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Determination of the Topography of Cytochrome b_5 in Lipid Vesicles by Fluorescence Quenching†

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ABSTRACT: Cytochrome b_5, a protein isolated from the endoplasmic reticulum by detergent extraction, interacts spontaneously with small unilamellar phosphatidylcholine vesicles. When the vesicles are made from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), the tryptophan fluorescence of the cytochrome is enhanced, and when they are made from 1-palmitoyl-2-(dibromostearoyl)phosphatidylcholine (BRPC), the fluorescence is quenched. A series of BRPC were synthesized with bromine atoms at the 6,7, 9,10, 11,12, or 15,16 positions. The vesicles synthesized from each of these lipids were similar in size to those made from POPC. The relative fluorescence intensities of the cytochrome b_5 in POPC and 6,7-, 9,10-, 11,12- and 15,16-BRPC were 100, 19.4, 29.4, 37.1, and 54.0, respectively. These data suggest that the exposed tryptophan(s) is (are) at a depth of 0.7 nm below the surface of the vesicle. Bromine is a collisional quencher; hence, these data may indicate the relative position of the lipid annulus around the protein rather than the depth of the protein below the average vesicle surface. Cytochrome b_5 contains three potentially fluorescent tryptophans, and determinations of fluorescent quantum yield indicate all three are fluorescent with an average quantum yield, when in POPC vesicles, of 0.21. Fluorescence lifetime measurements by the demodulation technique indicated heterogeneity of fluorescence lifetimes in all vesicles. The lifetimes in the BRPC vesicles ranged from 2.0 to 2.4 ns compared to a value of 3.3 ns in POPC. Quenching of fluorescence in vesicles composed of mixtures of POPC and a BRPC indicated that the quenchable tryptophan(s) was (were) well shielded from the bromo lipid with perhaps only a 75° angle of approach. This study suggests that specifically brominated lipid can be used to determine the depth and exposure of tryptophans in membrane binding domains of proteins.

Cytochrome b_5, a membrane protein found in the endoplasmic reticulum, has long been known to be involved in the biosynthesis of unsaturated fatty acids (Holloway, 1983), and it has also been shown to be involved in other aspects of lipid metabolism (Paultauf et al., 1974; Nagai et al., 1983; Grinstead & Gaylor, 1982; Vatsis et al., 1982) and in the metabolism of xenobiotics (Brunstrom & Ingelman-Sundberg, 1980; Waxman & Walsh, 1983). In spite of these important roles, the structure of cytochrome b_5 in the membrane is still the subject of some controversy.

Using photoactivatable phospholipids, Takagaki et al. (1983a,b) showed the orientation of the hydrophobic domain depends upon the reconstitution conditions. The hydrophobic domain can either span the bilayer (in the "nontransferable form") or fold back the carboxyl terminus to the external surface (in the "transferable" form). Enoch et al. (1979)) also made these distinctions, but their more recent reports have suggested that both forms had the carboxyl terminus on the external surface (Dailey & Strittmatter, 1981).

The membrane binding domain of cytochrome b_5 contains the amino acid sequence Pro-Ser-Trp-Trp-Thr-Asn-Trp-Leu (Fleming et al., 1978), and tryptophan fluorescence has been used to explore topology. Fleming et al. (1979) used fluorescence energy transfer from tryptophan residue(s) in the hydrophobic domain to trinitrophenyl and to dansyl groups coupled onto the vesicle surface. The fluorescent tryptophan(s)

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in both vesicle-bound cytochrome b_5 and vesicle-bound nonpolar segment was (were) calculated to have an average location 2–2.2 nm below the surface of the vesicle bilayer. Kleinfeld et al. (1982) using energy transfer from cytochrome b_5 to a variety of anthoyloxy fatty acids also concluded that tryptophan fluorescence originated near to the center of the bilayer.

The tryptophan fluorescence of cytochrome b_5 has been under investigation in our laboratory, and we showed that the fluorescence was quenched when the protein was incorporated into 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine (Leto et al., 1980). Later, quenching was also seen with 1-palmitoyl-2-(9,10-dibromostearoyl)phosphatidylcholine (9,10-BRPC)[1] and 1-palmitoyl-2-(6,7-dibromostearoyl)phosphatidylcholine (6,7-BRPC) (Holloway et al., 1982). These brominated lipids were synthesized to determine the depth of the fluorescent tryptophan in the lipid bilayer. We have recently synthesized 11,12-BRPC and 15,16-BRPC to extend this series.

In this paper we compare the quenching efficiency of tryptophan fluorescence by 6,7-BRPC, 9,10-BRPC, 11,12-BRPC, and 15,16-BRPC and compare the quenched intensity to that seen when the protein is incorporated into 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). If the assumption can be made that the tryptophan will be maximally quenched when it is within the Van der Waals radius of the bromine atoms (Steiner & Kirby, 1969), then we suggest that the order of quenching efficiency indicates there is more quenchable tryptophan fluorescence originating near the surface of the vesicle than deep within the lipid bilayer. A preliminary report of this work has been presented (Holloway et al. 1984).

