Intramembrane Position of the Fluorescent Tryptophanyl Residue in Membrane-Bound Cytochrome b_5†

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ABSTRACT: We have developed a method to measure the intramembrane position of the fluorescent tryptophanyl residue in whole cytochrome b_5 and the nonpolar membrane binding segment when these molecules are bound to phospholipid vesicles [Koppel, D. E., Fleming, P., & Strittmatter, P. (1979) *Biochemistry* (preceding paper in this issue)]. The method utilizes excitation energy transfer from the donor tryptophanyl residue in the protein to trinitrophenyl or dansyl acceptor groups on the surface of the phospholipid bilayer. It was determined that the single fluorescent tryptophanyl residue in vesicle-bound cytochrome b_5 and the nonpolar segment is located approximately 20–22 Å below the surface of the bilayer. This position represents a minimum depth of penetration of this portion of the cytochrome in the membrane.

Cytochrome b_5 is an amphipathic molecule composed of a hydrophilic heme peptide segment that is joined by a short, apparently flexible sequence to a nonpolar segment (Spatz & Strittmatter, 1971; Visser et al., 1975). Considerable data, in addition to the known existence of the two major structural domains, indicate that the nonpolar portion of this cytochrome inserts into the membrane to anchor the whole protein and orient the heme peptide at the aqueous–lipid interface (Strittmatter et al., 1972; Rogers & Strittmatter, 1973, 1975). There has been, however, no detailed information on the depth or the arrangement of the nonpolar segment in the hydrocarbon region. The importance of such knowledge is not limited to an understanding of the spatial arrangement of the cytochrome b_5 molecule per se but extends to the sequence of interactions of the heme protein as an electron acceptor for NADH:cytochrome b_5 reductase and donor for stearyl-CoA desaturase.

Recent information on the structure of the complete bovine cytochrome prompted the development of a distance probe based upon excitation energy transfer to identify the depth of one tryptophanyl residue in the nonpolar segment below the surface of a lipid bilayer. In addition to the known primary structure of the heme peptide segment (Ozols & Strittmatter, 1969; Mathews et al., 1972), the primary structure of the nonpolar peptide segment has been determined (Fleming et al., 1978). This latter amino acid sequence reveals three tryptophanyl residues in a predominantly nonpolar structure. It has been shown (Fleming & Strittmatter, 1978) that only Trp-109 is fluorescent. The single excitation energy donor within the cytochrome has been utilized here to determine the distance between this residue and trinitrophenyl or dansyl groups placed at the head group region of a phospholipid membrane with quantitative excitation energy transfer experiments. The method we have devised is described in detail in the preceding paper (Koppel et al., 1979). In this communication we describe the application of this method to the specific cytochrome b_5 problem.

Materials and Methods

Steer liver cytochrome b_5 was isolated and purified from fresh calf liver as described previously (Strittmatter et al., 1978) and stored at −20 °C. The carboxyl-terminal nonpolar segment of cytochrome b_5 was prepared as follows. Cytochrome b_5 (1–2 mM in 0.02 M Tris–acetate, pH 8.1, and 0.1 M NaCl) was enzymatically cleaved by the addition of 0.1 molar equiv of trypsin (Worthington) dissolved in water and brought to pH 8 with Tris base. This mixture was incubated 18 h at 4 °C and then separated by passage through a 2 × 55 cm column of P-60 (Bio-Rad) equilibrated with 0.05 M $NaHCO_3$, 0.05 M NaCl, and 0.02% NaN_3, pH 8.1 (buffer A). The nonpolar peptide is aggregated and elutes with the void volume. The concentration of nonpolar peptide was determined by optical density at 280 nm with 19 600 M^{-1} cm^{-1} as the extinction coefficient.

Dansyldodecylamine (DDA)[1] was prepared by reacting 0.10 mmol of dodecylamine (Aldrich), 0.02 mmol of triethylamine (Aldrich), and 0.11 mmol of dansyl chloride (Pierce) in 1 mL of chloroform for 2 h at 22 °C. The product was isolated by preparative thin-layer chromatography on silica gel G using chloroform as the developing solvent. Analytical thin-layer chromatography using the same system showed a single fluorescent spot (R_f = 0.75). No additional spots were seen upon charring the plate after chromic acid spray.

Vesicles containing 95% dimyristoylphosphatidylcholine and 5% dimyristoylphosphatidylethanolamine were prepared in buffer A as described previously (Enoch et al., 1977). Phospholipid concentrations were determined by the method of Chen et al. (1956). Labeled lipid [^{14}C]dimyristoylphosphatidylcholine (Analabs) was added to the vesicle preparations to facilitate subsequent concentration measurements.

Trinitrophenyl-labeled vesicles were made by adding a limiting amount of trinitrobenzene sulfonate (50 mM in 2% $NaHCO_3$) to a sample of vesicles and incubating at 30 °C for 30 min. The concentration of the trinitrophenyl derivative of dimyristoylphosphatidylethanolamine in each sample was determined from the optical density at 340 nm with 15000 M^{-1} cm^{-1} as the extinction coefficient. The same amount of cytochrome b_5 was then bound to each population of trinitro-

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[1] Abbreviations used: DDA, dansyldodecylamine; TNP-PE, *N*-(trinitrophenyl)dimyristoylphosphatidylethanolamine; NPP, nonpolar peptide segment (residues 91–133) of bovine cytochrome b_5.

