Fluorescent Probe Studies of Biomembranes and Model Systems—the Design and Use of Anionic Site Reporters

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ABSTRACT
The fluorescence properties of a homologous series of fluorescent alkylamines are described. The binding of the probes to erythrocyte membranes increases with the length of the alkyl chain. The probes are shown to interact more strongly with membranes than with protein and lipid model systems. The binding of the probes to the membrane is sensitive to the cation concentration of the medium.

INTRODUCTION
The application of fluorescent probe techniques to the study of biological membranes can be extended by the use of specifically designed reporter molecules [1,2]. While anionic probes like 1-anilino-8-naphthalene sulfonate (ANS) have been used extensively, cationic probes have found relatively few applications in membrane studies. We have earlier described the use of the fluorescent alkylamine, dansyl cadaverine (V), as a probe of membrane anionic sites [3]. In this report, we present the fluorescence properties of a series of homologous probes where the distance between the fluorophore and the amino group is systematically varied.

MATERIALS AND METHODS

Synthesis of probes. The fluorescent probes were synthesised by dansylation of the appropriate alkylamine. An illustrative procedure has been described elsewhere [3]. All compounds used were characterised spectroscopically and were homogenous by chromatography.

Proteins were obtained from Sigma Chemical Co., erythrocyte membranes were prepared by the method of Dodge et al [4]; lipids were extracted by the Folch, Lees and Stanley procedure [5].

Fluorescence measurements. Spectra were recorded using a manual Perkin Elmer Model 203 Spectrofluorimeter and are uncorrected. The excitation wavelength was 340 nm. For energy transfer measurements, the excitation was 290 nm. Matched
1 cm cuvettes were used. The fluorescent probe concentration was 20 μg/ml. Protein/lipid concentrations were fixed at 200 μg/ml. Erythrocyte membrane protein concentration was determined by the procedure of Lowry, Rosenbrough, Farr and Randall [6]. Lipid dispersions were prepared by sonication. All solutions were prepared in 10mM tris-HCl buffer at pH 7.4. The ion binding experiments were carried out with a membrane preparation that had been washed repeatedly and then resuspended in double distilled water. All solutions for this experiment were prepared in double distilled water. The probe and protein concentrations were 20 μg/ml and 120 μg/ml respectively. The ion concentrations used were 10 mM NaCl and 0.5 mM CaCl₂.

RESULTS AND DISCUSSION

Fig. 1 shows the fluorescence spectra of the probes II, V and X in the presence and absence of erythrocyte membrane. Binding to the membrane is accompanied by an enhancement in the emission intensity and

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**Fig. 1.**

Uncorrected fluorescence spectra of probes II, V and X. Excitation 340 nM. Probe concentration 20μg/ml. Protein concentration 145 μg/ml. (○) II in distilled water. (x) II + erythrocyte membranes. (△) V in distilled water. (■) V + erythrocyte membranes. (●) X in distilled water. (◇) X + erythrocyte membranes.
FIG. 11

Scatchard plot for the fluorescence of V in the presence of (a) membranes in distilled water, (b) membranes in 10 mM NaCl and (c) membranes in 0.5 mM CaCl₂. X axis units and Y axis units are litre g⁻¹ and moles gm protein respectively. (Probe) is the concentration of bound probe.

The decrease of fluorescence in the presence of ions can clearly be attributed to the decrease in the values of the occupancy numbers.

TAB. 11

Occupancy numbers and dissociation constants for probe V at two sites

<table>
<thead>
<tr>
<th>Sample</th>
<th>Site A</th>
<th>Site B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n(mmol/g)</td>
<td>K_d(M)</td>
</tr>
<tr>
<td>Free probe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe + NaCl₂</td>
<td>45.075</td>
<td>123.50</td>
</tr>
<tr>
<td>Probe + CaCl₂</td>
<td>21.25</td>
<td>81.73</td>
</tr>
<tr>
<td></td>
<td>10.05</td>
<td>105.79</td>
</tr>
</tbody>
</table>

It is pertinent to note that the fluorescent alkylamines are capable of exhibiting changes in their emission intensities at cation concentrations as low as 0.1 mM for monovalent ions and 0.01 mM for divalent ions. This
a blue shift of the emission maximum, suggesting that the fluorophore has been transferred to an environment of low polarity or reduced solvent accessibility [7]. These changes are dramatically enhanced by increasing the length on the alkyl chain on the probe. TAB. I summarises the data.

**Fluorescent characteristics of Probes in different Systems**

<table>
<thead>
<tr>
<th>Protein or lipid</th>
<th>λ_max (nM)</th>
<th>PROBES</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>V</td>
<td>X</td>
</tr>
<tr>
<td>Erythrocyte membrane</td>
<td>505</td>
<td>495</td>
<td>490</td>
</tr>
<tr>
<td>Whole brain lipid</td>
<td>520</td>
<td>520</td>
<td>490</td>
</tr>
<tr>
<td>BSA</td>
<td>540</td>
<td>540</td>
<td>490</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>540</td>
<td>540</td>
<td>540</td>
</tr>
<tr>
<td>Trypsin</td>
<td>540</td>
<td>540</td>
<td>540</td>
</tr>
<tr>
<td>Lecithin</td>
<td>540</td>
<td>535</td>
<td>515</td>
</tr>
</tbody>
</table>

obtained in the presence of purified proteins, lecithin micelles and brain lipid dispersions. Probes II and V interact very weakly with dipalmitoyl lecithin and brain lipids; their interaction with proteins, including BSA, is minimal. Probe X, however, binds to BSA and lecithin. This is probably due to its greater lipid solubility.

FIG. 1a shows the Scatchard plot [8,9] for probe V binding to the membrane. Over a wide range of concentrations, the data represent one class of binding sites and give apparent dissociation constants (K_a) of 2.8 x 10^{-5} M, 2.0 x 10^{-5} M and 1.6 x 10^{-5} M for probes II, V and X respectively. Thus, the binding affinity of the probes does not increase appreciably with increasing chain length.

The changes in fluorescence result mainly from the number of sites occupied on the membrane. The occupancy numbers (n) for II, V and X are 10.7, 45 and 188 moles/gm protein, respectively. The larger number of sites available to the higher homologs could be due to enhanced lipid solubility.

FIG. 1b and c represent the Scatchard plots obtained for the binding of V to membrane in 10 mM NaCl and 0.5mM CaCl_2. In the presence of the cations, there is a deviation from linearity suggesting binding sites heterogeneity. The data can be fitted to two classes of binding sites TAB. II summarises the n and K_a values for V in buffer, 10mM NaC and 0.5mM CaCl_2.
allows cation binding to be monitored at lower concentrations than those possible with ANS [10].

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REFERENCES