Materials and Methods

Isolation of Cytochrome b_5. Cytochrome b_5 was isolated by the method of Ozols (1974) except that an additional gel filtration step in deoxycholate was introduced.

Preparation of 15-Octadecenoic Acid. *cis*-11-Tetradecenol (5 g) (Sigma Chemical Co., St. Louis, MO) was converted to the mesylate (Baumann & Mangold, 1964), which was subjected to two cycles of malonic ester synthesis (Spener & Mangold, 1973) to yield, after chromatography upon silicic acid, 15-octadecenoic acid (1.8 g). The material was characterized by gas chromatography of the methyl esters, generated by diazomethane treatment, on OV-17 and Silar 10C (Applied Science Laboratories, College Park, MD). In addition, the 15-octadecenoic acid was reduced by catalytic hydrogenation and was also oxidized by periodate–permanganate (Scheuerbrandt & Bloch, 1962) to locate the position of the double bond. The cleavage fragments from the oxidation were compared by gas chromatography with the dicarboxylic acid cleavage fragments from oleic acid and from nervonic acid (15-tetracosenoic acid).

Bromination of Octadecenoic Acids and Synthesis of BRPC. The various octadecenoic fatty acids petroselenic (6-octadecenoic), oleic (9-octadecenoic), *cis*-vaccenic (11-octadecenoic) (all from Nu Check Prep Inc., Elysian, MN), and 15-octadecenoic acid were converted to their dibromo derivatives by treatment with 1 equiv of bromine in hexane at −10 °C. The resultant fatty acids, which were free of the parent unsaturated fatty acid by gas chromatography, were used to synthesize the corresponding BRPCs (Boss et al., 1975) and were then purified by medium-pressure (100 psi) chromatography on oxalate-treated silicic acid. The following criteria were used for estimating the purity of the BRPC: thin-layer chromatography in chloroform–methanol–water (65:25:4), gas chromatographic analysis on OV-17 of the fatty acid methyl esters generated by transmethylation with methanolic HCl and the phosphate:bromide ratio (after digestion). The bromide content of the samples was estimated by incubation of the lipid sample, or samples of 1,12-dibromododecane, in 5% methanolic potassium *tert*-butoxide at 125 °C. After 1 h the solution was acidified and made 0.25% in Triton X-100, and the free bromide was estimated by the gold chloride method (Tietz, 1970).

Preparation of Lipid Vesicles. Cyclohexane solutions of the PC (60 mg) were lyophilized overnight, and vesicles were prepared by sonication in 10 mM Tris HCl–0.1 mM EDTA (pH 8.0 at 20 °C) in an ice bath, except for the 15,16-BRPC, which was kept in a bath at 20 °C during sonication. The vesicles were isolated by centrifugation (Barenholz et al., 1977). The optimum centrifugation times and speeds for the BRPC vesicles were determined by sequential centrifugations of the sonicated lipid and monitoring of the upper portions of the centrifuge tube by 90° quasi-elastic laser light scattering (Nicomp Instruments Inc., Santa Barbara, CA) until a homogeneous population was obtained. By this procedure an optimal speed of 20000 rpm (257000*g*) for 20 min in a ty 65 rotor was determined.

Characterization of Lipid Vesicles. The lipid vesicles were characterized by quasi-elastic laser light scattering and by gel filtration. Gel filtration of a mixture of POPC, 6,7-BRPC, and 15,16-BRPC on a mixed-bed column of Sephacryl 300 and Sephacryl 1000 was performed, and the fractions containing the lipid were converted to the fatty acid methyl esters and analyzed by gas chromatography. The ratio of oleate:6,7-dibromostearate:15,16-dibromostearate across the elution profile was determined. On this column the vesicles had an elution volume of 24 mL, and the void volume and included volume were 13 and 31 mL, respectively. Trapped-volume measurements were made with 6-carboxyfluorescein (Weinstein et al., 1977), except that the external fluorophore was removed by centrifugation on "minicolumns" (Fry et al., 1978).

The densities of vesicles made from 6,7-BRPC and 15,16-BRPC were compared by sucrose density gradient centrifugation. A mixture of 0.5 μmol of the two lipid vesicles was applied to a step sucrose density gradient (26, 30, and 38% w/w in 10 mM Tris-HCl–50 mM KCl, pH 8.0 at 20 °C). The gradients were centrifuged in a ty 65 rotor at 40000 rpm for 16 h at 20 °C, and fractions were collected. The lipid-containing fractions were located by light scattering in a Perkin-Elmer MPF44A, and each fraction was extracted by the Bligh & Dyer (1959) procedure. The chloroform-rich layer was evaporated, an internal standard of heneicosanoic acid was added, and the sample was methylated and analyzed by gas chromatography to determine the relative percentages of 6,7- and 15,16-dibromostearic acids.

