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BINDING OF DANSYL PROPRANOLOL TO THE (Ca^{2+} + Mg^{2+})-ATPase

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We have studied the binding of dansyl propranolol to lipid bilayers and to the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum. The fluorescence emission spectra for dansyl propranolol bound to the ATPase system can be fitted to the sum of three peaks, characteristic of probe bound to lipid and to protein and free in solution, respectively. Titrations show that binding to the lipid component of the ATPase system is comparable to binding to simple lipid bilayers. Binding constants obtained using fluorescence spectroscopy for binding to lipid bilayers agree with constants obtained from microelectrophoresis measurements. Binding to sites on the ATPase can be described either in terms of the aqueous concentration of dansyl propranolol or in terms of the mole fraction of dansyl propranolol in the lipid phase of the membrane. Both descriptions suggest extensive binding to annular sites at the lipid/protein interface of the ATPase. Binding at other sites on the ATPase might also be present. Binding of dansyl propranolol to the ATPase results in a marked inhibition of activity. At high Ca^{2+} concentrations, inhibition fits to a non-competitive model of inhibition, described by a K_i of 5 μM. We attribute this effect to binding at annular sites. At lower Ca^{2+} concentration, a decrease is observed in the apparent affinity of the ATPase for Ca^{2+} which can be attributed to a build-up of positive charge on the membrane as a result of binding.

Introduction

Relatively little is yet known about the protein/lipid interface of membrane proteins. The nature of the surrounding or annular phospholipid is known to be an important factor in determining the activity of at least some membrane proteins: the activity of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum, for example, is markedly dependent on the chemical structure and physical state of the surrounding phospholipid [1–5]. Despite the importance of phospholipid in determining the activity of the ATPase, the ATPase shows relatively little selectivity in the binding of phospholipids differing in headgroup or fatty acyl chain length, although it does show a relatively large preference for lipid in the liquid crystalline phase over that in the gel phase [4,5]. However, we have shown that there is selectivity in the binding of a variety of other hydrophobic molecules to the phospholipid/protein interface, as shown by their relative abilities to displace brominated derivatives of phospholipids from around the ATPase. Thus, cholesterol is excluded from the annulus around the ATPase, whereas fatty acids can displace phospholipids from the phospholipid/protein interface of the ATPase, but relatively poorly, and alkyl alcohols and alkylamines can bind relatively strongly at the phospholipid/protein interface (Ref. 6, and unpublished observations). As well as annular binding sites on the ATPase, we have detected a set of non-annular sites on the ATPase from the fluorescence quenching caused by

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

brominated derivatives of cholesterol, fatty acids, amines etc. [6]. Elsewhere, we have shown that it is possible to use the environmental sensitivity of the fluorescence emission of the dansyl group in dansylundecanoic acid to quantitate these non-annular sites [7]. The fluorescence emission for dansylundecanoic acid bound to the ATPase system can be fitted to the sum of two peaks, one characteristic of dansylundecanoic acid bound to a lipid bilayer and a second peak attributed to dansylundecanoic acid bound directly to the ATPase. Analysis of titrations with dansylundecanoic acid gives three binding sites per ATPase attributed to the non-annular sites on the ATPase [7]. If the above analysis is correct, a very different pattern of binding should be observed for dansyl derivatives of amines, since our previous studies suggest extensive binding of amines at annular sites. Simple dansyl derivatives of amines such as dansyldecylamine cannot readily be used in these studies since only small shifts in fluorescence emission occur when such compounds bind to membrane systems, presumably because they bind with the dansyl group located close to the phospholipid/water interface (Burden, E.A. and Lee, A.G., unpublished observations). Instead, we have studied the binding of 1-[[2-(5-dimethylaminonaphthal-ene-1-sulphonyl)aminoethyl]amino]-3-1-naphthaleneoxy-2-propanol, referred to as dansy propranolol. This was introduced as a fluorescent analogue of a β-blocker [8] but probably shows extensive, nonspecific binding to membranes (see Ref. 9).

Materials and Methods

Lipids were obtained from Lipid Products and dansyl propranolol from Calbiochem. (Ca^{2+}+ Mg^{2+})-ATPase was prepared from female rabbit (New Zealand White) hind leg muscle as described in East and Lee [5]. Polyacrylamide gels showed the presence of essentially pure ATPase (over 95%), and the protein/phospholipid molar ratio was 1:30. ATPase activity was measured as described in the previous paper [10] with the free Ca^{2+} concentration varied with EGTA and $CaCl_2$. Protein was estimated using the extinction coefficient given by Hardwicke and Green [11]. Fluorescence spectra were recorded on a Perkin-Elmer MPF 44A fluorimeter and digitised with a 12-bit A/D converter before storage in a Z-80 based microcomputer system for smoothing according to the method of Savitsky and Golay [12,13]. Dansyl propranolol was added as a methanol solution, the final methanol concentration never exceeding 1%. Methanol at this concentration had no effect on ATPase activity. The buffer for fluorescence measurements was 40 mM Hepes/0.1 M NaCl/0.1 mM EDTA (pH 7.2).

Fluorescence emission spectra were fitted to a sum of skewed Gaussian peaks of the form:

$$A = A_0 \exp\left[-\ln 2 \left\{ \frac{\ln\left(1 + 2b(X - X_0)/\Delta X_{1/2}\right)}{b} \right\}^2 \right]$$

in the limit

$$2b(X - X_0)/\Delta X_{1/2} > -1$$

and for

$$2b(X - X_0)/\Delta X_{1/2} \leqslant -1, A = 0$$

Here A_0 is the maximum peak height, X_0 is the wavelength at the peak maximum, $\Delta X_{1/2}$ is the peak width at half-height and b is the skew parameter [7].

Measurements of electrophoretic mobility were made on a Rank Bros. Mark 1 microelectrophoresis apparatus. Care was taken to focus at the stationary layer. The buffer was 10 mM sodium phosphate/10 mM NaCl/0.1 mM EDTA. Zeta potentials were calculated from the measured electrophoretic mobilities by the Helmholtz-Smoluchowski equation, and the zeta potentials were used to calculate binding constants for dansyl propranolol exactly as for other amine derivatives, as described in the previous paper [10].

For fluorescence titrations of dansyl propranolol with liposomes of dioleoylphosphatidylcholine, fluorescence intensities were measured for increasing concentrations of dansyl propranolol, sufficient time (approx. 2 min) being taken before each measurement for equilibrium to be attained. The fluorescence data were fitted to the equation:

$$F = \alpha([A]_{total} - [A]_{bnd}) + \beta[A]_{bnd} \quad (1)$$

where α and β are coefficients describing the fluorescence intensity of probe in aqueous solution and bound to the membrane, respectively. Under conditions where the effects of charge can be ignored, $[A]_{bnd}$ is given by the simple binding equation:

$$[A]_{bnd} = \left\{C - \left(C^2 - 4nE_0[A]_{total}\right)^{1/2}\right\}/2 \quad (2)$$

with

$$C = K_d + nE_0 + [A]_{total} \quad (3)$$

where K_d is the dissociation constant for binding, n is the number of binding sites and E_0 is the phospholipid concentration.