0006-2960/79/0418-5458$01.00/0

Table I: Values of Parameters Used in Theoretical Calculation of Energy Transfer for Two Donor-Acceptor Pairs

donor	acceptor	$J^a \times 10^{15}$ ($cm^3\ M^{-1}$)	$\beta^b \times 10^{-8}$ ($Å^6$)	$R_0{}^c$ (Å)	$EA_D{}^d$	$EA_A{}^d$	$\overline{\cos^2 \theta_D{}'}^e$	$\overline{\cos^2 \theta_A{}'}^e$
NPP[f]	TNP-PE	15.32	15.68	31.9	0.070	0.069[g]	0.61	0.61
NPP[f]	DDA	4.26	4.35	25.7	0.070	0.038	0.61	0.54

[a] J is the overlap integral for the normalized emission and absorption spectra of the donor and acceptor, respectively. [b] β equals $R_0{}^6/\kappa^2$ where $R_0{}^6 = (8.785 \times 10^8)\kappa^2\phi_D n^{-4}$ ($J \times 10^{15}$), κ^2 is the orientation factor for dipole-dipole interaction [see Dale & Eisinger (1974)], ϕ_D is the donor quantum yield in the absence of acceptor, and n is the refractive index of the lipid bilayer. The values used for calculation of β were $n = 1.455$ (Cherry & Chapman, 1969; Seufert, 1970; Yi & MacDonald, 1973) and $\phi_D = 0.522$ [using $\phi = 0.14$ for tryptophan in water at 25 °C; Eisinger (1969)]. [c] Calculated in the limit, $\kappa^2 = 2/3$. [d] EA_D and EA_A are the emission anisotropies for the donor and acceptor, respectively, at typical experimental conditions, Excitation was at the long-wavelength edge of the absorption band for each chromophore. [e] Calculated from $\overline{\cos^2 \theta'} = [1 + 2(EA/0.4)^{1/2}]/3$ analogously to eq 49 by Dale et al. (1979). The value, 0.4, is the fundamental emission anisotropy. [f] The same values given here for NPP were used for whole cytochrome b_5. [g] Determined for *N*-dansyldimyristoylphosphatidylethanolamine (see the text).

phenyl-labeled vesicles at lipid/protein molar ratios of 200–600 at 30 °C.

When DDA was used as the acceptor, it was added as an ethanolic solution (15 mM) with rapid mixing to the protein–lipid complex at a dilution such that the final concentration of ethanol was <0.6%. After 60 min of intermittent mixing, an aliquot sample of the mixture was brought to 0.5% in Triton X-100, and the concentration of dansyldodecylamine was determined by optical density at 335 nm with an extinction coefficient of 4600 $M^{-1}\ cm^{-1}$ (Waggoner & Stryer, 1970).

The fluorescence emission spectrum of each sample was recorded at an excitation wavelength of 285 nm using a Perkin-Elmer MPF-3 spectrofluorometer. Fluorescence measurements were made with 5 μM protein concentrations in 3-mm^2 cuvettes at 30 °C. Quenching efficiencies were calculated from the fluorescence intensities at 335 nm vs. a standard sample containing no acceptor groups. In experiments with DDA as the acceptor, the increase in dansyl fluorescence emission at 520 nm relative to a standard with no donor was proportional to the decrease in tryptophan fluorescence at 335 nm. Therefore, the efficiency of energy transfer was determined by quenching of tryptophan fluorescence for all experiments. A small correction for light scattering (<5%) was made by using a blank sample containing vesicles only. The apparent efficiency of energy transfer was corrected for inner filter effect at 335 nm as shown:

$$\langle E \rangle = 1 - \frac{s \ln 10}{\sinh (s \ln 10)} \frac{1 - \langle E_{app} \rangle}{10^{-l}} \qquad (1)$$

where s and l equal the absorbance at 335 nm along the fluorescence collection axis across half of the width of the incident beam and half of the width of the cuvette, respectively. An inner filter correction is not required for the excitation wavelength. All samples and acceptor-free reference samples have essentially the same optical density at 285 nm (~0.08).

Fluorescence polarization measurements were done by observing the emission at 90° to the excitation beam and calculating the emission anisotropy, EA (Jablonski, 1962), by

$$EA = \frac{I_{VV} - \frac{I_{HV}}{I_{HH}} I_{VH}}{I_{VV} + 2\frac{I_{HV}}{I_{HH}} I_{VH}} \qquad (2)$$

where I is the fluorescence intensity corrected for light scattering and the subscripts V and H indicate the orientation, vertical and horizontal, of the excitation and emission polarizers. The quantity I_{HV}/I_{HH} in eq 2 is a correction for instrument error as described by Chen & Bowman (1965) and Azumi & McGleen (1962). Excitation was at 305 nm for the polarization measurements of tryptophan fluorescence.

Since it was not possible to measure the mobility of the nonfluorescent acceptor TNP-PE, we assumed that it would be similar to that of dansylphosphatidylethanolamine. The fluorescence polarization of the latter compound bound to dimyristoyllecithin vesicles is 0.1 at 30 °C (Faucon & Lussan, 1973). This value was used to estimate the mobility of TNP-PE as listed in Table I.