Fluorescence Measurements. Fluorescence measurements were made with an SLM 4800 (SLM Instruments, Urbana, IL). When fluorescence lifetimes were measured with an excitation wavelength below 300 nm, the ethanol–water mixture in the Debye-Sears ultrasonic modulation tank was prepared from freshly distilled ethanol to remove fluorescent

[1] Abbreviations: PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; BRPC, 1-palmitoyl-2-(dibromostearoyl)-phosphatidylcholine; 6,7-BRPC, 1-palmitoyl-2-(6,7-dibromostearoyl)-phosphatidylcholine; 9,10-BRPC, 1-palmitoyl-2-(9,10-dibromostearoyl)phosphatidylcholine; 11,12-BRPC, 1-palmitoyl-2-(11,12-dibromostearoyl)phosphatidylcholine; 15,16-BRPC, 1-palmitoyl-2-(15,16-dibromostearoyl)phosphatidylcholine; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

contaminants present in all commercially available absolute ethanol. A solution of *p*-terphenyl in distilled ethanol, which is known to have a homogeneous lifetime of 1.05 ns (Lakowicz et al., 1981), was used in the "reference position".

Quantum Yield of Cytochrome b_5 Fluorescence. The quantum yield of tryptophan fluorescence in cytochrome b_5 was obtained by the method of Chen (1972). Emission spectra of cytochrome b_5, cytochrome b_5 plus POPC vesicles, aqueous tryptophan, or *p*-terphenyl in HPLC-grade cyclohexane were measured at one fixed detector voltage and amplifier gain on the SLM 4800 with conditions identical with those used to obtain the manufacturer's correction factors. Rhodamine G in ethylene glycol was used as a quantum counter. Excitation was at 295 nm, to avoid tyrosine absorption, and emission scans were all performed with the same cuvette from 304 to 500 nm. Ten determinations at each wavelength interval were averaged; then, the entire scan was repeated 10 times, and the data at each point were summated. Correction factors were used to eliminate wavelength dependence of the instrument and inner filter effects. The spectra were corrected for base-line drift and Raman scattering. The spectra were then integrated. The absorption spectra of the solutions were measured in an Aminco DW2 spectrophotometer. The absorption due to the heme chromophore in cytochrome b_5 was also corrected for by using literature values for apocytochrome b_5 (Strittmatter, 1960).

Results

Synthesis of BRPC. The synthesized 15-octadecenoic acid had the predicted structure based upon the following criteria. The retention time of the methyl ester was the same as that of methyl oleate in two systems, upon catalytic reduction the product had the same retention time as methyl stearate, and upon oxidation the major product (93%) had the same retention time as the product obtained from oxidation of nervonic acid and was therefore a C15 dicarboxylic acid.

Bromination of all four octadecenoic acids was complete, and the purified BRPC were greater than 99% pure by thin-layer chromatography. Analysis of the ratio of palmitate to dibromostearate in each BRPC by gas–liquid chromatography gave equivocal results because of the reduced response of the flame ionization detector to halogenated compounds and/or the thermal decomposition of the brominated fatty esters. The average values obtained for this ratio were 1.3, 1.2, 1.2, and 1.4 for the 6,7-, 9,10-, 11,12-, and 15,16-BRPC, respectively. All four BRPC gave similar ratios. More confidence can be placed in the bromine:phosphate ratios; for the 6,7- 9,10-, 11,12-, and 15,16-BRPC, these were 2.05, 1.85, 2.26, and 1.92, respectively. It can be concluded that there are no major differences in the degree of bromination of the different BRPC. This is an important conclusion as the differential quenching of cytochrome b_5 fluorescence by the different BRPC will be compared.

Characterization of BRPC Vesicles. The sizes of the vesicles prepared by sonication and centrifugation were compared by several techniques. Although the available 90° quasi-elastic laser light scattering apparatus is not eminently suitable for such a study, it was concluded that the diameters of the 6,7-, 9,10-, and 11,12-BRPC vesicles were 1.31, 1.36, and 1.25 times larger than those made from POPC. These values must not be considered to be accurate comparisons of the actual diameters but do suggest the vesicles are not grossly different in size.

Gel filtration of a mixture of three types of vesicles, POPC, 6,7-BRPC, and 15,16-BRPC, was performed. As the retention times on gas chromatography of the methyl esters of oleate, 6,7-dibromostearate, and 15,16-dibromostearate are different, it was possible to simultaneously determine the relative proportions of each lipid across the elution profile of the chromatogram. The ratios of the three fatty acid methyl esters were constant, indicating the three vesicles had very similar Stokes radii.

The trapped volumes ($\mu L/\mu mol$) for POPC and the 6,7-, 9,10-, 11,12-, and 15,16-BRPC were 0.17, 0.22, 0.21, 0.30, and 0.19, respectively; a value of 0.193 has been reported previously for POPC vesicles by using trapped ferricyanide or glucose (Roseman et al., 1978). All these data indicate that the BRPC upon sonication produced vesicles that had similar dimensions to those made from POPC.

