Fluorescence Studies of the β-Adrenergic Receptor Topology†

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ABSTRACT: The nature of the propranolol binding site of the β-adrenergic receptor has been examined by utilizing the intrinsic fluorescence of propranolol as a probe. Additionally, the spatial relationship between the propranolol binding site and membrane tryptophan has been examined by utilizing I^- quenching of intrinsic tryptophan fluorescence, chemical modification of membrane tryptophan, and singlet–singlet energy transfer between membrane-bound propranolol and tryptophan. Propranolol, at concentrations consistent with specific β-receptor binding, protected ~42% of the membrane tryptophan fluorescence from I^- quenching. Further, in the presence of propranolol, the apparent quenching constant (k_q) was altered from 3.6 to 21.8 M^{-1}. Reaction of the membrane fragments with 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent I) in the presence and absence of propranolol indicated that low concentrations of propranolol protected ~45% of the membrane tryptophan from the reagent. The singlet–singlet energy transfer from tryptophan to propranolol was determined by sensitized emission. The distance between these two species was found to be <20 Å. These results have been interpreted to indicate that propranolol, when bound to the β-adrenergic receptor, is situated such that its naphthyl moiety is inserted into a tryptophan-rich hydrophobic pocket of the receptor.

It has recently been demonstrated that the intrinsic fluorescence of the β-adrenergic antagonist propranolol [1-(isopropylamino)-3-(1-naphthyloxy)-2-propranol] serves as a useful probe for the β-adrenergic receptor. In two systems which have been studied, frog (*Rana catesbeiana*) erythrocyte membrane fragments and frog cornea whole epithelial cells, the fluorescence emission of propranolol undergoes a red shift upon binding to its receptor, producing two emission maxima at 340 and 356 nm (Cherksey et al., 1980; Cherksey & Zadunaisky, 1980). These two components were shown to represent the free and bound propranolol, respectively. Propranolol fluorescence was found to be stereospecifically displaced by (−)-isoproterenol but not by the biologically inactive (+) isomer. In these studies, it was possible to demonstrate by using steady-state fluorescence depolarization that the receptor–propranolol complex of the frog erythroctye membrane is rotationally constrained at physiological temperatures. The rotational constraint was reversibly released in the range 6–10 °C and irreversibly released by drugs which disrupt the cell cytoskeleton.

During the course of the above studies, it was noted that the fluorescence emission of membrane protein tryptophan was diminished in the presence of low concentrations of propranolol. This effect might be expected if some population of membrane tryptophan was in close proximity to the naphthyl moiety of propranolol. The presence of tryptophan in protein hydrophobic binding regions has previously been reported for a number of enzymes and for bacterial chemosensory and transport proteins (Parsons & Hogg, 1974; Robertson et al., 1977). Thus, it would not be surprising for a hydrophobic site on the β receptor to resemble these previously defined sites.

We examine here the spatial relationship between the propranolol binding site and membrane tryptophan by utilizing three different approaches. Initially, the quenching of intrinsic tryptophan fluorescence with I^- in the presence and absence of propranolol was determined. This was followed by experiments in which the membrane tryptophan was chemically modified with Koshland's reagent I, 2-hydroxy-5-nitrobenzyl bromide (HNBB), a specific reagent for tryptophan (Koshland et al., 1964). Finally, the magnitude of the singlet–singlet energy transfer from membrane tryptophan to the naphthyl moiety of receptor-bound propranolol was determined.

Materials and Methods

Chemicals. (±)-Propranolol (as the hydrochloride), tryptophan, and the (+) and (−) isomers of isoproterenol were obtained from the Sigma Chemical Co. (St. Louis, MO). In some samples of propranolol an impurity was found to be present which fluoresced with an excitation maximum at 290 nm. This was removed by recrystallization from 1-propanol. The pure material was found to be homogeneous on thin-layer chromatography with five different solvent systems. Pure propranolol was found to fluoresce with a single excitation at 319 nm and an emission maxima of 340 nm as previously reported (Cherksey et al., 1980).

Quinine sulfate, fluorescein, and 2-hydroxy-5-nitrobenzyl bromide (HNBB) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade or better.