Binding of dansyl propranolol to sites on the ATPase can be analysed in a conventional manner, except that it is necessary to take account of the fact that some dansyl propranolol will be bound to the lipid component of the system. For a single class of protein-binding site, we can write:

$$K_d^{lipid} = \frac{\left([lipid] - [A]_{bnd}^{lip}\right)\left([A]_{total} - [A]_{bnd}^{lip} - [A]_{bnd}^{prot}\right)}{[A]_{bnd}^{lip}} \quad (4)$$

and

$$K_d^{prot} = \frac{\left([protein] - [A]_{bnd}^{prot}\right)\left([A]_{total} - [A]_{bnd}^{lip} - [A]_{bnd}^{prot}\right)}{[A]_{bnd}^{prot}} \quad (5)$$

where square brackets represent concentrations (mol/l), and the superscripts lip and prot refer to the concentrations of dansyl propranolol bound, respectively, to phospholipid and protein sites. Under conditions where only a small fraction of the lipid sites are occupied, these equations can be solved in a closed form to give:

$$[A]_{bnd}^{prot} = \left\{-B - \sqrt{B^2 - 4AC}\right\}/2A \quad (6)$$

where

$$B = -K_d^{prot} - [protein]\left\{1 - [lipid]/\left(K_d^{lip} + [lipid]\right)\right\}$$

$$-[A]_{total}\left\{1 - [lipid]/\left(K_d^{lip} + [lipid]\right)\right\}$$

$$A = 1 - [lipid]/\left(K_d^{lip} + [lipid]\right)$$

and

$$C = [protein][A]_{total}\left\{1 - [lipid]/\left(K_d^{lip} + [lipid]\right)\right\}$$

In the case of two classes of protein-binding site, equations of the form of (5) have to be written for each site, and a closed solution of the problem is no longer possible. The binding equations were then solved by the Bolzano method [14], and the fluorescence data for the protein-bound component were fitted to the equation:

$$F = \alpha\left([A]_{total} - [A]_{bnd}^{prot1} - [A]_{bnd}^{prot2} - [A]_{bnd}^{lip}\right)$$

$$+ \beta_1[A]_{bnd}^{prot1} + \beta_2[A]_{bnd}^{prot2} \quad (7)$$

where the superscripts refer to dansyl propranolol bound to lipid sites and to the first and second class of protein binding site, respectively, and β_1 and β_2 are coefficients describing the fluorescence intensity of dansyl propranolol bound to sites 1 and 2, respectively. Fluorescence data were fitted to these equations by a derivative-free, non-linear least-squares technique [15]. The ratio of the square root of the residual sum of squares to the mean fluorescence value was taken as a measure of the coefficient of variation.

The above analysis is the obvious one for binding to sites on the ATPase exposed to the aqueous medium. If, however, binding sites on the ATPase are located at the lipid/protein interface or are otherwise within the hydrophobic interior of the membrane, then it may be more appropriate to describe binding in terms of concentrations within the membranes rather than in terms of concentrations in the aqueous phase. Thus in previous studies we have described the binding of fatty acids to the ATPase in terms of the mole fraction of the fatty acid within the lipid phase of the membrane [6], and binding of phospholipids at the lipid/protein interface has been described in terms of binding constants relative to that for dioleoylphosphatidylcholine [5].

Binding at the lipid-protein interface (annular sites) will result in displacement of phospholipid by dansyl propranolol, so that we can write for a single site on the ATPase,

$$A + lipid \cdot protein \rightarrow A \cdot protein + lipid \quad (8)$$

where A represents dansyl propranolol. The relative binding constant for dansyl propranolol and lipid is then:

$$K_{A/lipid} = \frac{[A \cdot protein][lipid]}{[A][lipid \cdot protein]} \quad (9)$$

The natural concentration unit for the above expression is mole fraction, where for example, the mole fraction, x_A, of A is:

$$x_A = \frac{[A]_{free}}{[A]_{free} + [lipid]_{free}} \quad (10)$$

where concentrations of A_{free} and $lipid_{free}$ are expressed in units of mole/litre and the subscripts free refer to concentrations of A bound to lipid and of lipid unbound to the ATPase, respectively. For a 1:1 displacement, the divisors in expressions for the mole fraction cancel, so that in Eqn. 9, concentrations become mol/l.

The concentration of lipid bound to annular sites on the protein can be expressed in terms of the bound concentration of dansyl propranolol:

$$[lipid \cdot protein] = N[protein] - [A \cdot protein] \quad (11)$$

where [protein] is the protein concentration and N is the number of annular binding sites per ATPase. This expression assumes that all annular sites are occupied, either by lipid or by dansyl propranolol. Similarly, the concentration of lipid not bound to the ATPase can be written as:

$$[lipid] = [lipid]_{total} - (N[protein] - [A \cdot protein]) \quad (12)$$

where $[lipid]_{total}$ is equal to the total lipid concentration.

Substitution of Eqns. 11 and 12 into Eqn. 9 gives a quadratic for [A · protein], the concentration of dansyl propranolol bound to annular sites on the ATPase. Binding to other, non-annular binding sites on the ATPase can be described using a conventional binding equation except that concentrations are expressed in terms of mole fraction of dansyl propranolol bound to the lipid phase of the membrane. Thus, by analogy to Eqn. 5, we have:

$$K_d^{prot} = \frac{([protein] - [A]_{bnd}^{prot})\, x_A^{lipid}}{[A]_{bnd}^{prot}} \quad (13)$$

where [protein] and $[A]_{bnd}^{prot}$ are expressed, for convenience, in mol/l (this is possible since divisors in expressions for mole fractions of sites cancel, as described above) and x_A^{lipid} is the mole fraction of dansyl propranolol free in the lipid phase, given by Eqn. 10.

The above equations can also be solved by the Bolzano method [14] and the fluorescence data again fitted to Eqn. 7 where $[A]_{bnd}^{prot1}$ and $[A]_{bnd}^{prot2}$ represent, respectively, the concentration of dansyl propranolol bound to annular and non-annular sites.