Sucrose density gradient centrifugation of a mixture of 6,7-BRPC vesicles and 15,16-BRPC vesicles was performed, and it was found that the density of the 15,16-BRPC vesicles was slightly greater (32.8% sucrose) than that of the 6,7-BRPC vesicles (29.2% sucrose). This result indicates that the two vesicle populations are similar and that the degree of bromination of the 15,16-BRPC is greater or equal to but no *less* than that of the 6,7-BRPC.

Differential scanning calorimetry indicated that large multilamellar liposomes made from 6,7-BRPC had no phase transition between 3 and 50 °C whereas those made from 15,16-BRPC had a broad phase transition centered at 12 °C. No phase transition could be detected in small unilamellar vesicles by fluorescence depolarization measurements with diphenylhexatriene. Diphenylhexatriene would have been able to monitor the phase transitions in BRPC if there had been one, as the fluorescence intensity and fluorescence lifetimes of the probe were 70 and 80%, respectively, of the corresponding values seen in POPC.

Vesicles made from all BRPC except the 15,16-BRPC appeared to be stable at 5 °C for several days whereas those made from 15,16-BRPC precipitated out of solution at 5 °C. Accordingly, for all studies the 15,16-BRPC vesicles were prepared and stored at room temperature when they were stable for several days.

Quantum Yield of Cytochrome b_5 Fluorescence. The results of the determination of the quantum yield are shown in Table I. The value of the quantum yield of aqueous octameric cytochrome b_5, when corrections are made for the absorbance of the nonfluorescent tryptophan in the polar portion of the molecule and of the heme, is 0.10. In POPC vesicles the quantum yield is 0.21.

Fluorescence Quenching Studies. As has been reported previously (Leto et al., 1980), the fluorescence intensity of soluble, detergent-free cytochrome b_5 is enhanced when the protein binds to POPC and is decreased when it binds to BRPC vesicles. As shown in Table II, the fluorescence enhancement increases in the order 6,7-BRPC < 9,10-BRPC < 11,12-BRPC < 15,16-BRPC < POPC. Fluorescence lifetime measurements were performed to further characterize the system but were subject to a number of problems. When cytochrome b_5 alone, in the octameric form, was investigated, there was poor agreement between the lifetimes determined by the phase and modulation methods. These differences were also seen with cytochrome b_5 bound to lipid vesicles (Table II).

In contrast, compounds with known homogenous lifetimes (indole and diphenylhexatriene in ethanol) gave values that only differed in phase and modulation by 0.2–0.5 ns. These differences in phase and modulation lifetimes for cytochrome b_5 suggested that cytochrome b_5 has a heterogeneous lifetime. This conclusion was supported by the ranking of the several lifetimes: the phase lifetime was always less than the modulation lifetime, and the lifetimes measured at higher modu-

Table I: Quantum Yield of Cytochrome b_5[a]

sample	integrated fluorescence	absorbance uncorrected	absorbance corrected	quantum yield
tryptophan	3555904	0.0233	0.0233	0.14
p-terphenyl	6494621	0.0028	0.0028	0.79
Cyt b_5	8946887	0.0662	0.0317	0.10
Cyt b_5 plus POPC	9950013	0.0316	0.0162	0.21

[a] The solutions of cytochrome b_5 (approximately 2 μM) were adjusted by dilution with 10 mM Pipes (pH 7.9 at 25 °C) to give approximately equal fluorescence intensities. A stock solution of tryptophan was similarly diluted. A cyclohexane solution of *p*-terphenyl was also prepared with approximately the same fluorescence intensity. The fluorescence spectra were recorded over the range 304–500 nm in an SLM 4800. Ten determinations of signal strength were taken at each wavelength interval and averaged to generate an emission spectrum. The entire spectrum was rescanned 10 times, and the spectra were summed and corrected for wavelength dependence of the instrument, inner filter effects, base-line drift, and Raman scattering. The resultant spectra were then integrated over the emission band 315–400 nm. Absorbance spectra were measured on an Aminco DW2. For samples that contained cytochrome b_5, a correction for heme absorbance was made by using the absorbance measured at 412 nm and the ratio of absorbances at 412 and 280 nm for apocytochrome b_5. The resultant value was also corrected, by multiplying by 0.75, for the tryptophan in the heme region, which absorbes but does not fluoresce. The value of 0.14 for tryptophan (Jameson & Weber, 1981) was used to calculate quantum yields.