Membrane Fragment Preparation. Erythrocyte membrane fragments were prepared from bullfrogs (*R. catesbeiana*) as previously described (Cherksey et al., 1980) and were suspended in a modified frog Ringers. The concentration of this preparation was calculated according to its protein concentration as determined by the Lowry assay (Lowry et al., 1951), using bovine serum albumin as the standard.

Fluorescence Binding Assay. The procedure utilized for the fluorescence binding assay has been described in detail elsewhere (Cherksey et al., 1980). Membrane fragments at a protein concentration in the range 0.05–0.10 mg/mL were placed in a 1.0-cm quartz fluorescence cell, and fluorescence emission spectra were obtained. Propranolol in increasing concentrations ranging from 1 to 10 pM was added to the membrane fragments in 0.01-mL aliquots and allowed to equilibrate at 25 °C for 15 min. The fluorescence emission spectrum with an excitation at 319 nm was obtained at each concentration of propranolol added.

Tryptophan and Propranolol Quenching. Potassium iodide, containing 0.1 mM sodium thiosulfate to prevent free iodine

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formation, was freshly prepared as a 6 M solution prior to each experiment. Fluorescence emission spectra at an excitation wavelength of 290 nm were obtained as the I^- concentration was increased from 10 mM to 0.5 M by serial addition. I^- concentrations were increased in 10 mM increments from 10 to 100 mM and then in 0.1 M increments to 0.5 M. Similarly, I^- quenching of a mixture of 100 nM tryptophan–100 nM propranolol was also determined.

To obtain the quenching curves for tryptophan native to membrane protein, we pipetted 2 mL of the frog erythrocyte membrane fragment containing solution, containing 0.05–0.1 mg of protein/mL, into a 1-cm quartz fluorescence cell. The fluorescence emission spectrum was obtained at the excitation maximum of 290 nm. Potassium iodide in the concentrations described above was then added in 10-μL aliquots directly into the fluorescence cell and allowed to equilibrate for 15 min at 25 °C. The fluorescence spectrum was then obtained. This procedure was repeated until further addition of I^- produced a <2% decrease in the relative fluorescence intensity.

The effect of altered ionic strength due to the addition of the KI in high concentrations was determined by maintaining constant ionic strength with sodium chloride. In these experiments, aliquots of the cell membrane solution were prepared so that the sum of the concentrations of sodium chloride plus potassium iodide was constant as the potassium iodide was increased from 10 to 300 nM. The emission spectra of these solutions were then obtained at an excitation wavelength of 290 nm. The results obtained when the ionic strength was maintained at a constant value did not differ significantly from those obtained with a variable ionic strength.

In another series of experiments, propranolol was added to the cell membrane containing solution to produce a final concentration of 10 nM. The quenching of the fluorescence due to the tryptophan was determined as described above. Additionally, the quenching of the propranolol fluorescence at an excitation wavelength of 319 nm was also determined from these same solutions.

Chemical Modification of Tryptophan. To chemically modify the tryptophan, we dissolved 10 mg of HNBB in 0.1 mL of acetone. This reagent was then added to 10 mL of membrane fragments in frog Ringers at a protein concentration of 0.5 mg/mL. The fragments were reacted with HNBB at a concentration of 1 nM. Reactions were carried out in the dark at room temperature (20–25 °C) for 20 min. At the end of this time, the solution was centrifuged at 16000*g* for 20 min. The supernatant was discarded, and the pellet was resuspended in 19 mL of modified frog Ringers. This washing was repeated twice. The fragments were finally resuspended at 5 times their original volume in modified frog Ringers. Fluorescence spectra were obtained for both the reacted and unreacted (control) cell membrane fragments at an excitation wavelength of 290 nm. Propranolol fluorescent binding assays were performed on the control and modified membrane fragments.

In a second series of experiments, cell membrane fragments were pretreated with 10 nM propranolol before being subjected to the chemical modification procedure with 1 nM HNBB. After reaction, the solution was centrifuged at 16000*g* for 20 min. After centrifugation, the pellet was resuspended in 10 mL of frog Ringers containing 10 nM of propranolol. The membrane fragments were washed twice with this solution. After the final washing, fluorescence spectra were obtained at excitations of 290 and 319 nm. The cells were then centrifuged at 16000*g* for 20 min, suspended in modified frog Ringers, recentrifuged at 16000*g*, and resuspended. This was continued until propranolol fluorescence was undetectable. Fluorescence binding assays were then performed, as described, on the reacted cell membrane fragments and on cell membrane fragments subjected to the same washing and centrifugation procedure but which had not been reacted with HNBB (controls).