For samples excited at 280 nm, the total optical density due to absorption by both protein tryptophan groups and dansyl propranolol exceeds approx. 0.1, beyond which the relationship between fluorescence intensity and concentration becomes non-linear. In the Perkin-Elmer fluorimeter, fluorescence emission from a vertical segment at the centre of the sample is focussed by a lens system onto the entrance slit of the emission monochromator. With this geometry, it is readily shown that the mean intensity of light absorbed by the observed segment is closely approximated by the intensity of light absorbed at the centre of the segment. Fluorescence intensities were therefore corrected according to the relationship:

$$F^{corr} = F\exp(-D^{total}x) \quad (14)$$

where D^{total} is the total optical density (optical absorbance) of the sample at 280 nm and x is the distance to the centre of the sample cuvette (0.5 cm). For the ATPase we used the extinction coefficient of Hardwicke and Green [11] and for dansyl propranolol we determined an extinction coefficient of 11 000 $M^{-1} \cdot cm^{-1}$.

Results

Binding to lipid bilayers

The binding of dansyl propranolol to liposomes of egg yolk phosphatidylcholine produces a positive surface charge on the liposomes. Fig. 1 shows the measured variation of zeta potential with concentration of dansyl propranolol at a fixed pH of 7.2, and as a function of pH at a concentration of 150 μM. Taking the hydrodynamic plane of shear of the liposomes to be 2 Å from the surface [10,16,17], we can calculate the corresponding surface potential, ψ_0, which can in turn be related to the number of positively charged molecules per unit area of membrane [10]. The variation in concentration of bound dansyl propranolol as a function of the total concentration of dansyl pro-

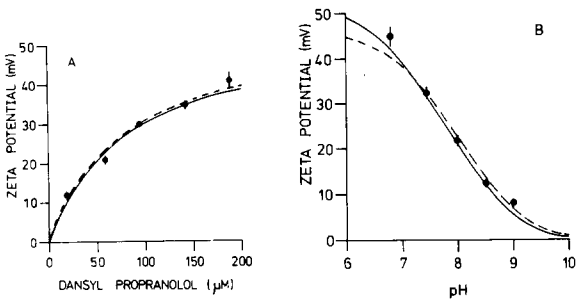

Fig. 1. Zeta potentials of liposomes of egg phosphatidylcholine (0.67 mM) in buffer (10 mM Tris-HCl/10 mM NaCl/0.1 mM EDTA) at 25°C as a function of (A) concentration of dansyl propranolol at pH 7.2 and (B) pH in the presence of 150 μM dansyl propranolol. Symbols represent potentials (mean ± S.D.) calculated from at least ten electrophoretic mobility measurements [10]. Curves represent the predicted zeta potentials calculated using the following parameters: $K^A = 1 \cdot 10^{-6}$, $\sigma^{max} = 1/90$ Å^2 and $pK = 9.1$, $\Delta pK = -1.5$ (solid line), $pK = 9.5$, $\Delta pK = -1.8$ (dashed line). In all cases, binding of Cl^- to the protonated form of the bound probe was assumed, with a K_d of 0.5 M [10].

pranolol gives us the binding constant as described in the previous paper [10]. To fit the experimental data, we need to know the pK of dansyl propranolol. By comparison with other related compounds of known pK, and on the basis of approximate calculations using Taft constants, we estimate this to be 9.1 (a higher value up to 9.5 also gives a good fit to the experimental data, although with a less reasonable, larger, ΔpK value – see later). The areas occupied in the surface by molecules of lipid and dansyl propranolol have been taken to be 60 and 30 Å^2, respectively: the fit is relatively insensitive to the value taken for the latter parameter. The variables remaining are then the dissociation constant for binding, K^A, the shift in pK on binding, ΔpK, and the maximum number of drug molecules that can bind per Å^2, σ^{max}. As for other amines such as the antihistamine methdilazine [10], Cl^- binding to the protonated form of the adsorbed probe was accounted for using a dissociation constant of 0.5 M. As found previously, it is not possible to determine K^A and σ^{max} independently, as equally good fits are obtained over a range of values for which σ^{max}/K^A is kept approximately constant [10,16]. Fig. 1 shows a fit to the data with $\sigma^{max} = 1/90$ Å^2, corresponding to a maximum binding stoichiometry of one drug molecule per lipid molecule, with the parameters listed in Table I. The ΔpK value of -1.5 is typical of the binding of a wide range of drug molecules to lipid bilayers [10]. Equally good fits to the data can be obtained with values for σ^{max} of 1/30 and 1/60, corresponding, respectively, to unlimited drug binding and a stoichiometry of 2:1 drug:lipid.

Binding of dansyl propranolol to lipid bilayers can also be studied using the increase in the intensity of fluorescence emission for this molecule that follows from binding. Fig. 2 shows a fluorescence titration of dioleoylphosphatidylcholine with dansyl propranolol in solutions containing 0.1 M NaCl. Using the binding constants derived from the electrophoresis data, we estimate that under the conditions of the fluorescence experiment the surface potential on the liposomes that results from binding does not exceed 10 mV, so that under these conditions the effects of charge can be ignored. Before attempting to fit the fluorescence data to a binding equation, it is necessary to establish that the fluorescence enhancements on binding to the bilayer are equal for the charged and the neutral forms of dansyl propranolol. It can be seen from the fluorescence experiments (Fig. 2) and calculated from the electrophoresis data (Table I) that binding will be essentially complete in the presence of 400 μM lipid for concentrations of dansyl propranolol up to approx. 6 μM. Under these conditions we find that fluorescence intensities are identical at pH values of 7.2 and 9.8. Since at pH 7.2, from the electrophoresis data, we calculate that approx. 60% of the

TABLE I

BINDING CONSTANTS FOR DANSYL PROPRANOLOL TO EGG PHOSPHATIDYLCHOLINE CALCULATED FROM ELECTROPHORESIS DATA

Binding of Cl^- to the protonated form of the adsorbed dansyl propranolol was accounted for with a K_d of 0.5 M. Temperature: 25°C.

σ^{max}(Å^{-2})	ΔpK	K^A (μM)
1/30	−1.5	2.0
1/60	−1.5	1.5
1/90	−1.5	1.0

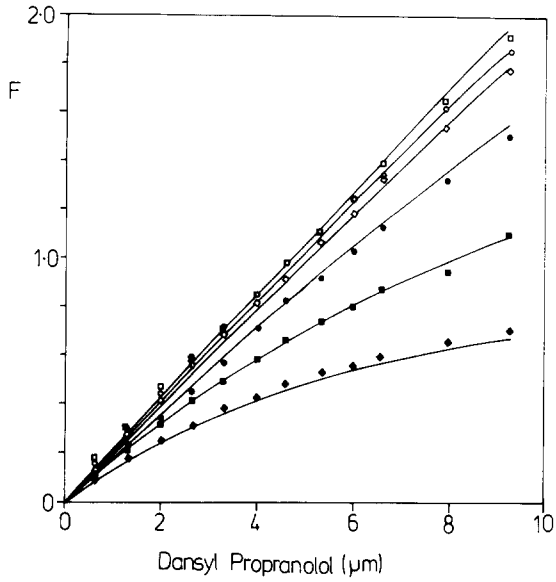

Fig. 2. Fluorescence titration for dansyl propranolol with dioleoyl phosphatidylcholine at 23°C in buffer (40 mM Hepes/0.1 M NaCl/0.1 mM EGTA (pH 7.2)) exciting fluorescence at 280 nm and measuring emission at 527 nm. Concentration of lipid (μM); ○, 1530; □, 230; ◇, 153; ● 76.5; ■ 38.3; ◆ 19.1.

