distinguish between probe free in solution and bound to lipid and protein sites (Figure 1A). Direct excitation, at 345 nm, of dansylundecanoic acid in the presence of bleached membranes (Figure 1B) results in an emission spectrum similar to that obtained in buffer (Figure 1A) but with increased intensity at short wavelengths, indicative of some binding to hydrophobic sites. This component becomes the major feature on excitation at 284 nm via energy transfer from protein tryptophan residues, resulting in an emission spectrum resembling that of dansylundecanoic acid bound to hydrophobic sites on BSA (Figure 1A). Enhancement of the short-wavelength component of the spectrum on excitation at 284 nm is consistent with the presence of protein-bound probe, since resonance energy transfer is strongly distance dependent. In the presence of native purple membrane, the emission spectrum of dansylundecanoic acid excited at 345 nm is again similar to that of probe in buffer, but excitation at 284 nm elicits only a small change in the spectrum (not shown). Reduced energy transfer between excited tryptophan and bound dansyl groups is to be expected in the purple membrane since protein tryptophan fluorescence is almost entirely quenched in the retinylidene protein (Kalisky et al., 1981; Oesterhelt, 1971).

Table II: Fluorescence Properties of Dansyl Probes

probe	A_0 (arb units)	λ_0 (nm)	w_λ (nm)	b
in buffer				
dansylundecanoic acid	0.53	573.5	117.8	0.208
dansyldodecanoic acid	0.53	573.5	117.8	0.208
dansylpropranolol	0.58	554.2	136.1	0.161
in dioleoylphosphatidylcholine				
dansylundecanoic acid	3.60	543.7	118.8	0.202
dansyldodecanoic acid	3.60	543.7	118.8	0.202
dansylpropranolol	4.00	540.7	119.9	0.226
in extracted lipids from bleached membranes				
dansylpropranolol	2.08	539.6	128.9	0.185

We have shown elsewhere (Lee et al., 1983; Rooney & Lee, 1986) that composite spectra of the type illustrated in Figure 1 can be resolved by least-squares curve fitting into components representing probe bound to protein, probe bound to bilayer lipid, and free probe in aqueous solution. The shapes of the emission spectra of dansyl fluorophors are well described by skewed Gaussians, with values for the spectral parameters A_0 (maximum intensity), λ_0 (wavelength of maximum emission), w_λ (half-width), and b (skew) varying with the nature of the probe environment. Using test spectra of known composition, we have shown that the contribution due to protein-bound probe can be recovered well if the parameters λ_0, w_λ, and b describing lipid-bound and free probes are known (Rooney & Lee, 1986).

Appropriate values of these parameters for the dansyl probes in aqueous solution were obtained by fitting the emission spectra of the probes in buffer to a single skewed Gaussian (Table II). Similarly, the spectra of probes in the presence of an excess of lipid can be fitted to obtain the corresponding parameters for lipid-bound probe, which are relatively insensitive to the chemical nature of the lipid head group or the length of the fatty acyl chains (Rooney & Lee, 1986); Table II shows that the emission maximum of dansylpropranolol bound to lipid extracted from bleached membranes is essentially identical with that obtained in the presence of dioleoylphosphatidylcholine (DOPC), although slightly broader and less skewed. Maximum fluorescence intensity for dansylpropranolol bound to the extracted lipid is reduced relative to that found in DOPC, however, perhaps indicating quenching by some component of the complex lipid mixture. Because the extracted lipid carries a high negative surface charge, the dansyl fatty acids do not bind strongly enough at pH 7.5 to isolate their lipid-bound spectra; the parameters obtained with DOPC were therefore used in the subsequent analyses of membrane spectra.

The composite spectra of dansylundecanoic acid bound to bacterioopsin were analyzed in terms of 12 parameters, 6 of which (λ_0, w_λ, and b for the lipid-bound and aqueous components) were fixed at predetermined values. The results of typical least-squares fits are shown in Figure 2 for excitation at 284 and 345 nm, and the estimated values for the unknown parameters (the parameters describing the shape of the protein-bound spectrum and the intensities of each component) are listed in Table III.