Energy Transfer. The energy transfer between tryptophan and propranolol was determined by measuring the sensitized emission of the propranolol with tryptophan excitation. Frog red blood cell membrane fragments were suspended in modified frog Ringers at a concentration of 0.1 mg of protein/mL of solution, and the corrected fluorescence spectra were obtained for agar (1 mg/mL), agar (1 mg/mL)–100 nM tryptophan, 100 nM tryptophan, 10 nM propanolol, and agar (1 mg/mL)–10 nM propranolol. The agar in the above solutions remained particulate rather than dissolving and served to scatter light as do the particulates in the membrane fragment solutions. Such scattering could introduce error into the results obtained in the spectral measurements used to calculate the efficiency of energy transfer. The differences in the fluorescence intensity between the non-agar- and agar-containing solutions provided a measure of the added contribution to the fluorescence intensity from scattering.

In a second series of experiments membrane fragments at a protein concentration of 0.1 mg/mL in frog Ringers were initially reacted with HNBB in the presence of 10 nM propranolol. After an incubation of 20 min, the membrane fragments were washed until they were essentially free of bound propranolol as determined by fluorescence spectroscopy. Fluorescence spectra were then obtained at an excitation of 290 nm to determine the extent of reaction of membrane tryptophan with HNBB. These reacted membranes were then incubated with 10 nM propranolol, and fluorescence spectra were taken at an excitation of 319 nm to determine the energy transfer between tryptophan which had previously been protected by propranolol during the reaction with HNBB and the bound propranolol.

Spectral Measurements. Fluorescence excitation and emission spectra were obtained on an Aminco Bowman SPF spectrophotofluorometer. Absorption spectra were recorded in a Cary 14 spectrophotometer. Quantum yields of compounds in modified frog Ringers were detemined via the following equation (Parker & Rees, 1966)

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

where Q_1 is the quantum yield, F_1 is the area of the corrected emission spectrum, A_1 is the absorbance at the exciting wavelength. Quinine sulfate in 0.1 N H_2SO_4 was utilized as the fluorescence standard by assuming an absolute quantum yield of 0.70 at 23 °C (Scott et al., 1970).

Results

As previously reported, free propranolol exhibits an excitation maximum at 319 nm and an emission maximum at 340 nm. In the presence of β receptor containing membrane fragments, a portion of the emission undergoes a red shift to 356 nm. This represents the bound propranolol while the remaining emission at 340 nm represents the drug free in solution (Cherksey et al., 1980).

The fluorescence quenching of pure tryptophan in distilled water and in frog Ringers by I^- followed the Stern–Volmer law as is shown in Figure 1. In the presence of 10 nM propranolol, tryptophan fluorescence quenching was also found to obey Stern–Volmer considerations. This is also shown in Figure 1. The rate constants were obtained from the linear Stern–Volmer plots and ranged from 8.4 to 16.1 M^{-1} as indicated in Table I.

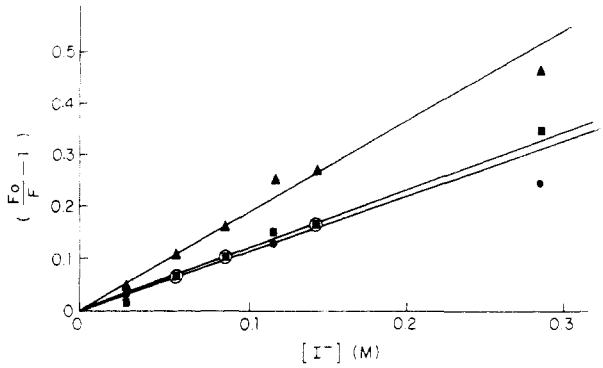

FIGURE 1: Fluorescence quenching of pure compounds. Quenching of the fluorescence of pure tryptophan in both water and frog Ringers was determined and plotted according to the Stern–Volmer equation. The quencher used in these studies was I^-. The quenching of tryptophan fluorescence in the presence of propranolol was also determined. Tryptophan in water (●); tryptophan in frog Ringers (■); tryptophan plus propranolol (▲).