bound dansyl propranolol will be in the charged form, whereas at pH 9.8 the percentage will be less than 1, we conclude that both forms have identical fluorescence quantum yields when bound to the membrane. The fluorescence data can therefore be simply fitted to the binding equations, Eqns. 1–3. The value of α describing the fluorescence intensity of dansyl propranolol in buffer was obtained from a linear least-squares fit of the fluorescence intensity in buffer versus concentration. Using this value of α, the results of a non-linear least squares fit at 23°C are shown in Fig. 2, and the resulting parameters are given in Table II. Clearly, the fit is good with a coefficient of variation of 0.35. Unfortunately, as found previously for similar titrations [17], the values for the number of binding sites n and the dissociation constant K_d are highly correlated, with an estimated asymptotic correlation of 0.94. It is therefore possible to fix n over a wide range of values and obtain equally good fits.

It is readily shown that the fluorescence and electrophoresis data are in agreement. Taking $\sigma^{max} = 1/90$ Å^2, corresponding to one dansyl propranolol binding site per lipid, the binding constants obtained from electrophoresis measurements (Table I) can be used to calculate an effective total binding constant under conditions where charge effects can be ignored:

$$K_{eff} = [\text{lipid}]([A]_{total} - [A]_{bnd})/[A]_{bnd}$$

Over the concentration range used in the fluorescence experiment, the calculated K_{eff} varies from 3 to 45 μM. This agrees well with the dissociation constant, K_d, of 33 μM obtained from the fluorescence data for $n = 1$. It is important to note that, although it is not possible to determine independently K_d and n from either set of experiments, the binding parameters that have been determined allow an accurate description of binding to lipid bilayers over the concentration ranges used in these experiments.

Binding to (Ca^{2+} + Mg^{2+})-ATPase

We have found that the fluorescence emission spectra of a variety of dansyl derivatives are sensitive to environment, with fluorescence emission

TABLE II

PARAMETERS OBTAINED FROM FLUORESCENCE TITRATIONS FOR DANSYL PROPRANOLOL AND DIOLEOYL PHOSPHATIDYLCHOLINE

Buffer was 40 mM Hepes/0.1 M NaCl/0.1 mM EGTA (pH 7.2). α is the fluorescence intensity of 1 μM dansyl propranolol in buffer. β is the fluorescence intensity of 1 μM dansyl propranolol when fully bound to dioleoyl phosphatidylcholine.

α	β	Number of sites per lipid, n	Dissociation constant K_d (μM)	Coefficient variation of fit
0.0138	0.214 ± 0.002	0.195 ± 0.013	2.35 ± 0.36	0.35
0.0138	0.240 ± 0.002	$n = 1$, fixed	32.7 ± 1.46	0.44

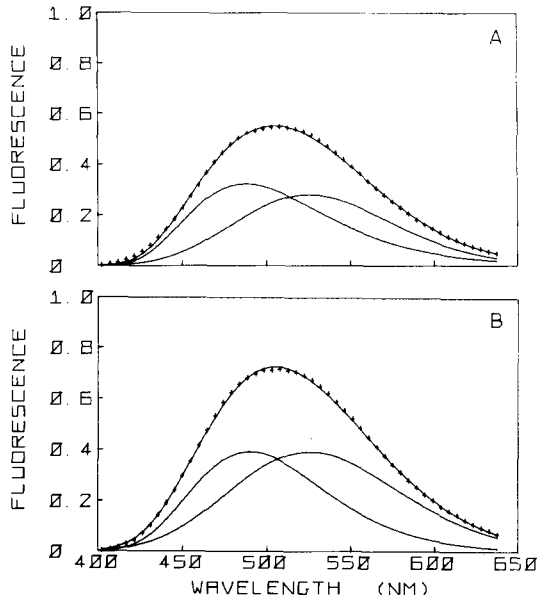

Fig. 3. Fluorescence emission spectra for dansyl propranolol (7.3 μM) bound to (Ca^{2+} + Mg^{2+})-ATPase (0.25 μM) (A) excited at 280 nm and (B) excited at 347 nm +, experimental points. Solid lines: best fit to the experimental data, and the two components of the fit, with the skew parameters, peak widths and peak positions given in Table III.

maxima shifting to shorter wavelength with decreasing solvent polarity [7]. In organic solvents the shapes of the emission spectra can be accurately described as skewed Gaussians, with the value of the skew parameter, b, increasing as the emission maximum shifts to shorter wavelength. When dansylundecanoic acid binds to the ATPase-lipid system, the fluorescence emission is composed of three components, two due to probe in buffer and bound to the lipid component of the membrane, respectively, and the third attributed to probe bound to the ATPase [7].

Addition of (Ca^{2+} + Mg^{2+})-ATPase to dansyl propranolol results in an enhancement of fluorescence with emission shifted slightly to shorter wavelength compared to the spectrum for dansyl propranolol bound to lipid alone (Fig. 3, Table III). The shift to shorter wavelength is more marked at an excitation wavelength of 280 nm than at 340 nm. The former excites the fluorescence of dansyl propranolol both directly and via energy transfer from the protein tryptophan groups, whereas the latter excites fluorescence only directly. We conclude from the dependence of the shape of the emission spectra on excitation wavelength that more than one component is present. Our procedure for fitting these spectra was then as follows.