Table III: Analysis of Fluorescence Spectra of Dansyl Probes Bound to Bleached Purple Membranes[a]

concn (μM)	components	λ_{ex} (nm)	A_0^{water} (arb units)	A_0^{lipid} (arb units)	$A_0^{protein}$ (arb units)	$\lambda_0^{protein}$ (nm)	$w_\lambda^{protein}$ (nm)	$b^{protein}$	CV[b]
				Dansylundecanoic Acid					
10.6	3	284	0.036 ± 0.004	0.065 ± 0.008	0.114 ± 0.002	478.1 ± 1.0	80.6 ± 2.6	0.192 ± 0.025	0.025
10.6	3	345	0.445 ± 0.006	0.082 ± 0.008	0.054 ± 0.008	468.5 ± 1.7	71.5 ± 5.0	0.101 ± 0.104	0.023
10.6	2	284	0.059 ± 0.003		0.137 ± 0.001	487.4 ± 0.4	101.3 ± 2.1	0.337 ± 0.026	0.026
10.6	2	345	0.482 ± 0.022		0.082 ± 0.003	494.0 ± 8.1	116.9 ± 27.2	0.281 ± 0.199	0.025
				Dansyldodecanoic Acid					
4.3	3	284	0.001 ± 0.002	0.044 ± 0.003	0.083 ± 0.001	473.8 ± 0.6	81.4 ± 1.6	0.237 ± 0.019	0.018
4.3	3	345	0.129 ± 0.003	0.038 ± 0.006	0.041 ± 0.001	473.5 ± 2.0	82.4 ± 5.7	0.226 ± 0.065	0.017
4.3	2	284	0.014 ± 0.001		0.095 ± 0.001	483.2 ± 0.3	104.1 ± 1.8	0.388 ± 0.021	0.024
4.3	2	345	0.141 ± 0.005		0.055 ± 0.001	492.5 ± 2.6	115.2 ± 9.2	0.322 ± 0.069	0.020
				Dansylpropranolol					
2.0	2	284		0.298 ± 0.002	0.519 ± 0.001	499.1 ± 13.2	104.5 ± 26.4	0.293 ± 0.064	0.025
2.0	2	345		0.917 ± 0.004	0.985 ± 0.003	501.1 ± 19.5	109.9 ± 31.8	0.241 ± 0.072	0.021
2.0	2	284	0.108 ± 0.002		0.684 ± 0.001	507.0 ± 12.2	113.9 ± 24.0	0.288 ± 0.052	0.027
2.0	2	345	0.334 ± 0.007		1.515 ± 0.006	512.6 ± 28.2	119.4 ± 42.2	0.226 ± 0.077	0.023
2.0	3	284	0.002 ± 0.001	0.295 ± 0.002	0.520 ± 0.001	499.2 ± 13.8	104.5 ± 26.5	0.293 ± 0.064	0.025
2.0	3	345	0.010 ± 0.003	0.897 ± 0.004	0.994 ± 0.003	501.3 ± 20.6	110.1 ± 37.2	0.241 ± 0.075	0.021

[a] Protein concentrations were 6.0 μM for the dansyl fatty acids and 5.4 μM for dansylpropranolol. Values of the parameters λ_0, w_λ, and b for probes in buffer and bound to lipid were fixed (see Table II). Best least-squares estimates of the variable parameters of the fits are given plus or minus the estimated standard deviation. [b] Coefficient of variation (CV) is the root-mean-square deviation of the fit divided by the mean fluorescence intensity of the experimental data.

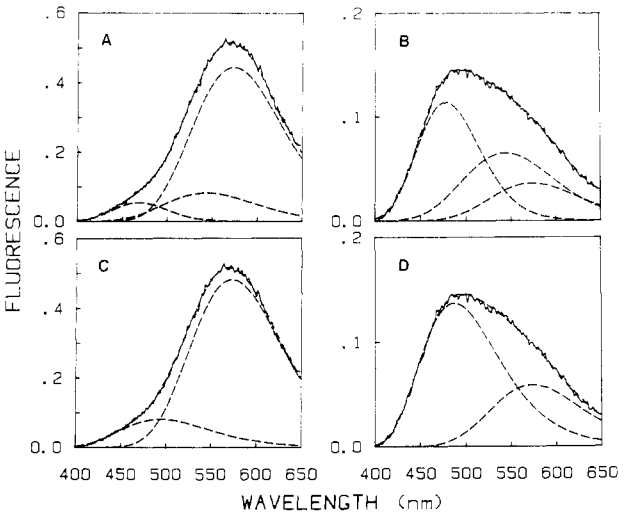

FIGURE 2: Fluorescence emission spectra of 10.6 μM dansylundecanoic acid in the presence of bleached membranes (6 μM protein) at pH 7.5, excited at 345 (A, C) or at 284 nm (B, D) analyzed in terms of contributions from probe in protein, lipid, and aqueous phases (A, B) or in terms of protein-bound and aqueous signals alone (C, D). Refer to Table III for details.