To calculate the surface density (σ) of excitation energy acceptors on the outer surface of the phospholipid vesicles, we used the equation

$$\sigma = \frac{PL_{ves}}{S_{ves}} \frac{A}{PL} \qquad (3)$$

where PL_{ves} is the number of phospholipid molecules per vesicle, S_{ves} is the outer surface area per vesicle, and A/PL is the acceptor to phospholipid molar ratio in the particular vesicle preparation. To calculate PL_{ves}, we used an average vesicle molecular weight of 2.5×10^6 (Watts et al., 1978; Aune et al., 1977) and phospholipid molecular weight of 678 to give a value of 3687 phospholipid molecules per vesicle. An average vesicle radius of 114 Å (Watts et al., 1978; Aune et al., 1977) gave a surface area of 1.633×10^5 $Å^2$/vesicle.

Estimation of the intramembrane position of the cytochrome b_5 tryptophanyl donor was performed as outlined in the preceding paper (Koppel et al., 1979) and is described in detail below.

Rhodamine-labeled cytochrome b_5 was prepared by incubating 400 μM cytochrome b_5 with a 25-fold molar excess of tetramethylrhodamine isothiocyanate isomer B (Baltimore Biological Laboratories) in 0.1 M sodium phosphate buffer at pH 7.7 for 3 h at 34 °C. The labeled cytochrome was isolated by gel filtration with a column of Sephadex G-25 in 0.02 M Tris–acetate and 0.1 M NaCl buffer at pH 8.1 and contained 3.5 rhodamine groups per heme. Vesicles containing DDA (lipid/DDA ratio of 200) and varying amounts of rhodamine-labeled cytochrome b_5 (lipid/protein ratio of 25–244) were prepared by adding DDA to protein-containing vesicles as described above. The fluorescence emission spectrum of each sample was recorded at an excitation wavelength of 340 nm and used to calculate quenching efficiencies from the fluorescence intensities at 520 nm.

Results and Discussion

The nonpolar peptide of cytochrome b_5 binds completely to phospholipid vesicles (Fleming & Strittmatter, 1978), and, therefore, in these experiments the protein–lipid complex could be used immediately after binding. Previous experience has shown that whole cytochrome b_5 binds incompletely to vesicles. It was determined that under the conditions of binding used in this study 90–95% of the protein was bound to vesicles. Since the fluorescence quantum yield of unbound cytochrome

b_5 is only approximately 30% of dimyristoylphospholipid-bound protein, the unbound cytochrome represents about 2–3% of the observed fluorescent intensities in the cytochrome b_5 experiments. No correction was made for the fact that this small amount of fluorescence would not be quenched by the vesicle-bound energy acceptors. All of the protein bound to dimyristoyllecithin vesicles in these experiments is oriented in the same direction, i.e., with the heme peptide (for whole cytochrome b_5) or the heme peptide linkage terminus (for NPP) facing the outer aqueous interface (Enoch et al., 1977; Fleming & Strittmatter, 1978). Furthermore, with dimyristoyllecithin, the protein is bound in the tight, nontransferable form of binding as described by Enoch et al. (1979).

The degree of binding of DDA to phospholipid vesicles was determined by passage of a sample through a Sephadex G-200 column equilibrated with buffer A at 30 °C. Analysis showed that 91% of the dansyl absorbance eluted with the vesicles, and it was then assumed that a similar degree of binding occurred in all samples. The reaction of trinitrobenzene sulfonate with dimyristoylphosphatidylethanolamine incorporated into vesicles as described under Materials and Methods has a half-time of approximately 5 min (data not shown). Since the vesicle bilayer is impermeable to the trinitrophenyl reagent on this time scale at the reaction temperature of 30 °C (Fleming & Strittmatter, 1978), it is reasonable to assume that the trinitrophenyl moiety is randomly distributed over the outer surface of the vesicles and over the vesicle population as a whole. The binding of DDA to the vesicles is apparently more rapid as measured by fluorescence changes (data not shown). Therefore, this probe was added to a rapidly stirred solution of vesicles to promote a homogeneous distribution of probe among the vesicle population. It was not possible to make an independent determination of the distribution of DDA among the vesicles but the nearly identical results obtained with both DDA and TNP-PE as described below indicate that DDA is distributed as evenly as TNP-PE.

The geometry of the system is defined in terms of the following: h, the depth in the membrane of the tryptophanyl donors relative to the position of the acceptor groups; A_{ex}, an excluded surface area associated with each donor molecule; R, the distance of the acceptors from the center of the vesicle [see Figure 1, Koppel et al. (1979)]. The energy transfer data were analyzed by the methods of Koppel et al. (1979) with the primary objective of determining the value of h. The data are first fit to a simplified theory in which κ^2, the dipole–dipole orientation factor, is assumed equal to 2/3 for all donor–acceptor pairs. One can then use a general formulation to assess the effects of the possible variations of κ^2 about 2/3.

In the limit of uniform κ^2, one can define and compute values of R_0, the Förster critical distance (see Table I). As demonstrated by Koppel et al. (1979), the theoretical form of $\langle E \rangle$ then reduces to a function of two dimensionless parameters:

$$r_0 = r_{min}/R_0 \tag{4}$$

where r_{min} is the distance of closest approach

$$r_{min} = \left(h^2 + \frac{R - h}{R} \frac{A_{ex}}{\pi} \right)^{1/2} \tag{5}$$

and n_0 is an effective number of acceptors

$$n_0 = \frac{R}{R - h} \pi R_0^2 \sigma \tag{6}$$

With R_0, σ, $\langle E \rangle$, and R known, one can calculate r_{min} and A_{ex} for assumed values of h. This calculation is facilitated by the introduction of a simple empirical representation of $\langle E \rangle$ [see eq 39 to 40, Koppel et al. (1979)] as a function of r_0 and n_0