Firstly, we fitted the spectrum of dansyl propranolol in buffer to a single skewed Gaussian peak, with the parameters given in Table III. Secondly, we also fitted the spectrum of dansyl propranolol fully bound to dioleoylphosphatidylcho-

TABLE III

FLUORESCENCE PROPERTIES FOR DANSYL PROPRANOLOL (7.3 μM) FROM LEAST SQUARES FITS OF SPECTRA

System	Component	Intensity	Peak position (nm)	Band width (nm)	Skew factor	S.D. of fit
Buffer	water	–	552.5	130.7	0.15	0.03
Dioleoylphosphatidylcholine (1.5 mM)	lipid	–	528.2	111.0	0.15	0.01
(Ca^{2+} + Mg^{2+})-ATPase excited at 280 nm (0.25 μM)	protein lipid	1.29 1.12	492.7 528.2	93.1 110.0	0.40 0.15	0.02
(Ca^{2+} + Mg^{2+})-ATPase excited at 347 nm (0.25 μM)	protein lipid	1.57 1.57	492.7 528.2	93.1 119.0	0.40 0.15	0.02
Sarcoplasmic reticulum (0.25 μM in protein) excited at 280 nm	protein lipid	1.20 1.52	492.7 528.2	93.1 115.2	0.40 0.15	0.02
Sarcoplasmic reticulum (0.25 μM in protein) excited at 347 nm	protein lipid	0.87 3.00	492.7 528.2	93.1 113.3	0.40 0.15	0.02

line (1.5 mM) to a single skewed Gaussian peak. Spectra for dansyl propranolol in the presence of lower concentrations of lipid could be fitted to the sum of two peaks, with peak widths, position and skew characteristic of lipid and aqueous environments, respectively. However, since the fluorescence from probe in the aqueous phase was relatively low, it was found to be possible to fit such spectra to a single peak centred at 528.2 nm and of variable width (Fig. 4), the peak therefore representing probe both bound to lipid and free in the aqueous phase. Spectra for dansyl propranolol in the presence of the ATPase were then fitted to the sum of two components, one with peak position and skew characteristic of probe in the lipid/buffer system and the second assumed to represent probe bound directly to the ATPase (Fig. 3, Table III). Upon exciting fluorescence at 280 nm, the intensity of the component assigned to protein-bound probe increases relative to that assigned to lipid-bound probe, consistent with our assignment: fluorescence energy transfer is strongly dependent on distance and so would be expected to be more efficient for probe bound directly to the ATPase than for probe bound to the lipid component of the system. The difference between excitation at 280 nm and 340 nm is smaller for dansyl propranolol than that previously observed for dansyl-undecanoic acid [7] because of the high absorbance of the propranolol moiety at 280 nm. Also consistent with our assignment is the observed smaller relative intensity of the 492.7 nm

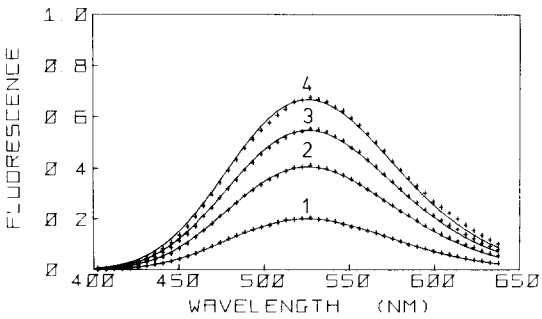

Fig. 4. Fluorescence emission spectra for dansyl propranolol in the presence of dioleoylphosphatidylcholine (19 μM) concentration of dansyl propranolol (μM); 1, 1.32; 2, 3.3; 3, 5.3; 4, 7.3 +, experimental points. Solid lines, non-linear least-squares best fits to the data for a single gaussian peak of width varying from 112 nm (spectrum I) to 115 nm (spectrum 4).

component for dansyl propranolol bound to sarcoplasmic reticulum (Table III). Although approx. 80% of the protein in sarcoplasmic reticulum is $(Ca^{2+} + Mg^{2+})$-ATPase, the lipid protein ratio of 90:1 compares to a ratio of 30:1 in our purified ATPase system: a greater relative binding to the lipid component would therefore be expected in sarcoplasmic reticulum.

It was not generally possible to obtain a consistent fit of these spectra to the sum of three peaks, with the peak widths, position and skew of two of the peaks fixed at values characteristics of probe bound to lipid and free in buffer, respectively, presumably because of the relatively small separation between the fluorescence emission spectra for probe in lipid and in buffer (Table III) and because of the low fluorescence intensity for probe in buffer. It was for this reason that the procedure outlined above was adopted, in which a single peak was used to represent probe in both lipid and buffer: experiments with liposomes showed that over the relevant concentration ranges this procedure was justified (Fig. 4). Importantly, good fits to the spectra were obtained with the same peak parameters for the peak assigned to protein-bound probe, throughout the course of fluorescence titrations with dansyl propranolol, exciting fluorescence at either 280 nm or 347 nm. The fluorescence intensities obtained from such fits for the peak attributed to protein-bound probe are plotted as a function of the concentration of dansyl propranolol in Figs. 5 and 6.

The data can be analysed either in terms of aqueous concentrations of dansyl propranolol or in terms of the concentration in the lipid component of the membrane. Figs. 5 and 6 show an analysis in terms of aqueous concentrations. It is first necessary to account for the appreciable binding of dansyl propranolol to the lipid component of the system. Assuming that binding to the lipid component is described by the same binding parameters as describe binding to dioleoylphosphatidylcholine (see below), binding will follow Eqn. 6 if there is a single class of protein binding site. Fits to this equation are relatively poor, particularly for fluorescence excited at 280 nm (Table IV), but it is clear that there must be a relatively large number of binding sites in the system. Comparison of observed and calculated fluorescence

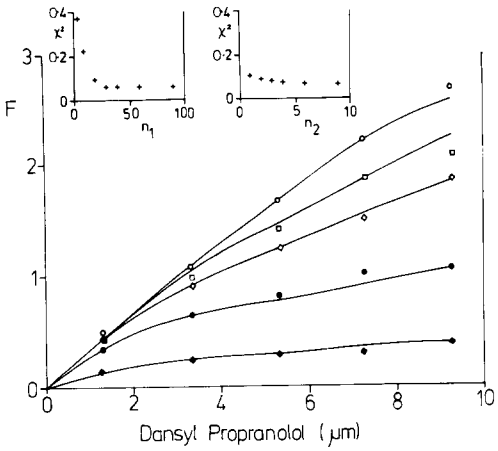

Fig. 5. Fluorescence titration for dansyl propranolol with $(Ca^{2+} + Mg^{2+})$-ATPase at 23°C, exciting fluorescence at 347 nm, plotting the height of the peak attributed to protein-bound probe obtained by non-linear least-squares fitting of spectra. Buffer: 40 mM Hepes/0.1 M NaCl/0.1 mM EDTA (pH 7.2). Concentration of ATPase (μM): ○, 0.75; □, 0.50; ◇, 0.35; ●, 0.15; ◆, 0.05. Points, experimental. Solid lines, non-linear least-squares best fit to the data in terms of aqueous concentrations (see text). Inserts, variation of residual sum of squares of fit (X^2) with numbers of binding sites n_1 and n_2.