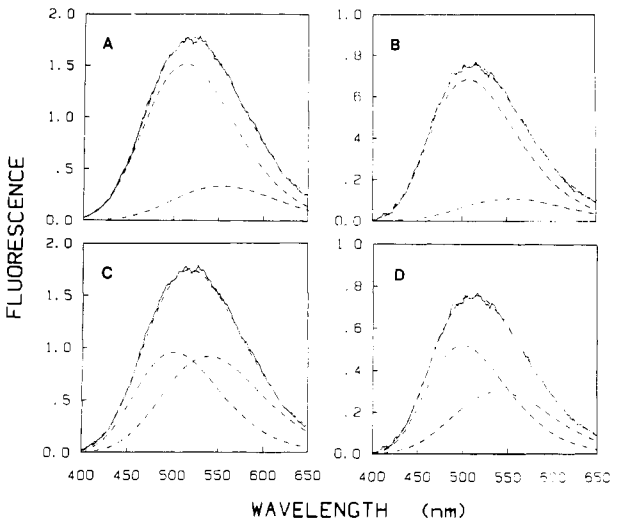

FIGURE 3: Fluorescence emission spectra of 2.0 μM dansylpropranolol in the presence of bleached membranes (5.4 μM protein), excited at 345 (A, C) or at 284 nm (B, D) and analyzed in terms of contributions from protein-bound and free probes (A, B) or protein-bound and lipid-bound probes (C, D). Refer to Table III for details.

It is clear that the relative contribution from the component at short wavelength is enhanced by excitation at 284 nm, as would be expected for probe in close association with protein. The differences in parameter estimates for the protein-bound probe at the two excitation wavelengths can probably be attributed to the small contribution of the protein-bound signal to the total fluorescence when the probe is excited at 345 nm rather than to heterogeneity of binding sites. It is of interest that the contribution assigned to lipid-bound probe, excited directly at 345 nm, is only about 15% of that expected in the presence of an equivalent concentration of extracted lipid, calculated from the known lipid to protein ratio in purple membrane (Blaurock & Stoeckenius, 1971), the dissociation constant for binding of dansylundecanoic acid to lipid bilayers (Rooney et al., 1983), and the net lipid surface charge density estimated by microelectrophoresis (Table I). Indeed, since the three-component fits are not significantly better than two-component fits to only protein-bound and aqueous components (Table III, Figure 2C,D), the small lipid contribution calculated from the three-component fit may in fact be an overestimate. Two-component fits with protein-bound and lipid-bound components are very poor. Similar results are obtained with dansyldodecanoic acid (Table III).

The cationic probe dansylpropranolol binds strongly to both purple and bleached membranes, with probe fluorescence again being quenched in the case of purple membranes. Emission spectra excited at 345 nm in the presence of bleached membranes are dominated by the membrane-bound component (Figure 3); the change in the spectrum on changing to an excitation wavelength of 284 nm is much less marked than in the case of the dansyl fatty acids because dansylpropranolol itself absorbs strongly at 284 nm. Good two-component fits to the spectra can be obtained in terms of protein-bound probe and either lipid-bound (Figure 3C,D) or free probe (Figure 3A,B), or three-component fits can be obtained with protein- and lipid-bound probes with a negligible contribution from free probe (Table III). The two-component fit to protein-bound probe and free probe can, however, be eliminated since the

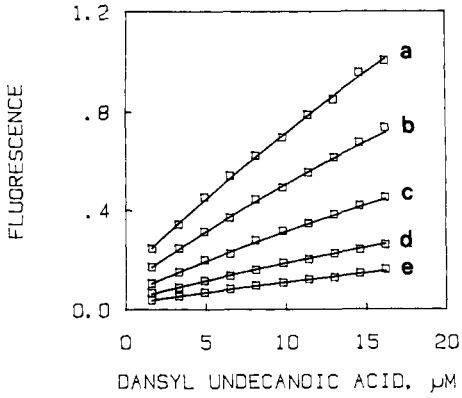

FIGURE 4: Fluorescence titrations of bleached membranes with dansylundecanoic acid. Buffer was 40 mM HEPES, 0.1 M NaCl, and 0.1 mM EDTA, pH 7.5 at 25 °C. Fluorescence was excited at 345 nm, and intensity was recorded at 460 nm. Points were experimental. Lines represent the best least-squares fit to eq 5, with K_d^p = 131 μM and n = 3.2 (Table IV). Protein concentrations were (a) 11.2, (b) 5.9, (c) 3.0, (d) 1.5, and (e) 0.7 μM.