$$\frac{1 - \alpha}{\langle E \rangle} = \sigma^{-1.1} \left[\frac{0.62}{\pi R_0^2} \frac{R - h}{R} \exp(-0.34 r_0 + 1.63 r_0^2) \right]^{+1.1} + 1 \tag{7}$$

incorporating the additional condition that some fraction α ($0 \leq \alpha \leq 1$) of the donors, for some reason or another, cannot be quenched. A plot of $1/\langle E \rangle$ vs. $\sigma^{-1.1}$ is shown in Figure 1 using the experimental values of $\langle E \rangle$ measured for both TNP-PE and DDA as acceptors for the NPP tryptophanyl donor. The theoretical lines drawn through these data are the best fit with a forced intercept of 1.0. The fact that the data reasonably fit lines with this intercept demonstrates that $\alpha \simeq 0$, i.e., that all donor molecules have an equal probability for energy transfer.

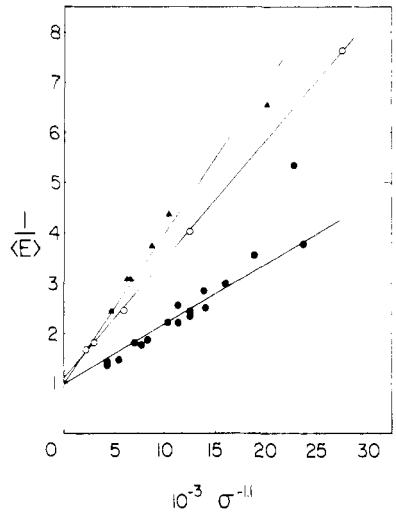

FIGURE 1: The variation of reciprocal energy transfer ($1/\langle E \rangle$) from a donor bound to dimyristoyllecithin vesicles as a function of the −1.1 power of surface density ($\sigma^{-1.1}$) of acceptors. The experimental details are given in the text under Materials and Methods. The data for respective donors and acceptors shown are as follows: (○) DDA to rhodamine-labeled cytochrome b_5; (▲) NPP to DDA; (●) NPP to TNP-PE. The line drawn through (○) is the best fit by a least-squares analysis. Lines through (▲) and (●) were drawn by inspection as the best fit to the data with an intercept of 1.0.

Our analysis assumes that all donor and acceptor molecules are oriented to the outer vesicle surface. The evidence that this is the case for TNP-PE was discussed above; that for cytochrome b_5 and NPP has been previously presented (Fleming & Strittmatter, 1978). To show that DDA is also oriented toward the outer surface of the vesicle, an experiment using DDA as the donor and rhodamine-labeled cytochrome b_5 as the acceptor was performed as described under Materials and Methods. The presence of the acceptor on the outside of the vesicle was ensured by the established position of the cytochrome b_5 heme peptide domain when the cytochrome is bound to vesicles. As shown in Figure 1 a least-squares best fit line through the data points obtained with this donor–acceptor pair intercepts the ordinate at 1.1 (i.e., $\alpha = 0.09$). This result indicates that essentially all of the DDA molecules were equally subject to excitation energy transfer to the acceptor and therefore were oriented at the same surface of the bilayer.

From the slopes of the straight lines in Figure 1 for transfer to DDA and TNP-PE, one can compute values of r_{min} for assumed values of h, using eq 7. The results of such a computation are plotted in Figure 2, along with the corresponding values of A_{ex} derived from eq 5 above. The results for the two

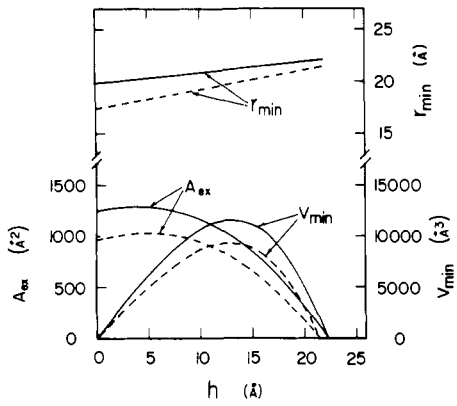

FIGURE 2: The variation of r_{min}, A_{ex}, and V_{min} as a function of h for either DDA (—) or TNP-PE (- - -) as the acceptor. The curves for r_{min} were calculated by using eq 29 and 41 from Koppel et al. (1979) using the parameters described in the text for each acceptor. The curves for A_{ex} and V_{min} were generated by using eq 5 and 7, respectively, given in the text.

different acceptor groups are in close agreement.

As expected, the values of r_{min} displayed in Figure 2 are relatively insensitive to the assumed value of h. They vary with h at all only through an effect of vesicle curvature contained in the factor $R/(R - h)$. Thus, even without additional information, we have an unambiguous estimate of r_{min} of 20 ± 2 Å. We can, however, put limits on the possible value of h by putting limits on the possible value of A_{ex}.

A_{ex} for the NPP may be estimated by the following reasoning. For the membrane-binding nonpolar segment of cytochrome b_5, the predicted secondary structure contains both segments of helix and extended β structure (Fleming et al., 1978; Dailey & Strittmatter, 1978). Also, the insertion of NPP into phospholipid bilayers reaches a saturation level when only seven or eight outer monolayer phospholipid molecules per protein molecule are present (Fleming & Strittmatter, 1978). When one is given this information, a reasonable estimate of the excluded area on the bilayer surface would be the cross-sectional area of a polypeptide α helix or extended β structure which can be surrounded laterally by seven to eight phospholipid molecules. Such a circular area would have a diameter of approximately 16 Å to give a value of A_{ex} equal to ~200 Å^2. Inspection of Figure 2 shows that this value of A_{ex} correlates with a depth (h) of 20 and 21 Å with TNP-PE and DDA acceptors, respectively.