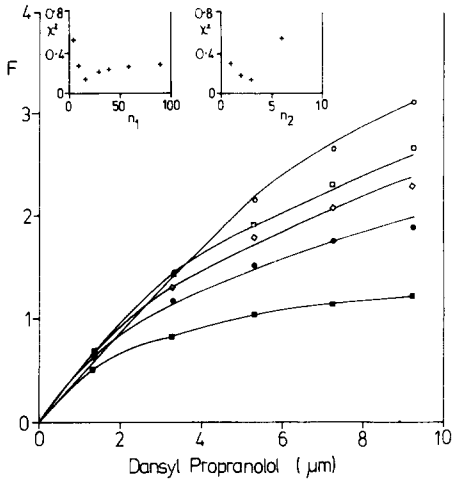

Fig. 6. Fluorescence titration for dansyl propranolol with $(Ca^{2+} + Mg^{2+})$-ATPase at 23°C, exciting fluorescence at 280 nm, plotting the height of the peak attributed to protein-bound probe obtained by non-linear least squares fitting of the spectra. Buffer as for Fig. 5. Concentration of ATPase (μM); ○, 2.0; □, 1.0; ◇, 0.75; ●, 0.50; ■, 0.25. Points are experimental. Solid lines, non-linear least-squares best fit to the data in terms of aqueous concentrations (see text). Inserts, variation of residual sum of squares of fit (X^2) with number of binding sites, n_1 and n_2.

intensities show that the fit is particularly bad at low concentrations of dansyl propranolol, where the calculated intensities are consistently lower than the observed values. This suggests the presence of two classes of protein site, one of high affinity giving rise to the relatively high fluorescence intensity observed at low concentrations of dansyl propranolol. In fitting the data to a two-site model, it is reasonable to assume that for spectra excited directly at 347 nm, the fluorescence en-

TABLE IV

BINDING PARAMETERS FOR DANSYL PROPRANOLOL TO $(Ca^{2+} + Mg^{2+})$-ATPase ANALYSED IN TERMS OF AQUEOUS CONCENTRATIONS

The dissociation constant of site 2 has been fixed at a low value, 0.002 μM.

Site 1			Site 2		Residual sum of squares X^2	Coefficient of variation
Fluorescence enhancement β_1	Dissociation constant (μM) K_d	number of sites n_1	Fluorescence enhancement β_2	number of sites n_2		
Excitation wavelength 347 nm						
0.41 ± 0.05	2.54 ± 1.37	22.41 ± 2.74			0.122	0.280
0.34 ± 0.02	7.89 ± 5.12	37.17 ± 15.9	β_1	6.01 ± 1.11	0.072	0.215
0.34 ± 0.02	5.63 ± 0.76	fixed, 30	β_1	5.70 ± 1.11	0.074	0.218
0.37 ± 0.02	4.68 ± 2.29	27.98 ± 6.42	β_1	fixed, 3	0.084	0.232
Excitation wavelength 280 nm						
0.43 ± 0.04	1.00 ± 0.66	12.29 ± 1.39			0.913	0.650
0.25 ± 0.04	9.78 ± 0.59	16.80 ± 1.7	0.57 ± 0.02	2.95 ± 0.27	0.155	0.268
0.28 ± 0.06	4.81 ± 2.43	fixed, 30	0.55 ± 0.02	3.17 ± 0.27	0.222	0.320

hancements on binding are equal for the two classes of site, since fluorescence intensities of dansyl derivatives are correlated with emission wavelengths [7] and the fluorescence emission spectra of the two classes of protein site must be very similar. However, for spectra excited at 280 nm where energy transfer from tryptophan is important, it is not safe to assume that the efficiency of transfer will be equal to the two sites. Indeed, previous fluorescence quenching results [6] have suggested that quenching of tryptophan residues in the ATPase is more efficient from non-annular sites than from annular sites. In this case, therefore, different fluorescence enhancements need to be allowed for the two classes of site. Best fits to the two site model (Eqn. 7) are shown in Fig. 5 for excitation at 347 nm and the best fit with correction for optical density effects (Eqn. 14) to the data for excitation at 280 nm is shown in Fig. 6 with the parameters listed in Table IV. It is clear that the two-site model is well able to fit the experimental data.

Because of inaccuracies in fitting the experimental spectra at low concentrations of dansyl propranolol, it is not possible to obtain data at sufficiently low concentration to define accurately the affinity of the high-affinity site in the two-site model. The dissociation constant for binding to the high-affinity site has therefore been fixed at a value (0.002 μM) such that binding to this site will be complete at the lowest concentration of dansyl propranolol employed in the titrations (Figs. 5, 6). Increasing the dissociation constant to 0.01 μM has no significant effect on the fits. For spectra excited at 280 nm, the improvement in the quality of fit between the single-site model and the two-site model is particularly marked. Since under these conditions the fitted value for the fluorescence enhancement of the high-affinity site is greater than that for the low-affinity site, it is likely that these conditions serve best to define the number of high-affinity sites. The number (three) is equal to the number found previously for the binding of dansyl undecanoic acid [7]. As shown in Fig. 6, the number of high affinity sites is reasonably well-defined and is certainly less than six and more than one. The number of low-affinity sites (n_2) is, however, much less well-defined, although clearly greater than five. The number of low-affinity sites should be better defined by the data from excitation at 347 nm, since then the fluorescence enhancements of the two sites are postulated equal. As shown in Fig. 5, under these conditions the number of high-affinity sites is poorly defined, but the number of low-affinity sites is greater than 20.

The data can also be described in terms of the concentration of dansyl propranolol in the lipid phase using the lipid-displacement model of Eqns. 8–13. Because the derivation of the equations assumes that all the annular sites are occupied either by phospholipid or by dansyl propranolol, the number of annular sites is limited to being less than the total number of phospholipids per ATPase (i.e., 30). Fluorescence intensities excited at 347 nm fit well to a single class of site, 29.9 in number with a relative binding affinity of approx. 4 (Table V). Introduction of a second site does not improve the quality of the fit. Fluorescence intensities at 280 nm excitation also fit reasonably well to a single class of site, with a relative binding constant of 0.7. As before, however, fluorescence intensities at low concentrations of dansyl propranolol tend to be underestimated, and a better fit is obtained in terms of two classes of binding site. The improvement in fit is, however, less than that observed for the data analysed in terms of aqueous concentrations. The two-site model fits to one class of site of high affinity and a second site of lower affinity, with a relative binding constant of approx. 4–10 (Table V).

Measured intensities for the fluorescence of the protein bound component are identical at pH 7.2 and 8.5, so that binding is not appreciably pH-dependent in this range.