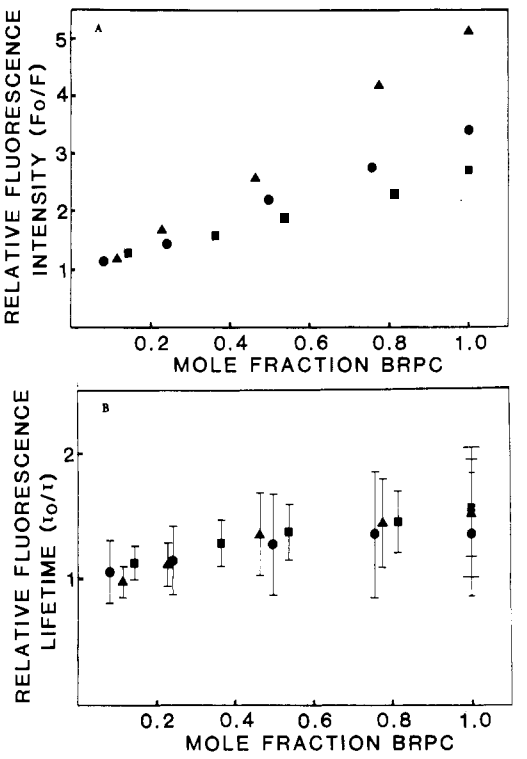


FIGURE 1: Effect of different BRPC in vesicles with POPC upon the relative fluorescence intensity and lifetime of cytochrome b_5. The vesicles containing different mol % of a BRPC with POPC were prepared and analyzed as described under Materials and Methods. The cytochrome b_5 (2 nmol) was mixed with the lipid vesicles (2 μmol of lipid phosphorus) in 10 mM Pipes–0.1 mM EDTA (pH 8.0 at 20 °C) in a final volume of 2 mL. The sample was allowed to stand at room temperature for 30 min, and its fluorescence intensity and fluorescence lifetimes were measured as described in the legend to Table I. The mixed lipid vesicles were prepared from (▲) 6,7-BRPC, (●) 9,10-BRPC, and (■) 11,12-BRPC. (A) Fluorescence intensity relative to that in POPC. The error bars are smaller than the symbols. (B) Fluorescence lifetime relative to that in POPC. The lifetime shown is the mean of that determined by the phase angle shift method at 6 Mhz and of that determined by the demodulation method at 30 Mhz.

lation frequencies were always shorter. These rankings have been shown, on theoretical grounds (Spencer, 1970) and experimental grounds (Lakowicz, 1983), to be consistent with systems that have heterogenous lifetimes. Further evidence came from a study of the wavelength dependence of lifetimes. It had been suggested by Weber (1981) that multiple lifetimes may be able to be deconvolved by determining the lifetimes at different wavelengths. A control experiment with diphenylhexatriene in DMPC showed no significant changes in lifetime as a function of wavelength whereas cytochrome b_5 alone or in POPC or DMPC showed a marked wavelength dependence, being shorter at shorter wavelengths (data not shown).

In spite of the inability to explain adequately the lifetime data, it does appear that the effect of the different BRPC on the average fluorescence lifetimes is much less than the effect on the fluorescence intensities. This unequal effect of a quencher on lifetime vs. intensity suggests that there may be considerable "static" quenching of fluorescence according to classic theory. To further evaluate the contributions of "static" and "dynamic" quenching, the fluorescence of cytochrome b_5 bound to vesicles composed of varying ratios of BRPC and POPC was measured. The vesicles were analyzed for composition by gas chromatography of the fatty acid methyl esters with both the ratio of dibromostearate:oleate and the ratio of palmitate:oleate. Figure 1a shows that over the whole concentration range 6,7-BRPC is the most efficient quencher of fluorescence intensity. Figure 1b shows the effect of these vesicles on fluorescent lifetimes, and it is apparent that the lifetimes are less affected than the fluorescent intensities over the whole concentration range. Although such data could, in

Table II: Fluorescence Intensity and Lifetimes of Cytochrome b_5 in Lipid Vesicles[a]

sample	relative fluorescence intensity (%)	fluorescence lifetime (ns)			
		phase angle shift		demodulation	
		6 MHz	30 MHz	6 MHz	30 MHz
Cyt b_5	55	1.64 ± 0.05	1.49 ± 0.05	2.14 ± 0.5	2.23 ± 0.01
Cyt b_5 + POPC	100	2.99 ± 0.10	2.59 ± 0.03	3.94 ± 0.6	3.59 ± 0.07
Cyt b_5 + 6,7-BRPC	19.4	1.38 ± 0.28	1.31 ± 0.05	4.17 ± 1.6	2.77 ± 0.25
Cyt b_5 + 9,10-BRPC	29.4	1.64 ± 0.31	1.22 ± 0.11	6.71 ± 0.9	3.17 ± 0.16
Cyt b_5 + 11,12-BRPC	37.1	1.56 ± 0.18	1.55 ± 0.06	4.95 ± 0.70	2.49 ± 0.08
Cyt b_5 + 15,16-BRPC	54.0[b]	1.79 ± 0.25	1.79 ± 0.03	ND	2.18 ± 0.19