Alternately, one can put limits on the possible combinations of h and A_{ex} by considering the molecular volumes corresponding to these two dimensions. V_{min} is defined as the volume of the portion of the protein molecule located between radii R and $R - h$ in the vesicle membrane. Since, in general, the protein molecule could extend above R and below $R - h$, V_{min} is the minimum value possible for the protein volume. Values of h and A_{ex} can be excluded if they predict a V_{min} greater than the total volume of a protein molecule.

As a reasonable estimate of V_{min} we take the volume between R and $R - h$ subtended by area A_{ex} at radius R [see Figure 1, Koppel et al. (1979)], i.e.

$$V_{min} = \frac{A_{ex}}{4\pi R^2}\left[\frac{4}{3}\pi R^3 - \frac{4}{3}\pi(R - h)^3\right] \quad (8)$$

$$= A_{ex}h\left(1 - \frac{h}{R} + \frac{h^2}{3R^2}\right)$$

Values of V_{min} calculated from eq 8 are also plotted in Figure 2. To estimate the actual volume of the NPP, we used a molecular weight of 4535 for those residues that may be within the membrane [residues 94–133; Fleming et al. (1978)] and an average partial specific volume of 0.74 to give a molecular volume of 5571 Å^3. Figure 2 shows that there are two separate ranges of values for h in which $V_{min} \leq 5571$ Å^3: a low range of h below 5 Å and another above 19 Å. The most likely interpretation is that the higher values of h above 19 Å are the correct ones, in good agreement with those obtained above (20 and 21 Å) from a consideration of A_{ex} only. In any case, the error introduced by assuming A_{ex} equals 0 would be small. As shown in Figure 2 as A_{ex} approaches 0, h increases by only about 1 Å to give depths of 21.4 and 22.2 Å with TNP-PE and DDA, respectively.

We have fit the data to a simplified theory of energy transfer in which κ^2, the orientation factor for the donor and acceptor of interest, was assumed equal to 2/3, and we have used empirical analytical expressions to achieve this result. We will now fit the data to a complete theory of energy transfer which takes into account the possible relative orientations of donor and acceptor and thus variations in κ^2. Equations 19–20d in Koppel et al. (1979) show that the orientation factor, κ^2, may be expressed as a function of $\overline{\cos^2\theta'}$ and $\langle\cos^2\theta''\rangle$, the dynamic and ensemble averaged reorientation parameters, for both the donor and acceptor. As the theory is formulated, $\overline{\cos^2\theta'}$ incorporates all reorientations of the chromophore groups about axes fixed relative to the macromolecule containing the chromophore group, including depolarization resulting from transition dipoles of mixed polarization as an effective reorientation. These relatively rapid reorientations are assumed to be azimuthally symmetric about an axis, with θ' defined as the corresponding equatorial angle. The relatively slow reorientations of the entire macromolecule within the membrane are characterized by equatorial angle θ'' relative to an axis perpendicular to the vesicle surface. Experimentally, $\overline{\cos^2\theta'}$ is estimated from the steady-state emission anisotropy determined at the long wavelength edge of the absorption band for each chromophore (see Table I). The resulting depolarization factors are time-averaged over the excited-state lifetimes of the chromophores and are thus expected to largely reflect the magnitude of the rapid chromophore reorientations, as desired, while largely excluding the effects of the slower macromolecule reorientations. Values for $\langle\cos^2\theta''\rangle$ are systematically varied from 0 to 1 to sample the static distributions of dipole orientations. Maximum and minimum values for $\langle E\rangle$ were obtained in all cases when both $\langle\cos^2\theta_D''\rangle$ and $\langle\cos^2\theta_A''\rangle$ were equal to either 0 or 1.

Because the error introduced by assuming $A_{ex} = 0$ is so small (see above), we will use this assumption in the following calculations and generate theoretical curves for $\langle E\rangle$ as a function of h. An example of a calculation using a significant value for A_{ex} with the general theory is also given below. Theoretical curves of $\langle E\rangle$ as functions of $h \leq 25$ Å for $\sigma = 4 \times 10^{-4}$ Å^{-2} are shown in Figure 3. The width of the curves represents the possible range of $\langle E\rangle$ due to the uncertainty of the orientations between donor and acceptor dipoles. The experimentally observed efficiency of transfer from NPP to TNP-PE (0.66) at this surface density indicated an h of 19–22 Å for the donor tryptophanyl residue. The energy transfer from NPP to DDA (0.39) at the same surface density indicated an h of 21–23 Å.