Table I: Fluorescence Quenching [a]

	k_q (M^{-1})	% of sites accessible
tryptophan (100 nM) in H_2O	8.4	100
tryptophan (100 nM) in frog Ringers	12.0	100
tryptophan (100 nM) + propranolol (100 nM) in frog Ringers	16.1	100
membrane fragments		
0.1 mg of protein/mL	3.6	89.3
+10 nM propranolol	21.8	47.6
tryptophan protected by propranolol		41.7

[a] All values are the mean of 10 determinations. Excitation at 290 nm. Emission at 330 nm.

When quenching experiments were repeated for native tryptophan in the cell membrane fragments, the results no longer followed a simple Stern–Volmer relationship. This is shown in Figure 2, as are the results of quenching experiments performed in the presence of 10 nM propranolol. In neither case did quenching follow simple Stern–Volmer considerations.

Lehrer (1971) has shown that in a protein containing more than one tryptophan residue quenching will follow a modified Stern–Volmer equation

$$F_0/\Delta F = \frac{1}{[I^-]\sum f_i k_{q_i}} + \frac{\sum k_{q_i}}{\sum f_i k_{q_i}} \qquad (2)$$

where F_0 is the fluorescence intensity in the absence of quencher, ΔF is the change in intensity in the presence of I^-, k_{q_i} is the apparent quenching constant, and f_i is the number of accessible tryptophan residues. A plot of $F_0/\Delta F$ vs. $1/[I^-]$ will yield a line with an 1/intercept of $\sum f_i k_i / \sum k_{q_i}$ which can be considered as the maximum accessible fluorescence and an intercept/slope of $\sum k_{q_i}$, the effective quenching constant for the sum of the quenching processes.

The results obtained for I^- quenching of the membrane tryptophan have been plotted according to the modified Stern–Volmer equation. This is shown in Figure 3. From these data, it is possible to calculate an effective quenching constant of 3.6 M^{-1} for tryptophan in the membrane fragments. In the presence of propranolol, the effective quenching constant is altered and becomes 21.8 M^{-1}. The intercept of the tryptophan quenching curve is 1.12. These data are summarized in Table I. This is taken to indicate that 89% of the tryptophan in the erythrocyte membrane fragments is accessible to the quencher. In the presence of propranolol, the intercept shifts

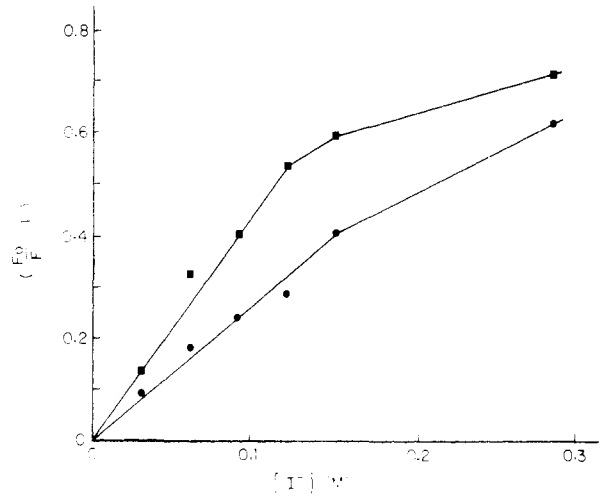

FIGURE 2: Quenching of membrane tryptophan fluorescence. Quenching of tryptophan fluorescence with KI was determined for the membrane fragment preparation (●) and in the presence of propranolol (■). The resulting curves deviate from the Stern–Volmer relationship. It is apparent that the curve obtained for the propranolol-treated membrane fragments differs from that obtained for the pure membrane fragment preparation.

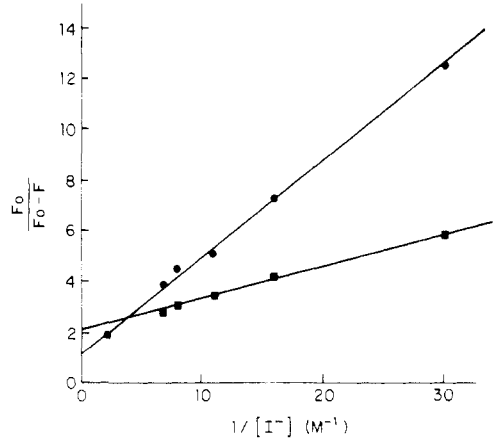

FIGURE 3: Modified Stern–Volmer plot for tryptophan fluorescence quenching. The data shown in Figure 2 have been replotted according to eq 2. The curves are not linearized. The inverse of the ordinate intercept represents the fractional quenching. It is seen that the native membrane fragments (■) have significantly more accessible tryptophan than do the propranolol-treated fragments (●). This is taken to indicate that there is a population of tryptophan which is protected from iodide quenching in the presence of propranolol.