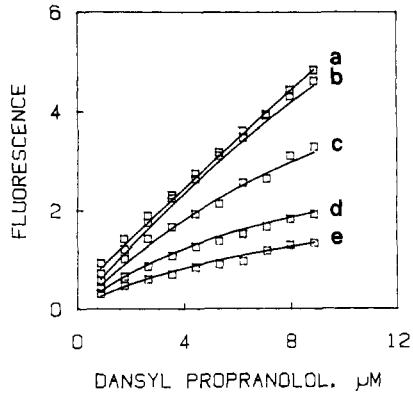

FIGURE 5: Fluorescence titrations of bleached membranes with dansylpropranolol. Experimental details as in the legend to Figure 4. Lines represent the best least-squares fit to eq 5, with K_d^p = 3.2 μM and n = 6.8 (Table IV). Protein concentrations were (a) 10.26, (b) 1.28, (c) 0.64, (d) 0.31, and (e) 0.16 μM.

change in intensity of the peak attributed to probe free in buffer on changing the excitation wavelength from 284 to 345 nm is 60% less than that observed for direct measurements of the fluorescence emission spectrum of dansylpropranolol in buffer. We conclude therefore that the correct spectral fit is provided in terms of protein- and lipid-bound components (Figure 3C,D and Table III). Interestingly, the size of the lipid-bound component is again smaller than would be expected for dansylpropranolol in the presence of an equivalent concentration of purple membrane lipids.

The fitted spectra in Figures 2 and 3 show that emission from membrane-bound probe at 460 nm arises very largely from protein-bound probe in the case of dansylundecanoic acid, with a small contribution from lipid-bound probe in the case of dansylpropranolol. To quantitate binding to protein, we therefore carried out fluorescence titrations with bleached membranes, using excitation and emission wavelengths of 345 and 460 nm, respectively. We used direct excitation of the dansyl group rather than excitation at 280 nm because energy transfer from tryptophan to all possible binding sites on the protein may not be the same. Figures 4 and 5 show representative sets of titration curves for dansylundecanoic acid and dansylpropranolol, along with the best fits to eq 5.

For dansylpropranolol, emission spectra indicate significant binding to the lipid component of the membrane. The analysis of the binding of positively charged dansylpropranolol to the lipid component of the membrane must take into account the

Table IV: Binding of Dansyl Probes to Bleached Membranes

β (arb units)	K_d^p (μM)	n	CV
	Dansylundecanoic Acid		
1.89 ± 0.08	131 ± 23	3.2 ± 0.5	0.021
1.98 ± 0.08	83 ± 6	2, fixed	0.024
1.89 ± 0.08	169 ± 10	4, fixed	0.021
	Dansyldodecanoic Acid		
1.06 ± 0.03	43 ± 4	3.0 ± 2	0.021
1.09 ± 0.04	27 ± 2	2, fixed	0.031
1.08 ± 0.03	63 ± 3	4, fixed	0.024
	Dansylpropranolol[a]		
0.91 ± 0.02	3.2 ± 0.8	6.8 ± 6	0.054
0.88 ± 0.02	2.3 ± 0.4	6, fixed	0.055
0.93 ± 0.02	4.6 ± 0.4	8, fixed	0.056

[a] Fitted assuming that dansylpropranolol bound to 30% of the available membrane lipid, which had an initial surface potential of −63 mV (see text for details).

surface charge on the membrane lipids measured by microelectrophoresis (−63 mV at pH 7.5, 0.1 M NaCl). As described above, this was done by using an effective binding constant $K_d^{l,eff}$. The titration data were fitted to eq 2, 3, and 5 to give best fit parameters β, K_d^p, and n describing the protein sites. Fits to the titration data could be obtained, assuming that all the lipid in the membrane sample was available for binding dansylpropranolol, but the calculated concentration of lipid-bound dansylpropranolol would correspond to a 2-fold greater intensity of emission than is actually observed (Figure 3). The intensity of the lipid-bound component in Figure 3 is in fact consistent with only 30% of the membrane lipid being available for binding, and the titration data can be fitted equally well under this condition (Figure 5), with the results listed in Table IV. In these calculations, $K_d^{l,eff}$ for lipid binding was calculated from [D] rather than from [D] − [D]$_f^p$; the effect was to overestimate n slightly. Thus, if the set of $K_d^{l,eff}$ is recalculated by taking into account the estimated binding to protein and the least-squares analysis is repeated, n falls to 6.3, rising again to 6.4 on a second cycle of iteration. These fits then overestimate the lipid-bound contribution to the spectrum of Figure 3, implying that the proportion of lipid available to bind dansylpropranolol is somewhat less than 30%. Further calculations, assuming 20% availability and correcting $K_d^{l,eff}$ iteratively, slightly underestimate the lipid-bound component of the spectrum, with n = 7.1 and K_d^p = 3.8 μM. We therefore take the estimate of n to fall in the range 6.3–7.1, with between 20% and 30% of the lipid participating in binding the probe.