It can be seen from the curves in Figure 3 that the values for $\langle E\rangle$ are relatively insensitive to h at $0 < h < 15$ Å for TNP-PE and $0 < h < 10$ Å for DDA. This insensitivity is in the range of values $r_0 < 0.5$ and, therefore, would be expected as shown in Figure 5 of the preceding paper (Koppel et al., 1979). As discussed in that paper, the accuracy of

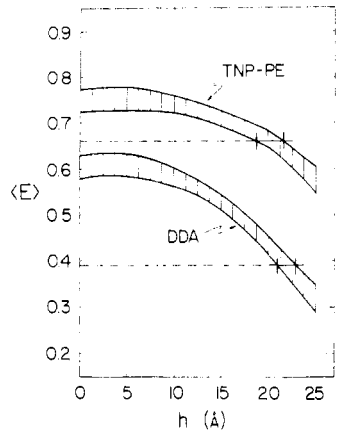

FIGURE 3: Variation of calculated energy transfer ($\langle E \rangle$) as a function of depth (h) of donor at an acceptor surface density of 4×10^{-4} Å^2. The upper curve represents the transfer to TNP-PE as the acceptor, and the lower curve is for DDA as the acceptor. The width of the curves is due to the uncertainty of the orientations of donor and acceptor. The curves were obtained with the complete theory described in Koppel et al. (1979). The horizontal lines indicate the observed energy transfer from membrane-bound NPP to the respective acceptor at this surface density.

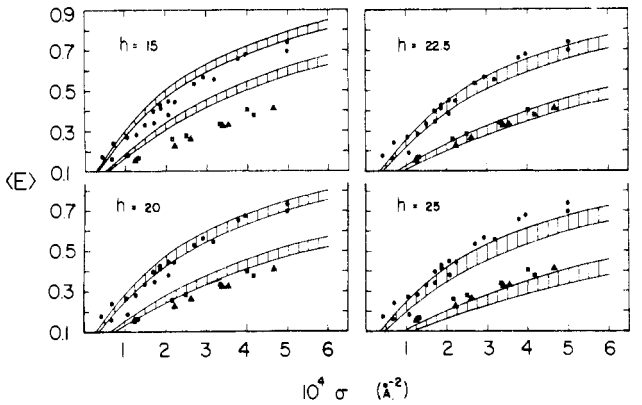

FIGURE 4: Variation of energy transfer ($\langle E \rangle$) with surface density (σ) of acceptors. For four depths of donor, h = 15, 20, 22.5, or 25 Å, the calculated energy transfer to TNP-PE (upper curve) and to DDA (lower curve) is shown. The width of the curves represents the range of values obtained from the different possible orientations of the donor–acceptor transition dipoles by using the complete theory described in Koppel et al. (1979). The same experimental data are presented in each graph for comparison. The respective donor–acceptor pairs are as follows: (●) NPP to TNP-PE; (■) cytochrome b_5 to DDA; (▲) NPP to DDA.

determined distance, h, is maximized at values of r_{min} equal to the limiting value of R_0. This is more nearly the case for the NPP–DDA pair where the limiting R_0 = 25.7 Å than for the NPP–TNP-PE pair with a limiting R_0 = 31.9 Å. For the present experimental system with cytochrome b_5, DDA is an ideal acceptor. For other experimental systems which may have an apparent h < 15 Å, it would be necessary to choose an acceptor with a smaller limiting R_0 or, alternately, place the acceptor in a position outside of the membrane or at the inner surface where the r_{min} would be greater.

The variation of efficiency of energy transfer with surface density of acceptor is shown for both acceptors in Figure 4. The calculated efficiency of transfer for four different values of h is shown. Again, the width of the curves represents the range of values obtained due to the uncertainty of the orientations of donor and acceptor. The data for transfer to DDA from either NPP or whole cytochrome b_5 show a best fit to the curve calculated for h = 22.5 Å and clearly do not fit the 15-, 20-, or 25-Å curves. The data for transfer from NPP to TNP-PE would fit a calculated curve for the region between

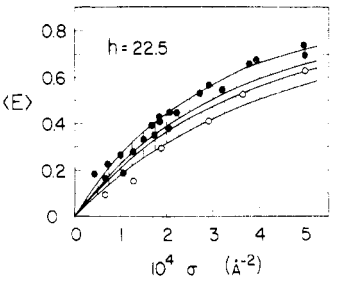

FIGURE 5: Comparison of energy transfer with and without excluded surface area for acceptor. The upper shaded curve shows the calculated energy transfer variation with surface density of TNP-PE for a donor depth (h) of 22.5 Å. The lower shaded curve shows the calculated energy transfer for the same depth and acceptor but with the TNP-PE being excluded from an 800-Å^2 surface area directly above the donor. Both curves were generated by using the complete theory described in Koppel et al. (1979). The experimental data are for the following donor–acceptor pairs: (●) NPP to TNP-PE; (○) cytochrome b_5 to TNP-PE.

20 and 22.5 Å but not 15 or 25 Å.

Such a limited range of values for h is possible in this case because the EA is relatively low for both donor and acceptor (see Table I). Other experimental systems where polarization measurements show that this is not the case would result in a greater range of possible values for h. These values of h agree very well with the values of h calculated by a simpler analytical expression above, thus demonstrating the validity of that method of data analysis.

As indicated above and in Figure 4, both NPP and whole cytochrome b_5 show the same efficiency of transfer to DDA. However, the transfer from cytochrome b_5 to TNP-PE was found to be less than that from NPP to TNP-PE. In fact, that data fit reasonably well a theoretical curve generated by using an r_{min} of 26 Å (not shown). One difference between the two acceptors is that DDA is a neutral molecule whereas TNP-PE is anionic. This fact, together with the known anionic nature of the heme peptide portion of cytochrome b_5 (Ozols & Strittmatter, 1969), provides a possible explanation for the observed lower energy transfer from whole cytochrome b_5 to TNP-PE. Because of the repulsion of like charges one might expect the anionic TNP-PE to be excluded from the surface area of the membrane that is juxtaposed to the anionic heme peptide portion of cytochrome b_5. The energy transfer assuming that TNP-PE was excluded from an area of 800 Å^2 [corresponding to the dimensions of the heme peptide; Mathews et al. (1972)] and that the donor was at a depth of 22.5 Å is shown in Figure 5. This curve provides a reasonable fit to the data except at the lower surface densities of acceptor where the concentrations of acceptor are more difficult to determine accurately.