In the above analysis of binding to protein sites, it has been assumed that binding to the lipid component of the ATPase-lipid system can be described by the same parameters as are used to describe binding to simple lipid bilayers. This assumption is readily tested. As described, fluorescence emission spectra are fitted to the sum of two peaks, one attributed to dansyl propranolol bound to sites on the ATPase and a second composite peak characteristic of dansyl propranolol in buffer and bound to the lipid component of the membrane. In Fig. 7, the intensity of this second peak at 527 nm, as obtained by fitting spectra at 347 nm excitation, is plotted as a function of the

TABLE V

BINDING PARAMETERS FOR DANSYL PROPRANOLOL TO (Ca^{2+} + Mg^{2+})-ATPase ANALYSED IN TERMS OF LIPID DISPLACEMENT

Annular sites			Non-annular sites			Residual sum of squares X^2	Coefficient of variation
Fluorescence enhancement β_1	Relative binding constant K	number of sites n_1	Fluorescence enhancement β_2	Dissociation constant (mole fraction units) K_d^2	number of sites n_2		
Excitation wavelength 347 nm							
0.28 ± 0.01	4.11 ± 0.43	29.9 [a]	–	–	–	0.119	0.234
Excitation wavelength 280 nm							
0.63 ± 0.02	0.69 ± 0.10	29.9 [a]	–	–	–	0.274	0.356
0.23 ± 0.01	10.1 ± 4.6	25.5 ± 2.5	0.82 ± 0.29	0.0005 ± 0.0005	2.2 ± 1.0	0.106	0.223
0.28 ± 0.01	4.1 ± 0.5	fixed = 29.9	0.78 ± 0.24	0.0001 ± 0.0001	1.9 ± 0.8	0.115	0.230

[a] Maximum allowed value for n_1 is 29.9.

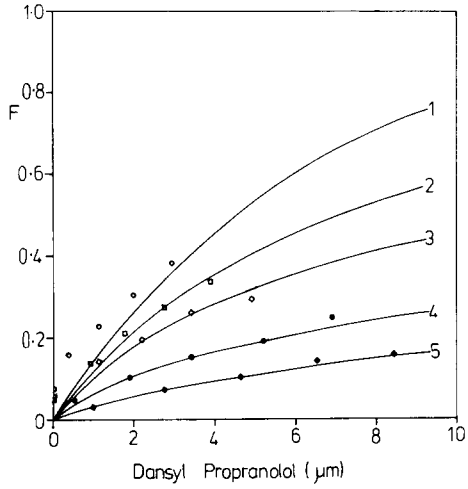

Fig. 7. Fluorescence intensity at 527 nm for dansyl propranolol bound to the lipid component of the ATPase system plotted as a function of the concentration of available dansyl propranolol, unbound to the protein sites. Concentrations of dansyl propranolol bound to protein sites were calculated from the two-site model (see text) with $n_1 = 3.0$, $K_d^1 = 0.002$, $n_2 = 30.0$, $K_d^2 = 4.7$. Experimental points for ATPase concentrations and equivalent lipid concentrations (μM) for a 30 : 1 lipid : protein ratio of: ○, 0.75 and 22.6; □, 0.50 and 15.1; ◇, 0.35 and 10.5; ●, 0.15 and 4.5; ◆, 0.05 and 1.5. The solid lines represent fluorescence intensities of dansyl propranolol bound to dioleoylphosphatidylcholine calculated using the parameters given in Table II, for concentrations (μM) of dioleoylphosphatidylcholine of: 1, 22.6; 2, 15.1; 3, 10.5; 4, 4.5; 5, 1.5.

'available' concentration of dansyl propranolol. The concentration of dansyl propranolol available for binding to the lipid is defined as the total concentration minus that bound to protein sites. The concentration bound to protein sites was calculated for the two-site model with the following parameters: $n_1 = 3.0$, $K_d^1 = 0.02$ μM, $n_2 = 30.0$, $K_d^2 = 4.7$ μM. These intensities are compared to the intensities expected for binding to an equivalent amount of pure lipid, calculated using the parameters derived for bilayers of dioleoylphosphatidylcholine (Table II). The close agreement suggests that binding to the lipid component of the ATPase-lipid system is indeed comparable to binding to a simple lipid bilayer.

The effect of dansyl propranolol on the activity of the ATPase is shown in Fig. 8 at a Ca^{2+} concentration that shows maximum enzyme activity [10]. Dansyl propranolol inhibits the ATPase, and the data fit to a simple inhibition equation:

$$v = V_{max}/(1 + [I]/K_i)$$

with $K_i = 5$ μM. Dansyl propranolol also inhibits the ATPase reconstituted with dimyristoleoylphosphatidylcholine. In the reconstituted system, the lipid/ATPase ratio is approximately 650/1 and

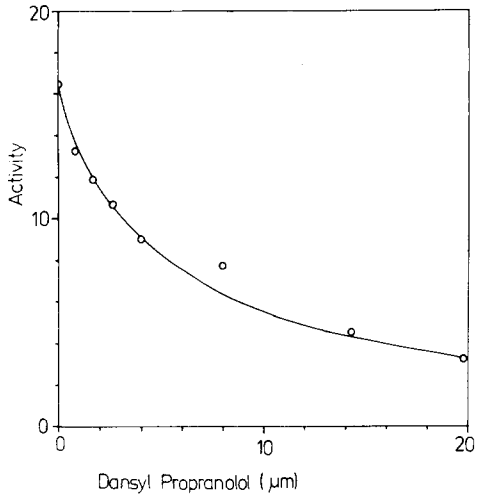

Fig. 8. The effect of dansyl propranolol on the activity (IU) of the $(Ca^{2+} + Mg^{2+})$-ATPase at 37°C in buffer (40 mM Hepes, pH 7.2) at a free calcium concentration of 7 μM. Points. Experimental. Solid line, calculated dependence $K_i = 5$ μM.

appreciable binding of dansyl propranolol will occur to the lipid component of the system. The data fit to a K_i of approx. 8 μM (data not shown).

A second effect of dansyl propranolol on the ATPase is to shift the apparent Ca^{2+} affinity of the enzyme to higher Ca^{2+} concentrations; as shown in Fig. 9, on addition of 12 μM dansyl propranolol, the concentration of Ca^{2+} necessary for half-maximum activity increased from 0.51 μM to 0.93 μM. As discussed in the previous paper [10], we attribute such shifts in apparent Ca^{2+} affinity to electrostatic repulsion resulting from the binding of positively charged drugs to sites on the ATPase protein and to sites in the surrounding lipid.

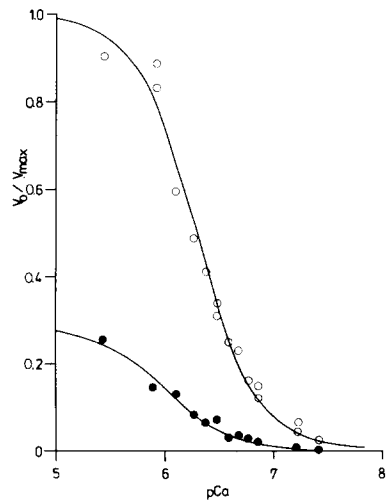

Fig. 9. Dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity on the free concentration of Ca^{2+} at 37°C and pH 7.2 in the presence (●) and absence (○) of 12 μM dansyl propranolol under the assay conditions described in Ref. 10. Curves are the results of non-linear least squares fit of the data to the Hill equation as described in the previous paper [10].