For the dansyl fatty acids, the effect of the negative surface charge on the membranes will be to increase the apparent dissociation constant for binding to lipid. In the case of dansylundecanoic acid, using the binding constants for interaction with egg phosphatidylcholine (Rooney & Lee, 1983), we can calculate a $K_d^{l,eff}$ of ca. 450 μM for binding to neutral lipid under the conditions of Figure 2; introduction of a surface potential of −63 mV will increase this to 1200 μM. Binding to lipid would thus be expected to be very weak, and indeed, fluorescence spectra and fluorescence titrations of extracted lipid with dansylundecanoic acid show very limited binding of the probe compared with that observed in DOPC in the experimentally accessible concentration range (data not shown). Thus, the titration data for both dansyl fatty acids were fitted, assuming negligible binding to the lipid phase of the membrane. This simplified the fitting procedure considerably, and the resulting best fit values for the protein parameters are listed in Table IV. Introduction of lipid binding, as for dansylpropranolol, made no significant difference to the

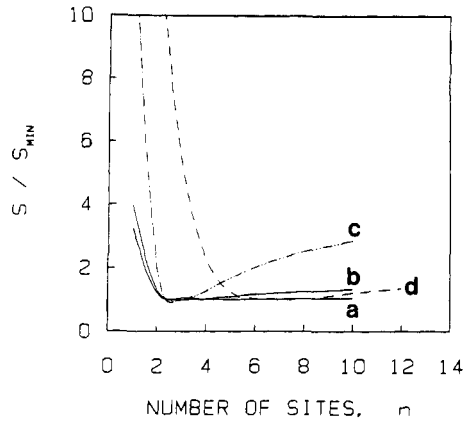

FIGURE 6: Dependence of goodness of fit of regression analysis on the fixed value of n, the number of binding sites. Goodness of fit is expressed as the residual sum of squares of the deviations (S) divided by the minimum value of S obtained for a given set of data, allowing n to vary. Curves: (a and b) two independent experiments with dansylundecanoic acid; (c) dansyldodecanoic acid; (d) dansylpropranolol.

best fit values of β, K_d^p, and n.

From the plots shown in Figures 4 and 5, it is clear that the titrations do not approach saturation of binding sites. This is unavoidable because of the limited aqueous solubility of the hydrophobic probes employed here. The restricted range of the data results in a relatively high degree of correlation among the parameters to be determined, as a change in one parameter may be compensated to a considerable extent by changes in other parameters, with only small changes in the goodness of fit to the data. To define possible ranges of values of n, K_d^p, and β that can be considered to give equally good fits to the data, we have therefore fitted the data, using a range of fixed values of n and allowing K_d^p and β to vary, and plotted the relative goodness of fit S/S_{min} as a function of n (Figure 6), where S is the residual sum of squares of the deviations obtained for any fixed value of n and S_{min} is the minimum value of S, obtained when n, K_d^p, and β are all allowed to vary. For dansylundecanoic acid it is clear from such plots that the number of binding sites must be greater than 2, but it is not clear what range of values of goodness of fit is acceptable, so an upper limit for the number of sites cannot be defined from a single titration. We have therefore carried out an analysis of this type for three different preparations of bacterioopsin: all give a minimum value of S for $n = 2$–4, and two such curves are illustrated in Figure 6. For dansyldodecanoic acid, plots of goodness of fit against n are steeper, so the value of n is better defined as being between 3 and 4. For dansylpropranolol it is clear that the number of sites is considerably greater than for the fatty acids, with n being between 6 and 8 (Figure 6).

Discussion

Little is yet known about the interactions that determine the three-dimensional organization of biological membranes. Many integral membrane proteins possess nonpolar segments that are long enough to span a lipid bilayer in an α-helical conformation, and bacteriorhodopsin probably contains seven such sequences (Engelman et al., 1982). The interaction of phospholipids with membrane proteins such as (Ca^{2+} + Mg^{2+})-ATPase (East et al., 1985) and (Na^+ + K^+)-ATPase (Esmann et al., 1985) shows little specificity, as expected for interaction with a hydrophobic surface made up of α-helical sequences of amino acids. If protein–protein interactions in the membrane are also dominated by hydrophobic interactions, then the free energy of separation of a protein aggregate into protein monomers fully solvated by lipid will be close to 0. Specific protein–protein interactions within the membrane would most likely involve charged residues and charge–charge interactions between the protein molecules. It is also highly likely, of course, that protein–protein interactions within a membrane will involve not only the transmembranous domains of the protein but also the cytoplasmic domains.

In the native purple membrane, trimers of bacteriorhodopsin form a two-dimensional hexagonal lattice, with considerable protein–protein contact within the trimers but with little or no contact between trimers (Henderson & Unwin, 1975). However, there are several experiments that suggest that the protein–protein interactions are relatively nonspecific and variable. Thus, an orthorhombic crystal form of the purple membrane can be obtained in vitro, in which, although the area occupied by a bacteriorhodopsin molecule is the same as that in the hexagonal lattice, the protein–protein interactions are markedly different (Michel et al., 1980). Cherry et al. (1978) have shown that bacteriorhodopsin is monomeric when reconstituted into a bilayer of lipid in the liquid-crystalline phase when the molar ratio of lipid to protein is greater than 40:1, but is aggregated at lower molar ratios of lipid to protein and when the lipid is in the gel phase. This argues against any great specificity in protein–protein interactions. Lastly, although there is some evidence for kinetic coupling between molecules of bacteriorhodopsin in the trimer (Korentein et al., 1985), bacteriorhodopsin has been shown to be active when solubilized in monomeric form in Triton X-100 (Casadio et al., 1980).