to 2.11, indicating that only 48% of the tryptophan was available to the quencher. This difference, 42%, represents the membrane tryptophan which is protected from the I^- by the bound propranolol.

Figure 4 illustrates the fluorescence quenching of 10 nM propranolol when free in solution and also when bound to membrane fragments. It is seen that with KI concentrations up to 0.1 M both curves coincide. Above this concentration, propranolol in solution continues to be quenched while that which is bound to the membrane fragments is no longer quenched but reaches a constant value. These results are compatible with the naphthyl moiety of propranolol being inaccessible to the quencher when propranolol is bound to the β receptor.

The effect of chemical modification of the membrane tryptophan was detemined by reacting membrane fragments with 1 μM HNBB. After reaction, the fluorescence emission intensity of the membrane tryptophan is diminished to <5% of the original intensity. When membrane fragments are reacted in the presence of 10 nM propranolol, the fluorescence

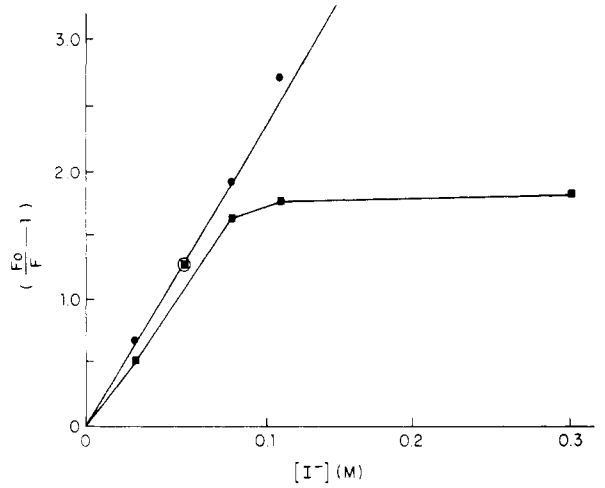

FIGURE 4: Quenching of propranolol fluorescence. Quenching of propranolol fluorescence by KI was determined and plotted according to the Stern–Volmer equation. It is seen that pure propranolol (●) in frog Ringers exhibits a linear quenching curve. In contrast, membrane-bound propranolol (■) exists in an environment such that it cannot come into contact with the quencher. This is evidenced by the plateau of the Stern–Volmer plot.

Table II: Chemical Modification of Tryptophan

	1 μM HNBB	1 nM HNBB
control	100[a]	100
reacted	4	15
+propranolol	4	63
protected by propranolol		45.2

[a] All values are expressed as percent of the control (mean of six determinations).

is also diminished to <5% of its original value. Emission spectra obtained at the excitation maximum for propranolol (319 nm) indicate that none of the ligand remained bound to the membrane fragments. Binding assays performed on the reacted membrane fragments showed no specific propranolol binding to the fragments, indicating that all binding sites had been destroyed.

When the procedure was repeated with 1 nM HNBB, the results were entirely different. After reaction, the fluorescence intensity of the membrane tryptophan was diminished to 15% of its original value. When membranes were reacted with HNBB in the presence of 10 nM propranolol, the fluorescence intensity fell to only 60% of its original value. This difference of 45% is taken to represent the tryptophan protected from reaction with HNBB by the bound propranolol.

The results obtained in the chemical modification experiments are shown in Table II. Comparison of these results with those obtained from the quenching experiments shows that in both procedures, propranolol protects essentially the same population of tryptophan.

When sensitized emission is determined, spectra from four different systems are necessary in order to correct for spectral interferences. The four samples for which spectra must be obtained are (a) pure donor and an acceptor analogue, (b) the donor–acceptor system of interest, (c) donor analogue and pure acceptor and, (d) donor analogue and acceptor analogue.