[a] Fluorescence intensities and lifetimes were measured at 25 °C with cytochrome b_5 (Cyt b_5) (2 nmol) either alone or with one of the PC (2 μmol of lipid phosphorus) in 10 mM Pipes–0.1 mM EDTA (pH 8.1) to a final volume of 2 mL. Measurements were made with an SLM 4800 spectrofluorometer under the following conditions: for intensity measurements, excitation 295 nm (slit 8 nm) and emission 340 nm (slit 8 nm); for lifetime measurements, excitation 295 nm (slits 16, 1, and 1 nm), emission 340 nm (slit 16 nm), and no polarizers. The lipid samples used were: POPC, 6,7-BRPC, 9,10-BRPC, 11,12-BRPC, or 15,16-BRPC. [b] In the experiments with 15,16-BRPC, 3 nmol of cytochrome b_5 and 0.6 μmol of PC were used in a final volume of 2 mL. With these different ratios, the relative fluorescence of the protein in 6,7-BRPC was 19.7% of that seen in POPC vesicles, and hence, the fluorescence intensities were concentration independent over this range.

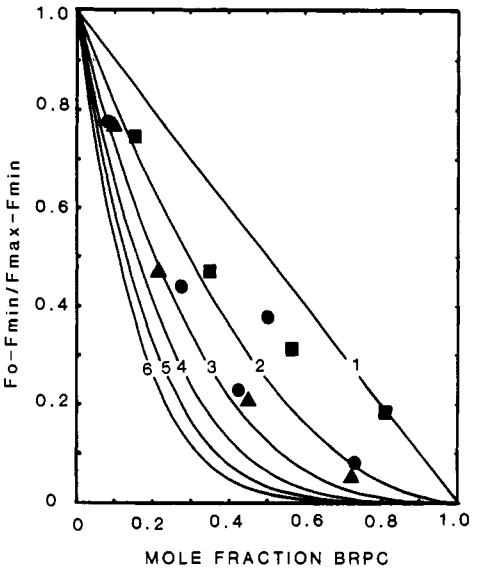


FIGURE 2: Effect of different BRPC in vesicles with POPC upon the relative maximum cytochrome b_5 fluorescence. The lines shown are the theoretical lines from the equation $F/F_0 = (1 - [BRPC])^n$. Curves for values of n from 1 to 6 are shown with increasing curvature. The symbols represent data obtained with mixed vesicles containing (▲) 6,7-BRPC, (●) 9,10-BRPC, and (■) 11,12-BRPC and are calculated from the data shown in Figure 1A.

theory, be used to calculate Stern–Volmer quenching constants, it was considered that the errors in lifetime precluded such an exercise; instead, the data were evaluated according to the techniques of London & Feigenson (1981a) where the following relationship is graphed:

$$(F - F_{min})/(F_0 - F_{min}) = (1 - [BRPC])^n$$

This is shown in Figure 2.

London and Feigenson used this analysis to determine "boundary" lipid, and it was required that the quenching be of a "static" nature with no appreciable dynamic component; this was predicted by the authors in view of the short lifetime of tryptophan. In our studies the variation of fluorescence lifetime with BRPC concentration violates the basic assumption of London and Feigenson, but a theoretical study by Keizer (1981), taking into account the "cageing" effect of the highly viscous lipid environment, generates a dynamic expression of the Stern–Volmer equation. This expression predicts that the London and Feigenson analysis will be approximately correct for both the fluorescence intensity and fluorescence lifetime measurements.

London & Feigenson (1981a) suggested that the n in the above equation can be thought of as the number of lipids close enough to the average fluorophore in the protein to result in quenching or else it is a measure of the overlap of fluorescent domains of the protein quenched by the neighboring molecules of BRPC. Thus, a small value of n indicates a small amount of overlap in domains quenched by neighboring BRPC molecules.

From Figure 2 it can be seen that the best fit for the 6,7-BRPC and 9,10-BRPC is with $n = 3$ whereas with 11,12-BRPC $n = 2$. Our calorimetric data with cytochrome b_5 in lipid vesicles (Freire et al., 1982) suggested that each cytochrome b_5 is in thermodynamic contact with 14 phospholipid molecules, and our fluorescent data suggest that 3 of these 14 sites, or a 72° angle of approach, is the angle for collisional quenching at the level of the 6,7 and 9,10 fatty acyl positions. Deeper in the membrane the tryptophan is less accessible to the bromine. Although we cannot derive association constants for lipid binding from our data, it can be seen that, from the similarity of the data for 6,7- and 9,10-BRPC (Figure 2) and the arguments of London & Feigenson (1981b), there is no major difference in affinity for cytochrome b_5 between the two lipids.