In order to understand better the interactions responsible for maintaining the structures of biological membranes, we have studied the binding of probe molecules at lipid–protein and at protein–protein interfaces in membranes, using environmentally sensitive fluorescence probes containing the dansyl group (Lee et al., 1983). Although we could demonstrate the binding of such probes to bacteriorhodopsin, considerable fluorescence quenching and absorption of fluorescence by the highly colored membranes makes detailed analysis difficult. We therefore chose to study the bleached membrane containing bacterioopsin.

Unfortunately, membranes containing bacterioopsin, whether derived from purple membrane by bleaching or isolated from *H. halobium* grown in the presence of nicotine ("brown" membrane) or retinal$^-$ mutants ("white" membrane), are much less well characterized structurally than purple membrane. Removal of the prosthetic group from bacteriorhodopsin leads to a conformational change in the protein, whereby the average tilt of the seven transmembrane helices increases by 20–28°, toward the plane of the membrane (Muccio & Cassim, 1979; Gibson & Cassim, 1985; Aldashev, 1985). This change in tertiary structure appears to disrupt the interactions responsible for maintaining the hexagonal crystalline lattice of the membrane, since the X-ray diffraction rings of isolated purple membrane patches are broadened by bleaching, showing an incomplete loss of order (Hiraki et al., 1978). Although bacterioopsin of brown membrane shows no evidence of crystalline order, this could be due, at least in part, to the fact that the brown membrane does not contain only bacterioopsin but also contains, for example, cytochromes whose presence would be expected to hinder crystallization. The white membrane isolated from one retinal$^-$ mutant has been reported to be crystalline (Mukohata et al., 1981) while that isolated from another has not (Marque et al., 1986).

The structure adopted by isolated purple membrane after bleaching is therefore uncertain. However, the low lipid to protein ratio in the isolated membrane makes some kind of

short-range order likely. This could explain the different results obtained from bleaching whole cells and from bleaching the isolated purple membrane. Oesterhelt et al. (1974) found that following bleaching of whole cells of *H. halobium*, the isolated membrane fraction containing bacterioopsin had a lower buoyant density than that for purple membrane. If, however, the purple membrane was isolated prior to bleaching, then after bleaching the membrane containing bacterioopsin had the same density as that for the purple membrane.

The lipid to protein ratio in isolated purple membrane and thus in the bleached membrane is too low to allow the presence of isolated, monomeric protein molecules, so some kind of aggregated species is most likely. In the absence of any firm data we have assumed that bacterioopsin adopts a trimer structure analogous to that reported for bacteriorhodopsin by Henderson and Unwin (1975), but our data would be consistent with any model that postulated intermolecular interactions between the α-helical segments of bacterioopsin molecules.

The results obtained here with dansylundecanoic acid and dansyldodecanoic acid indicate only very limited binding of these fatty acids to the lipid bilayer portion of the membrane, binding being at most 15% of that expected for an equivalent amount of a lipid such as dioleoylphosphatidylcholine in a simple lipid bilayer. The major phospholipids in the purple membrane are negatively charged phosphatidylglycerol phosphate and glycolipid sulfate (Kushawa et al., 1976; Kates et al., 1982), and the effect of the negative surface potential will be to reduce binding of the fatty acid probes. Dansylpropranolol, a tertiary amine, would be expected to bind strongly to a negatively charged membrane, and the fluorescence emission spectra of bleached membranes in the presence of dansylpropranolol show a lipid-bound component. The amount bound is, however, much less than would be expected in the presence of an equivalent concentration of purple membrane lipids and, in conjunction with the titration results, indicates that dansylpropranolol interacts with only ca. 30% of the bleached membrane lipid. The molar ratio of lipid to protein in the membrane is 10:1 (Blaurock & Stoeckenius, 1971). The total circumference of the lipid–protein interface for a trimer of bacteriorhodopsin can be estimated to be ca. 180 Å, so about 42 lipid molecules are required to form a complete annular shell around the trimer. Thus, since up to six lipids may be accommodated in the space in the center of the trimer, it is necessary that at least 85% of the remaining lipids are shared between the annular shells of two adjacent trimers. There is not likely to be any phospholipid present in a bulk phospholipid phase not in contact with protein (Glaeser et al., 1985). This should also be the case in the bleached membrane, where the circumference of the lipid–protein interface may in fact increase slightly due to the concerted tilt of the protein helical segments. In the purple membrane, adjacent bacteriorhodopsin trimers are separated by only a single layer of lipid molecules in the regions close to helices 2 and 4 [according to the numbering of Engelman et al. (1980)]; there is evidently more space available between the trimers in the regions close to helices 1 and 3 (Glaeser et al., 1985). It is possible, therefore, that it is the lipid in this region that is free to bind dansylpropranolol whereas the remaining lipid, more tightly packed between protein trimers, is not free to do so.