The efficiency of energy transfer is calculated from the fluorescence intensities of these spectra (Fairclough & Cantor, 1978) by

$$E = \frac{f_b(\lambda_D,\lambda_A) - f_c(\lambda_D,\lambda_A) - f_a(\lambda_D,\lambda_A)}{A_b^D(\lambda_A)/A_b^A(\lambda_D)[f_c(\lambda_D,\lambda_A) - f_a(\lambda_D,\lambda_A)]} \quad (3)$$

where f_a, f_b, and f_c are the fluorescent intensities of the samples described above after correction for scatter (f_d), where the fluorescence is measured with excitation at λ_D and emission at λ_A, and A is the optical absorbance of the sample at the indicated wavelength.

The efficiency of singlet–singlet energy transfer from a fluorescing donor to an absorbing acceptor molecule is related to the distance R between this pair by

$$R = (1/E - 1)^{1/6}R_0 \quad (4)$$

where R_0 is the Förster critical distance at which $E = 0.5$ and R is the distance between the donor and acceptor molecules.

The critical distance is calculated, according to Förster (1960), as

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi}{128\pi^5 n^4 N} J \quad (5)$$

where ϕ is the quantum yield of the donor, n is the refractive index of the medium (taken to be 1.33), N is Avagodro's number, and κ^2 is an orientation factor. If the system is assumed to have random directional distributions, the value of κ^2 is $^2/_3$ (Förster, 1951). κ^2 can vary between 0 and 4 depending on the geometry of the system. J is the "overlap integral", defined (Förster, 1948) as

$$J = \int f_d(\nu)\epsilon_a(\nu)\, d\nu/\nu^4 \quad (6)$$

where f_d is the fluorescence of the donor at frequency ν (in cm^{-1}) and ϵ_a is the molar absorptivity of the acceptor. The integration is over all frequencies.

In practice, the overlap integral is obtained as an approximation from the summation (Cantley & Hammes, 1975) over wavelength

$$J = \sum_\lambda f_d(\lambda)\epsilon_a(\lambda)\lambda^4\Delta\lambda / \sum_\lambda f_d(\lambda)\Delta\lambda \quad (7)$$

where $f_d(\lambda)$ and $\epsilon_a(\lambda)$ are as defined as above, but with respect to wavelength. The summation was carried out over 2-nm intervals.

The energy transfer from cell tryptophan to bound propranolol was determined. The emission spectrum of tryptophan coincides with the excitation spectrum of the propranolol. Propranolol was found to have a molar absorbtivity (ϵ) of 6590 mol^{-1} cm^{-1} and a quantum yield of 0.25. The average optical absorbance of the membrane fragments used in these studies was 0.43 ± 0.066. The sensitized emission of propranolol was obtained. From these experiments, the efficiency of energy transfer was calculated by using eq 3 to be 0.94, when the results were corrected to account for only the membrane tryptophan which is actually involved in the propranolol binding.

The overlap integral (J) was calculated according to eq 6 and determined to be 0.4226×10^{-15} $cm^6 \times 10^{-3}$/mol. This value is typical for tryptophan energy transfer (Fairclough & Cantor, 1978). From these data, the Förster critical distance was calculated according to eq 5 to be 20 Å. From eq 4, the distance between tryptophan and propranolol was calculated to be 13.4 Å.

Attempts were made to determine the energy transfer after reaction with 1 nM HNBB in the presence of 10 nM propranolol. In a number of these experiments it was found that the remnants of HNBB interfered with the energy-transfer measurements. In five experiments which were successful, the results were in agreement with the above data. No significant

difference was found when the results were compared by using Student's t test.

Discussion

Quenching of membrane tryptophan fluorescence using iodide was found to deviate from the Stern–Volmer relationship (Figure 2) but did follow a model for a heterogeneous tryptophan population (eq 2 and Figure 3). The data from the quenching experiments have been summarized in Table I. Approximately 89% of the tryptophan fluorescence could be quenched with iodide ion. When propranolol was bound to the fragments, only 48% of the tryptophan fluorescence is quenchable. This indicates that propranolol binding protects ~42% of the tryptophan from iodide quenching.