The complete set of data described above indicates that the fluorescent tryptophanyl residue in membrane-bound cytochrome b_5 is 20–22 Å below the surface positions of the aromatic moieties of DDA or TNP-PE. The fact that these two acceptors have different spectral characteristics and therefore different characteristic distances of transfer (R_0) but gave essentially the same result lends confidence to the method.

Because the values for the spectral overlap integral (see Table I) are different for the two acceptors, the variation of calculated energy transfer as a function of quantum yield of the donor is different for each acceptor. This provided a means to confirm a previous observation (Fleming & Strittmatter, 1978) that only one of the four tryptophanyl residues in cytochrome b_5 is fluorescent. The apparent "best fit" r_{min} for various assumed quantum yields of donor(s) is shown in Figure 6. If a single tryptophan were fluorescent, the quantum yield

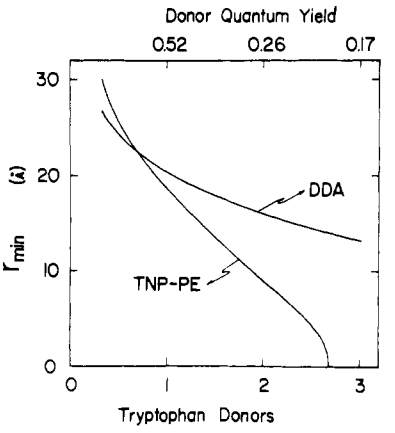

FIGURE 6: Dependence of r_{min} between surface acceptors and nonpolar peptide donor on quantum yield of donor fluorophor. The "best fit" apparent r_{min} is plotted against quantum yield of donor(s) for both acceptor molecules, TNP-PE and DDA. Values for r_{min} were calculated from eq 4, with R_0 corresponding to the appropriate ϕ_D and r_0 determined graphically from a plot of $n_0^{50\%}$ (the value of n_0 at which $\langle E \rangle = 0.5$) vs. r_0^2 [see Figure 4 in Koppel et al. (1979)]. Values of $n_0^{50\%}$ were determined by assuming, for simplicity, an absence of curvature (i.e., $R >> h$), in which case $n_0^{50\%} = \pi R_0^2 \sigma^{50\%}$. Values for $\sigma^{50\%}$ of 2.66×10^{-4} and 6.2×10^{-4} Å^{-2} were used for the TNP-PE and DDA acceptors, respectively. A single fluorescent tryptophanyl residue in NPP is assigned a quantum yield of 0.52; for two fluorophors of equal quantum yield each would have a value of 0.26, etc.

for that donor would be 0.522, for two fluorophors of equal quantum yield each would have a value of 0.26, etc., and the abscissa in Figure 6 is labeled accordingly. The two acceptors give essentially the same result only for the case of a single donor within the NPP. This result is consistent with the finding that the fluorescence of NPP can be eliminated by oxidizing one tryptophanyl residue in the peptide (Fleming & Strittmatter, 1978).

A schematic representation of the donor and acceptor molecules used in these experiments is shown in Figure 7. The aromatic moieties of the two acceptors were placed at the surface of the membrane within the area which would be the glycerophosphate region of a phospholipid membrane. The emission wavelength maximum at 520 nm for vesicle-bound DDA indicates that its environment is similar in polarity to that of methanol and therefore located in the glycerophosphate region of the membrane (Waggoner & Stryer, 1970). We have assumed that, because of the structural similarity between TNP-PE and dansylphosphatidylethanolamine, the trinitrophenyl moiety would be positioned as the dansyl group is when these phospholipid derivatives are bound to a phospholipid bilayer (Waggoner & Stryer, 1970). The nonpolar peptide portion of cytochrome b_5 is shown inserted within the membrane to a depth of approximately 21 Å. This represents the depth of the fluorescent tryptophanyl residue below the positions of the acceptors and a minimum depth for the peptide which may extend farther into the membrane. The uncertainty of the position of the acceptor chromophores within the phospholipid head group region limits the accuracy of the determined donor depth below the aqueous interface. Estimates of the glycerophosphorylcholine region of the bilayer using several different techniques show a thickness of approximately 8 Å (Small, 1967; Levine & Wilkins, 1971; Hauser et al., 1976; Worcester, 1976). If the acceptors were positioned at the inner edge of this head group region, the range of possible depths of the cytochrome b_5 tryptophanyl residue below the aqueous interface would extend to almost 30 Å. In the absence of more definitive data on the exact position of the acceptor chromophore, we prefer to use the most direct interpretation

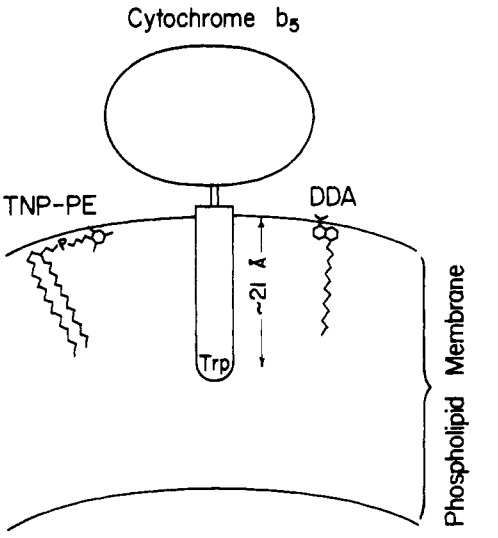

FIGURE 7: Diagram of cytochrome b_5 and fluorescent energy acceptors inserted in a phospholipid membrane. The relationships of size are proportionately to scale. The fluorescent tryptophanyl residue in cytochrome b_5 is placed approximately 21 Å below the surface of the membrane.