Fluorescence emission spectra for both dansyl fatty acids and dansylpropranolol bound to bleached membrane show components characteristic of protein-bound probe. For both dansylundecanoic acid and dansyldodecanoic acid the experimental data are best fitted by assuming three to four sites per monomer, while for dansylpropranolol, the best fit is with six to eight sites. In the structural model deduced for bacteriorhodopsin (Henderson & Unwin, 1975; Hayward & Stroud, 1981), there are six intermolecular contact regions between α-helical domains per trimer. Since the protein traverses the bilayer, the contact regions could accommodate six hydrophobic molecules on each side of the membrane, giving four potential binding sites per monomer. Since this is equal to the number of binding sites deduced for the dansyl fatty acids on bacterioopsin, it is possible that the fatty acids are binding at the protein–protein interfaces (nonannular sites). The number of binding sites estimated for dansylpropranolol is considerably greater and approaches the number (10) of phospholipids per monomer in the membrane. It seems likely, therefore, that dansylpropranolol binds at the lipid–protein interface (annular sites).

In our analysis of binding to protein sites we have assumed that probes bind only to a single class of sites on the protein. Analysis is also possible in terms of a two-site model, with 10 annular sites and 4 nonannular sites. The fluorescence titration data for the dansyl fatty acids can be fitted to such a model but predict either that the fluorescence intensity for dansyl fatty acid bound at annular sites is very low (for which there is no evidence) or, if fluorescence intensities for probe bound at annular and nonannular sites are constrained to be equal, that the affinity of the probes for the annular sites is at least 50-fold lower than that for the nonannular sites, so that over the concentration range employed, the annular sites are essentially unoccupied. The data demonstrate, therefore, that the dansyl fatty acids must bind very weakly to annular sites, in contrast to dansylpropranolol, which binds strongly at such sites. The number of sites (six to eight) occupied by dansylpropranolol could be composed of four nonannular sites and two to four annular sites, but then the number of annular sites seems rather small. Since a fit of the titration data for dansyl propranolol to 12 sites (8 annular and 4 nonannular) is significantly worse than that to 6–8 sites, it seems most likely that binding of dansylpropranolol to the nonannular sites is weaker than that to the annular sites.

The pattern of binding of the fluorescence probes to bleached membranes is identical with that proposed previously for binding to the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum, where it was suggested that dansylundecanoic acid bound to a small number of sites at protein–protein interfaces in ATPase oligomers whereas dansylpropranolol bound to a large number of sites at the lipid–protein interface (Lee et al., 1982, 1983).

The increased binding to protein of dansyldodecanoic acid as compared to dansylundecanoic acid corresponds to an increase in free energy of binding of 2.7 kJ/mol, identical with the increment per methylene group found for the partitioning of alcohols into lipid bilayers (Jain & Wray, 1978). This then demonstrates the importance of hydrophobic interactions in binding at the nonannular sites and suggests that protein–protein interactions within the trimer of bacteriorhodopsin are predominantly hydrophobic. Predictions of protein secondary structure based on the amino acid sequence of bacteriorhodopsin are still somewhat controversial but agree in placing numbers of negatively charged residues in the aqueous phase adjacent to the membrane surface (Engelman et al., 1982). It might be expected that apposition of charged regions at protein–protein contacts would be unfavorable, and indeed, in some of the preferred structural models for bacteriorhodopsin, the linking sequences in the intermolecular contact

regions contain few or no charged residues (Engelman et al., 1980; Trewhella et al., 1983). Such models would be consistent with stronger binding of positively charged molecules than of negatively charged molecules at the lipid–protein interface.

Finally, we note that spin-labeled fatty acids have been used extensively as probes for the lipid-bilayer region of biological membranes, but the results presented here suggest that such probes may also sample sites at protein–protein interfaces. Chignell and Chignell (1975) have observed that a variety of spin-labeled fatty acids give highly immobilized ESR spectra when incorporated into purple membrane, without the gradient of increasing motion toward the center of the bilayer that is typical of most membranes. Further, a break occurs in Arrhenius plots of ESR spectral parameters at 29 °C, a temperature that has been shown to correspond not to a change in the lipid component of the membrane but more likely to a minor change in protein–protein interaction (Tsuda et al., 1984). It seems likely, therefore, that spin-labeled fatty acids also bind extensively at protein–protein interfaces in the purple membrane.