Discussion

There has been much speculation as to how hydrophobic drugs could affect the activities of membrane proteins. We show here that the primary effect of a fluorescent drug analogue, dansyl propranolol, on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase arises from binding at the lipid/protein interface of the ATPase with secondary effects on the Ca^{2+} affinity of the ATPase, possibly arising from charge effects.

Because of the environmental sensitivity of the fluorescence emission of the dansyl group, it is possible to distinguish between dansyl propranolol molecules bound to the lipid and to the protein components of the ATPase system. We find that binding to the lipid component of the system is comparable to binding to simple lipid bilayers. Binding to the protein component can be described either in terms of the aqueous concentration of dansyl propranolol or in terms of the concentration within the lipid component of the membrane. If the binding sites on the ATPase are exposed to the aqueous medium, then a description in terms of aqueous concentrations seems most appropriate. For binding sites at the lipid/protein interface of the ATPase, however, binding of dansyl propranolol must result in displacement of phospholipid molecules from the annulus, and a description in terms of the relative binding constants of dansyl propranolol and phospholipid is then more appropriate. If high concentrations of dansyl propranolol are bound to the lipid component of the membrane, binding to non-annular sites on the ATPase can be described in terms of the mole fraction of dansyl propranolol in the lipid phase of the membrane: description in terms of mole fraction in the lipid phase and

moles/litre in the aqueous phase are not equivalent, because of the different standard states implied by the different choice of concentration units.

Both descriptions of binding suggest the presence of a class of site of large number per ATPase. Although this number cannot be accurately determined, the minimum number of sites is approx. 20 per ATPase. This can be compared to the number of annular phospholipid-binding sites on the ATPase which has been postulated to be about 30 [1]. It is likely, therefore, that these sites are the annular sites on the ATPase. Binding of dansyl propranolol to these sites is characterised by a dissociation constant of approx. 5 μM or a binding constant relative to phospholipid of approx. 4–10. A relative binding constant greater than unity indicates stronger binding of dansyl propranolol at the lipid/protein interface than of phospholipids: although the phospholipid composition of the ATPase system is quite complex [1], we have shown that phospholipid-binding constants depend little on the structure of the phospholipid [5], so that relative displacement of one class of phospholipid rather than another by dansyl propranolol is unlikely. Since in the lipid-displacement model, binding at the lipid-protein interface is calculated in terms of the mole fraction of dansyl propranolol in the lipid phase of the membrane, the relative binding constant is dependent on the accuracy of the binding constant for dansyl propranolol to lipid.

The two descriptions of binding are not equivalent. Although the two sets of best-fit parameters give very similar concentrations of dansyl propranolol bound to annular sites at high concentrations of dansyl propranolol, the lipid displacement model shows a non-hyperbolic dependence on aqueous concentration, and predicts more binding at low concentrations of dansyl propranolol than does the alternative model.

In terms of aqueous concentrations, binding to the ATPase cannot be described well in terms of a single class of binding site on the ATPase, but is well fitted in terms of two classes of site (Table IV). As well as the annular sites described above, a second class of site of high affinity is apparently present, with about three of these sites per ATPase. In previous studies we have shown the presence of three non-annular sites per ATPase for dansyl undecanoic acid [7]. However, in terms of the lipid-displacement model, introduction of a second set of sites results in only a small improvement in the quality of fit (presumably attributable to the more rapid rise in the concentration of dansyl propranolol bound to the annular sites found with this model). The presence of a second class of site for dansyl propranolol must therefore remain, at this stage, rather uncertain.

The ionization state of the dansyl propranolol bound to the ATPase is unknown. However, the observation that binding is unaltered by changing pH from 7.2 to 8.5 suggests that the pK of the bound drug could be the same as the bulk pK, with no large shift of the kind found on binding to lipid bilayers.

Binding of dansyl propranolol to annular sites on the ATPase must necessarily result in the displacement of the phospholipid molecules originally occupying these sites. Since it is known that the activity of the ATPase is sensitive to the chemical structure of the surrounding phospholipids, it is likely that replacing phospholipid molecules with dansyl propranolol in the annulus will lead to a large decrease in ATPase activity. Indeed, it is observed that the inhibition constant describing the effect of dansyl propranolol on the activity of the ATPase (5 μM) is equal to the binding constant at the annular sites determined by fluorescence spectroscopy (Fig. 8).

In the previous paper we have shown that a second effect of drugs on ATPase activity could arise through charge effects. Binding of a positively charged drug molecule to the membrane will produce a more positive surface potential, reducing the Ca^{2+} concentration in the vicinity of the Ca^{2+} binding site of the ATPase, and so producing a decrease in the apparent affinity of the ATPase for Ca^{2+}. Although, as shown in the previous paper, any calculations are very model-dependent, it is clear that charge effects arising from binding of dansyl propranolol to the membrane are sufficient to explain the observed shifts [10].

We find that dansyl propranolol inhibits the ATPase both in bilayers of the original sarcoplasmic reticulum lipids and when reconstituted with dimyristoleoylphosphatidylcholine. The activity of the ATPase is known to be sensitive to the fatty acyl chain length of the surrounding phos-

pholipid, with the short-chain lipid dimyristoleoylphosphatidylcholine supporting lower activity than longer-chain lipids such as dioleoylphosphatidylcholine [4,5]. We have shown that addition of cholesterol and fatty acids can increase the activity of the ATPase reconstituted with dimyristoleoylphosphatidylcholine to levels comparable to those observed with dioleoylphosphatidylcholine [6,7]. The failure of dansyl propranolol to exhibit such an effect reflects the strong binding of dansyl propranolol at annular sites on the ATPase and its strong inhibitory effect at such sites.

If, as seems likely, the activities of other membrane proteins are also sensitive to the chemical structure of their surrounding (annular) phospholipid, then the pattern of inhibition observed here could be a general phenomenon. Any drug that binds strongly at annular sites on a membrane protein could then be expected to cause inhibition because of the considerable structural differences between phospholipids and drug molecules. We have shown here that a proper analysis of such inhibition requires recognition of the three possible phases for the drug molecule – in water and bound to lipid and to sites on the protein. The effect of drugs therefore depends on the strength of binding to the lipid phase of the membrane and on the relative affinities of drug and phospholipid molecules for annular sites on the target protein. The most effective drug molecules would be expected to be hydrophobic. As shown in the previous paper [10], many drugs showing local anaesthetic effects bind extensively to lipid bilayers and it is thus possible that the local anaesthetic effects of these drugs follow from displacement of annular lipids from sites around the Na^+ channel in the nerve membrane.

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