Also noteworthy is the finding that when the propranolol is present, the apparent quenching constant is seen to increase. This could be due to two different effects. It may be that the population of tryptophan protected by propranolol and therefore removed from the quenchable population has an extremely low quenching constant. This appears to be a reasonable hypothesis due to hydrophobic nature of the environment in which some fraction of the tryptophan might exist. When this fraction is removed from the population the apparent quenching constant would therefore be increased. A second explanation for the increased quenching constant would be that the protein might undergo a conformational change upon the binding of propranolol further exposing tryptophan residues to the aqueous environment. This could also account for the increased apparent quenching constant which is found upon propranolol binding.

The results of the quenching studies have been confirmed by the reaction of membrane tryptophan with HNBB. It is seen that when 1 μM HNBB is reacted with the membrane fragments, the effects are relatively nonspecific. Reaction with 1 nM HNBB is more successful. When fragments are reacted with this lower concentration of HNBB, only 15% of the tryptophan fluorescence remains. This is similar to the 11% of the tryptophan fluorescence which was found to be unquenchable. In the presence of propranolol, 63% of the tryptophan remains unreacted. This represents an ~45% protection of the membrane tryptophan by bound propranolol. This is in excellent agreement with the 42% of the tryptophan which is protected in the iodide quenching experiments. The results of these two experiments provide convincing evidence of the intimate nature of the propranolol–tryptophan interaction. It is possible to picture the naphthyl moiety of the propranolol sitting on the surface of the receptor thereby blocking access of either iodide or HNBB to the underlying tryptophan. Alternatively, it is possible to postulate a hydrophobic pocket containing tryptophan into which the naphthyl moiety is inserted.

The red shift seen in the propranolol emission spectrum upon binding to the membrane fragments argues strongly for the hydrophobic nature of the propranolol binding site. Indeed, the red shift has been used to assess the interaction of high concentrations of propranolol with membrane lipids (Dachary-Prigent et al., 1979). The fluorophore of the propranolol molecule is the naphthyl moiety which is hydrophobic in nature. If the naphthyl moiety enters an environment which is more hydrophobic than the surrounding aqueous buffer, upon binding, the Stokes shift should increase. The exact nature of the hydrophobic environment cannot be determined solely from the environmentally produced red shift. Two alternative explanations are possible. Firstly, the effect could be produced by interaction of the naphthyl moiety with the membrane lipids. Secondly, the naphthyl moiety could be entering into a hydrophobic region of the receptor protein. This latter explanation is favored by the specificity of the propranolol–membrane fragment interaction which is evidenced by the Scatchard plots (Cherksey et al., 1980) for propranolol binding. Interaction with the membrane lipid should be nonspecific. Additionally, the results obtained from the quenching experiments and from chemical modification of membrane tryptophan indicate an intimate relationship between this amino acid and propranolol. These results can best be explained by the association of the naphthyl moiety with a specific site on the receptor rather than by the nonspecific interaction with the membrane lipids.

The possible involvement of a hydrophobic pocket, containing the tryptophan residues, is confirmed by the experiments on iodide quenching of propranolol fluorescence (Figure 4). When propranolol is bound to the receptor, its fluorescence cannot be quenched. This is indicated by the plateau of the Stern–Volmer plot. If the naphthyl group was sitting on the membrane surface, it would be partially exposed to the aqueous environment. In this case, the iodide ion could still approach the naphthyl moiety at its exposed surface and would continue to quench the propranolol fluorescence. This might result in an altered quenching constant but certainly would not cause the plateau seen in the Stern–Volmer plot. This plateau is better explained if the naphthyl moiety is protected completely from the iodide ion. This would be accomplished *only* if the naphthyl group was totally buried in a hydrophobic pocket of the receptor protein.

The energy transfer from tryptophan to propranolol was found to occur with extraordinarily high efficiency. The efficiency which was calculated from these experiments corresponds to nearly 95% transfer. If a dipole–dipole resonant energy-transfer mechanism is postulated, the Förster critical distance (eq 5) may be calculated. This distance is found to be ~20 Å. When this value is used, the calculated distance from membrane tryptophan to bound propranolol is ~13 Å. These calculations are based on the fluorescence attributable only to that fraction of tryptophan which was found to be unquenchable and nonreactive when propranolol is bound. Because of the magnitude of the energy transfer, the assumption of a Förster mechanism must be questioned. At the relatively short distances which separate the tryptophan from the propranolol, other mechanisms of energy transfer may be operative. Stronger interactions, such as exchange mechanisms, might be expected at these distances and would better account for the magnitude of the energy-transfer efficiency. No evidence of any other mechanism being operative in this system has been obtained. If other mechanisms were operative, the value obtained by using the Förster theory would be a lower limit for the distance between tryptophan and propranolol. It is nevertheless clear that the distance must be <20 Å (Förster, 1960).