Discussion

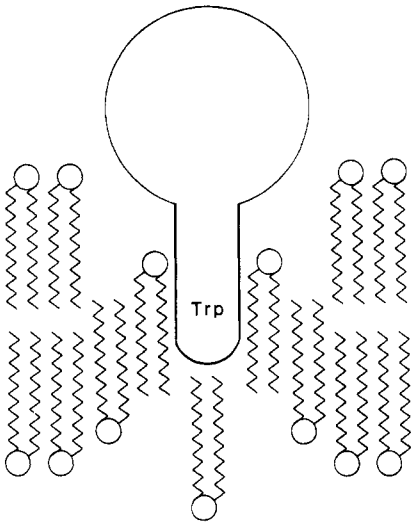
The use of fluorescence quenching with quenching agents located at discrete depths in the membrane is not a new technique. The present approach does however offer some advantages over previously used methods. Quenching of fluorescence by means of energy transfer has been used by two groups with cytochrome b_5 itself (Fleming et al., 1979; Kleinfeld et al., 1982) and by several other groups with other systems. With these studies there is the inherent problem of knowing the position of the quencher. It was originally shown by Waggoner & Stryer (1970), on the basis of fluorescence maxima in different solvents, that fluorescent chromophores can be selectively placed in different transverse regions of the bilayer. Specific studies with anthroyloxy fatty acids have given more precise data on placement but also showed that there were some exceptions; 11-(anthroyloxy)undecanoic acid and 2-(anthroyloxy)stearic acid behaved abnormally in some membranes (Haigh et al., 1979; Chalpin & Kleinfeld, 1983).

We chose bromine as a quencher of tryptophan fluorescence. Bromine is reported to be a collisional quencher and so should interact over shorter distances and, hopefully, locate the intramembrane trytophans more precisely. Another advantage of bromine is its small molecular volume, about the same as a methyl group, which is known to be well tolerated in membranes. As shown some years age (Roseman et al., 1978) and more recently (Lytz et al., 1984), bromine does not drastically perturb the membrane. The studies described in this paper reach the same conclusion. The different vesicles made from the several BRPC had similar dimensions to POPC vesicles, suggesting that the bilayer properties are not overly perturbed by the presence of bromine atoms. We would propose that the relative positions of the bromine atoms reflect the acyl-chain positions of the bromines. This was confirmed, in preliminary studies (unpublished results),[2] with the 9,10-, 11,12-, and 15,16-BRPC by X-ray diffraction experiments. The electron-density profile characteristic of phospholipid bilayers had two additional peaks of electron density with a separation expected for the two bromine atoms. Similar electron-density profiles were observed in X-ray studies of a monobromo PC (Lytz et al., 1984).

In order to compare the quenching by the different BRPC and so determine the depth of the fluorescent tryptophan(s) in the bilayer, the relative degrees of bromination of the BRPCs must be known. As all the BRPC were synthesized by the same procedure, it was expected that all would have the (theoretical) value of two bromine atoms per lipid. Although fatty acid analysis did not yield this value, because of experimental difficulties, a chemical technique for assaying bromine in organic molecules gave values between 1.85 and 2.26. The data for the 6,7- and 15,16-BRPC are particularly critical as these gave extreme values for quenching. The following is a comparison of the physical characteristics of the two vesicles: (a) bromine:phosphate by fatty acid analysis, 1.5 and 1.4 (uncorrected); (b) bromine:phosphate by bromide assay, 2.05 and 1.92; (c) trapped volumes, 0.22 and 0.19 $\mu L/\mu mol$; (d) indistinguishable Stokes radii by gel filtration;

[2] T. E. McIntosh, S. A. Simon, and P. W. Holloway, unpublished results.

Chart I



(e) equilibrium sucrose density centrifugation, 29.2 and 32.8% sucrose.

In order to use the different BRPC to determine the position of a membrane-embedded tryptophan, it is important to know which tryptophans are responsible for the observed fluorescence. Cytochrome b_5 fluorescence originates in tryptophan in the membrane binding domain. The amino acid sequence data indicate three tryptophans are present at positions 108, 109, and 112, but Fleming et al. (1978) have presented evidence that only one of these is fluorescent, specifically 109. The value of the quantum yield of aqueous cytochrome b_5 from Table I is 0.10 if all three tryptophans are fluorescent. If one tryptophan residue were fluorescent, its quantum yield would have to be 0.30. The quantum yield of cytochrome b_5 in POPC vesicles is 0.21, and if this were due to a single fluorescent tryptophan, its quantum yield would have to be 0.63; in other lipids it would be even larger. Clearly, these values are much higher than literature values of tryptophan quantum yield for which values of up to 0.15 are quoted. On the other hand, a system with THREE fluorescent tryptophans is consistent with other literature values for the quantum yield of tryptophan in other proteins and in aqueous solution. Our observation of fluorescent lifetime heterogeneity with cytochrome b_5 is in agreement with multiple tryptophans, although even a single tryptophan may show multiple lifetimes (Szabo & Rayner, 1980).