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Synthesis of Prodan–Phosphatidylcholine, a New Fluorescent Probe, and Its Interactions with Pancreatic and Snake Venom Phospholipases A_2†

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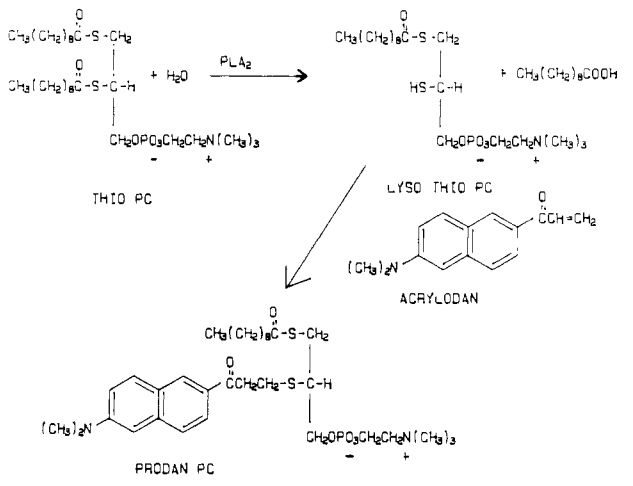
ABSTRACT: A new fluorescent probe, prodan–PC, was synthesized by incubating thio-PC, a thiol ester analogue of phosphatidylcholine [1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine], with acrylodan, a fluorescent thiol-reactive reagent [6-acryloyl-2-(dimethylamino)naphthalene], in the presence of phospholipase A_2, which served to generate lysothio-PC in situ. Prodan–PC (PPC) showed maximum absorption in ethanol at 370 nm. The fluorescence emission spectrum showed maximum emission at 530 nm in water and at 498 nm in ethanol. In the presence of a saturating amount of phospholipase A_2, the emission maximum shifted to about 470 nm. PPC showed a critical micellar concentration around 5 μM, with evidence of premicellar aggregation above 1 μM. Binding of PPC to *Crotalus adamanteus* phospholipase A_2 was evidenced by an increase in emission at 480 nm and an increase in fluorescence anisotropy. An apparent dissociation constant of 0.323 μM was calculated for this enzyme complex. Binding was dependent on the presence of calcium ion and was abolished by blocking the active site with *p*-bromophenacyl bromide. Binding was also followed by energy transfer from tryptophan in the enzyme to PPC. Apparent dissociation constants for PPC complexes with phospholipases A_2 from *Naja naja naja* and porcine pancreas and the prophospholipase A_2 from porcine pancreas were 0.509, 0.107, and 0.114 μM, respectively. PPC was shown to inhibit the activity of pancreatic phospholipase A_2 in thio-PC–sodium cholate mixed micelles. Inhibition studies were complicated because PPC can also serve as an activator of the snake venom enzymes. The unusually high affinity of PPC for phospholipase A_2 is discussed in terms of its use as a fluorescent probe and in the design of high-affinity inhibitors of the enzyme.

Several years ago we synthesized a chiral thiol ester analogue of phosphatidylcholine (thio-PC)[1] as a substrate for a continuous spectrophotometric assay of phospholipase A_2 (PLA_2) (Hendrickson et al., 1983). The availability of a very polarity-sensitive thiol-reactive fluorescent reagent, acrylodan (Prendergast et al., 1983), suggested the synthesis of a new fluorescent phosphatidylcholine analogue by treatment of thio-PC with PLA_2 in the presence of acrylodan (Scheme I). The new fluorescent probe, prodan–PC (PPC), seemed attractive for the study of phosphatidylcholine interaction with PLA_2. This probe has good absorbance, has high quantum yield, and is very sensitive to the polarity of its environment. Emission of the prodan moiety shows a dramatic shift to shorter wavelength in nonpolar environments due to charge separation in the excited state (Weber & Farris, 1979). Its absorption maximum at 370 nm is sufficiently close to the emission maximum of tryptophan (about 344 nm) so that it might be useful in energy-transfer studies with PLA_2. PLA_2 from porcine pancreas has a single tryptophan in or near the active site. PLA_2 from *Crotalus adamanteus* and *Naja naja naja* have more than two tryptophan residues, some of which may be in a hydrophobic surface area of the enzyme surrounding the active site (Verheij et al., 1981). Here we report the synthesis of prodan–PC, some of its properties, and some

Scheme I



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[1] Abbreviations: PC, phosphatidylcholine; thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; PLA_2, phospholipase A_2; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; prodan, 6-propionyl-2-(dimethylamino)naphthalene; PPC, prodan–PC, a thioether adduct of acrylodan and lysothio-PC; HPLC, high-performance liquid chromatography; cmc, critical micellar concentration; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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