By combining the results of the quenching, chemical modification, and energy-transfer experiments, it is possible to construct a picture of the β receptor. In general, the β agonists are characterized by three features: (a) the amine, (b) the 2-hydroxyl, and (c) the catechol moiety. It is interesting that all three groups are hydrophilic. Features common to all agonists and the antagonists are the amine and the hydroxyl group. It is assumed that these two groups principally define the interaction with the β receptor (George et al., 1971). Because of their hydrophilic nature, it can be assumed that these groups interact with sites which are exposed to the aqueous environment. Hence, it may be postulated that the amine and hydroxyl groups interact with sites on the receptor

which are on the exposed surface of the receptor protein. The structural difference between the agonists and the antagonists must therefore reside in the catechol moiety. The antagonists are characterized by replacement of the catechol with a strongly hydrophobic group. It is noteworthy that the catecholamines are inactivated physiologically by catechol *O*-methyltransferase which converts the catechol hydroxyl groups to methoxy groups. This, of course, changes the nature of the catechol from hydrophilic to hydrophobic. It would seem that the hydrophobicity–hydrophilicity of this terminus is crucial to the biological activity exhibited by the compound.

The results of the fluorescence studies reported here make it possible to understand the above relationships. It is apparent that the hydrophobic center of the antagonist enters a pocket in the receptor which cannot be occupied by the hydrophilic catechol. In some manner as yet undetermined, the physical occupancy of this hydrophobic pocket of the receptor prevents the activation normally brought about by the surface interactions of the catecholamine. The reason that such a hydrophobic site would exist on the β receptor is certainly not understood. Such a site might be simply a consequence of protein folding and be devoid of natural function. At present, no natural substrate for this hydrophobic "propranolol receptor" has been recognized. This of course does not rule out the possibility that a natural substrate might exist.

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Small Unilamellar Vesicles Containing Glycophorin A. Chemical Characterization and Proton Nuclear Magnetic Resonance Studies†

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ABSTRACT: Glycophorin A, a major glycoprotein of the red blood cell, is reconstituted in small lipid vesicles (250–300 Å in diameter) by using cholate detergent solubilization followed by rapid removal of cholate on a molecular sieve column. The extent of glycophorin incorporation is found to be critically dependent on the amount of cholate used, with higher amounts yielding vesicles with higher percentages of glycophorin. Vesicles with as much as 1 molecule of protein per 20 molecules of lipid can be prepared. Data on the vesicles obtained by using hydrolytic enzymes such as neuraminidase and trypsin, combined with amino acid analysis, suggest that glycophorin is incorporated in a transbilayer fashion with a high fraction of the molecules oriented with the carbohydrate-containing amino terminus to the vesicle exterior. Interaction of the protein with the hydrophobic portion of the bilayer is apparent in proton nuclear magnetic resonance spectra, and lipid line-width increases have been used to characterize the strength and stoichiometry of interaction. Glycophorin is found to affect directly as many as 40 lipid molecules per molecule of protein; however, the magnitude of the effects is not large.

The interaction of protein and lipid constituents of membranes is an important aspect of many membrane-centered phenomena. A variety of techniques have been employed, and a variety of lipid–protein systems studied, in an effort to elucidate the molecular details of those interactions [for a review, see Chapman et al. (1979)]. It is now apparent that a number of integral membrane proteins may have hydrophobic segments of appropriate length to span a lipid bilayer when in an α-helical configuration. A protein which is well characterized, is known to be transbilayer, and has a single such hydrophobic segment would seem an ideal choice for the study of the basic properties of postulated lipid–hydrophobic helix interactions.

Glycophorin A (GPA),[1] the major glycoprotein in human erythrocyte membranes, would seem an ideal protein. This

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[1] Abbreviations used: GPA, glycophorin A; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DSS, 5,5-dimethylsilapentane-2-sulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; R_1, spin–lattice relaxation rate (T_1^{-1}); R_2, spin–spin relaxation rate (T_2^{-1}).

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