Other data also support the suggestion of fluorescence from multiple tryptophans. Jameson & Weber (1981) showed that as the pH of an aqueous solution of tryptophan was increased the fluorescence intensity and fluorescence lifetime increased in parallel and the quantum yield also increased. If the quantum yield of a tryptophan were indeed 0.63, its lifetime should be over 12 ns. This is far longer than the longest lifetime reported by Jameson & Weber (1981) and does not agree with the value of 3.3 ns that we determined for cytochrome b_5 bound to POPC vesicles. If all three tryptophans are fluorescent, the quantum yield and lifetime data for cytochrom b_5 agree quite well with literature values for other tryptophan-containing proteins.

Although cytochrome b_5 appears to be more complex in its fluorescence behavior than previously suggested, the greater quenching by 6,7-BRPC can be interpreted as indicating that *at least one* fluorescent tryptophan is located at approximately 0.7 nm below the surface of the membrane. The experiments with mixtures of BRPC and POPC suggest that the accessibility of this tryptophan is relatively restricted, with perhaps a 72° angle of approach. Only one tryptophan need be in contact with the bromine atom because energy transfer from the other tryptophan(s) could occur. The observation that the fluorescence does not go to zero even with 100% 6,7-BRPC suggests that one of the three fluorescent tryptophans may not be exposed on the surface of the hydrophobic domain (within the bilayer) or one of the fluorescent tryptophans may be poorly "coupled", due to misaligned dipoles, to the other two so that incomplete energy transfer occurs. Alternatively, there could be a discrete population of cytochrome b_5 that does not have its tryptophan residues at this position, perhaps because of motion of the cytochrome b_5 perpendicular to the plane of the membrane.

The estimated depth of the fluorescent tryptophan of 0.7 nm is much less than the value of 2 nm reported previously (Fleming et al., 1979; Kleinfeld et al., 1982). If the binding of the tryptophan is the same in all systems studied and the components of the vesicles are not influencing the geometry of the system, then the different results must be due to the nature of the quenching molecule. Both previous studies were obtained with energy-transfer methods using vesicles that had been "doped" with less than 1% of the quencher or fluorophore. Because these processes are long-range interactions, they will necessarily be reporting the depth of the tryptophan below the "average" surface of the vesicle, which would be 2 nm. In the present study quenching is occurring by a predominantly static interaction with a molecule adjacent to the tryptophan, and it appears that the tryptophan is 0.7 nm below the polar head group of this adjacent lipid molecule. If this polar head group defines the "local" membrane surface, then the tryptophan is 0.7 nm below the local surface. An alternative description would be that of the surface of the vesicle is perturbed in the region of the tryptophan and that this perturbation may, or may not, perturb the bilayer. It is of interest that there have been examples recently in which foreign molecules so perturb the bilayer that "interdigitation" occurs (McIntosh et al., 1983). It is possible that the polar head group of the cytochrome b_5 causes such a change in packing and promotes interdigitation; such a model is shown in Chart I.

The possibility exists that the bromine atoms in 6,7-BRPC are binding to the tryptophan and are displacing the protein closer to the surface than in normal membranes. The experiments with the mixed lipids indicated there was no differences in affinity between 6,7- and 9,10-BRPC and so exclude any special binding to 6,7-BRPC. Furthermore, the increase in quenching with increasing amounts of 6,7-BRPC would be expected to show some cooperativity as more BRPC appeared in the lipid annulus. If binding were occurring with 6,7-BRPC, it should also occur with the other BRPC, and so 15,16-BRPC should be a very good quencher, if indeed the tryptophan were in the middle of the bilayer.

One other possibility has been considered. If the cytochrome b_5 is in rapid motion perpendicular to the plane of the membrane, then there will be, at any one time, a distribution of protein at different depths. One would suspect, a priori, that more of the tryptophan would be at an average depth equal to the center of the bilayer. If, however, the motion were like a damped bobbing cork, then the majority of the protein would be found at the limits of the excursion, where the bilayer becomes less mobile (near carbon 6). This too would result in greater quenching by the 6,7-BRPC.

We suggest that the BRPC are monitoring the position of the cytochrome b_5 in the bilayer, just as the energy-transfer quenchers are. The difference between the two types of processes, local vs. long distance, produces the different results. The BRPC are monitoring the position of the cytochrome b_5

in the immediate lipid environment rather than relative to the average surface of the bilayer. If conformational changes or changes in "exposure" of intrinsic membrane proteins do occur in different metabolic situations, these changes may be observable by use of the specific brominated BRPC.

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Registry No. 6,7-BRPC, 81138-96-7; 9,10-BRPC, 81124-54-1; 11,12-BRPC, 96110-16-6; 15,16-BRPC, 96110-17-7; POPC, 6753-55-5; Cyt b_5, 9035-39-6; tryptophan, 73-22-3; *p*-terphenyl, 92-94-4; 6-octadecenoic acid, 4712-34-9; 9-octadecenoic acid, 2027-47-6; 11-octadecenoic acid, 143-25-9; 15-octadecenoic acid, 15737-21-0.

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