of the results that the tryptophanyl donor is about 21 Å below the "surface" of the membrane. A depth of 21 Å would place the fluorescent tryptophanyl residue near the middle of a phospholipid bilayer where interactions with the lipid would be confined to the hydrocarbon terminal sections of the phospholipid acyl chains. The increased emission quantum yield and emission wavelength maximum of 335 nm for membrane-bound cytochrome b_5 are consistent with the fluorescent tryptophanyl residue of the protein being within this hydrophobic region of the bilayer. A more complete characterization of the structure of this portion of cytochrome b_5 within the membrane awaits a definitive placement of the carboxyl terminus and other residues either at a surface or within the phospholipid bilayer.

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Equilibrium Binding of [^{3}H]Tubocurarine and [^{3}H]Acetylcholine by *Torpedo* Postsynaptic Membranes: Stoichiometry and Ligand Interactions†

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ABSTRACT: Studies are presented of the equilibrium binding of [^{3}H]-*d*-tubocurarine (dTC) and [^{3}H]acetylcholine (AcCh) to *Torpedo* postsynaptic membranes. The saturable binding of [^{3}H]dTC is characterized by two affinities: K_{d1} = 33 ± 6 nM and K_{d2} = 7.7 ± 4.6 μM, with equal numbers of binding sites. Both components are completely inhibited by pretreatment with excess α-bungarotoxin or 100 μM nonradioactive dTC and competitively inhibited by carbamylcholine with a K_I = 100 nM, but not affected by the local anesthetics dimethisoquin, proadifen, and meproadifen. The biphasic nature of [^{3}H]dTC binding was unaltered in solutions of low ionic strength and by preparation of *Torpedo* membranes in the presence of *N*-ethylmaleimide, a treatment which yields dimeric AcCh receptors. dTC competitively inhibits the binding of [^{3}H]AcCh and decreases the fluorescence of 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium (Dns-Chol) in a manner quantitatively consistent with its directly measured binding properties. It decreases the initial rate of ^{3}H-labeled *Naja nigricollis* α-toxin binding by 50% at 60 nM with an apparent Hill coefficient of 0.58. The stoichiometry of total dTC, AcCh, and α-neurotoxin binding sites in *Torpedo* membranes was determined by radiochemical techniques and by a novel fluorescence assay utilizing Dns-Chol as an indicator, yielding ratios of 0.9 ± 0.1:0.9 ± 0.2:1, respectively. The biphasic equilibrium binding function is not unique to dTC since other ligands inhibited [^{3}H]AcCh binding in a biphasic manner with apparent inhibition constants as follows: gallamine triethiodide (K_{I1} = 2 μM, K_{I2} = 1 mM); Me_2dTC (K_{I1} = 500 nM, K_{I2} = 10 μM); decamethonium (K_{I1} = 100 nM, K_{I2} = 1.6 μM). Carbamylcholine, however, inhibited [^{3}H]AcCh binding with a single K_I = 100 nM. The observed competition between those ligands and [^{3}H]AcCh cannot be completely accounted for by competitive interaction with two different affinities, and the deviations are discussed in terms of the positive cooperativity of the [^{3}H]AcCh binding function itself. It is concluded that dTC binds only to the AcCh sites in *Torpedo* membranes and that those sites display two affinities for dTC but only one for AcCh.

The binding of acetylcholine (AcCh)[1] or other cholinergic agonists by nicotinic cholinergic receptors results in increased permeability of the plasma membrane to cations. Reversible antagonists of this response have been classified as either competitive or noncompetitive on the basis of their alteration of steady-state agonist dose-response relations. *d*-Tubocurarine (dTC) inhibits agonist-induced depolarizations of vertebrate skeletal muscle (Jenkinson, 1960) and of *Electrophorus* (Higman et al., 1963) and *Torpedo* (Moreau & Changeux, 1976) electroplax in a manner consistent with competitive antagonism, while local anesthetic aromatic amines and the piperidine alkaloid histrionicotoxin are examples of noncompetitive antagonists (Bartels & Nachmansohn, 1965; Kato & Changeux, 1976). While the classification of antagonist mechanisms based upon the analyses of depolarization responses may be fortuitous [see, for example, Hubbard et al.

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received July 19, 1979.* This research was supported by U.S. Public Health Service Grant NS 12408 and by a grant from the Sloan Foundation. R.R.N. is supported by U.S. Public Health Service Predoctoral Training Grant GM 02220, and J.B.C. is a recipient of U.S. Public Health Service Research Scientist Career Development Award NS 00155.

[1] Abbreviations used: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; DFP, diisopropyl phosphofluoridate; Dns-Chol, 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium; dTC, *d*-tubocurarine; meproadifen, 2-(diethylmethylamino)ethyl 2,2-diphenylvalerate; TPS, *Torpedo* physiological saline (250 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM NaP_i, pH 7); TPS–0.1% BSA, TPS supplemented with 0.1% bovine serum albumin.

0006-2960/79/0418-5464$01.00/0