THE ROTATIONAL DIFFUSION OF CYTOCHROME b_5 IN LIPID BILAYER MEMBRANES

INFLUENCE OF THE LIPID PHYSICAL STATE

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ABSTRACT A derivative of the integral membrane protein, cytochrome b_5, has been prepared in which the native heme group has been replaced by the structurally similar rhodium(III)-protoporphyrin IX. This metalloporphyrin has a finite triplet yield with a single exponential decay time of 22 μs in water. After insertion of the metalloporphyrin into the protein, its triplet-state decay becomes strongly nonexponential with at least three equal amplitude components with time constants varying over a range of 100. The derivatized protein has been incorporated into unilamellar liposomes prepared from dimyristoyllecithin, and the rotational diffusion of the protein in the lipid bilayer has been studied at temperatures above and below the lipid phase transition temperature via triplet absorbance anisotropy decay. The anisotropy decay curves are biphasic both above and below the lipid phase transition. The rotational diffusion constant is found to be $2.4 \times 10^5\ s^{-1}$ at 35°C, and $1.1 \times 10^4\ s^{-1}$ at 10°C, both being calculated from the fast decay component. The ratio of the limiting anisotropy to the initial anisotropy is 0.6 at both temperatures. This implies a cone of restricted motion of 34° for the protein in the bilayer.

INTRODUCTION

Since the proposal of the "fluid mosaic" model for biological membrane structure by Singer and Nicolson (1), much interest has been aroused in the translational and rotational Brownian motion of membrane components (2, 3). Many cellular processes that occur at the membrane level, such as active and passive transport across membranes, electron transport, enzymatic and immunologic processes, and receptor events, are possibly dependent upon the diffusion of membrane proteins in the plane of the lipid bilayer. Although considerable evidence has accumulated in recent years for the relative high mobility of proteins in membranes (typical diffusion constants are 10^{-8}–$10^{-10}\ cm^2\ s^{-1}$) (4–19), relatively few of these studies have been done on "reconstituted" systems in which the lipid and protein components are well defined (19). In this communication, we wish to report our measurements on the rotational diffusion of cytochrome b_5 (an integral membrane protein from microsomal membranes) incorporated into bilayer membranes formed from dimyristoyllecithin. This is a one-protein and one-lipid system in which it is possible to study the rotational diffusion of the protein in the gel and

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liquid crystalline phases of the lipid. We have prepared a derivative of cytochrome b_5 in which the heme group has been replaced by the heme analogue, rhodium(III)-protoporphyrin IX. The derivative has a relatively high triplet yield (unlike the completely quenched heme) that allows the use of triplet absorbance anisotropy to study the rotational Brownian motion of the protein in the bilayer. The replacement of the heme group by rhodium(III)-protoporphyrin IX is expected to produce minimal structural changes in the protein due to the structural similarities of the two metalloporphyrins (20), and thus should offer a general method for similar studies on other heme proteins.

MATERIALS AND METHODS

Cytochrome b_5 was isolated and purified from bovine liver microsomal membranes by the use of detergents, as described by Spatz and Strittmatter (21). The apocytochrome b_5 was prepared from detergent-solubilized cytochrome b_5 according to the procedure of Strittmatter (22). Rhodium(III)-protoporphyrin IX was prepared as described by Hanson et al. (20). Rhodium(III)-protoporphyrin IX was incorporated into apocytochrome b_5 by incubation of the apoprotein with a 10-fold excess of the metalloporphyrin in 0.1 M sodium phosphate, pH 7.5, for 3 d at 4°C. The reaction mixture was then filtered through a column of Sephadex G-25 to separate the unbound metalloporphyrin from the rhodium(III)-protoporphyrin IX-apocytochrome b_5 complex (Rh-cytochrome b_5). The absorption spectrum of the Rh-cytochrome b_5 so prepared is shown in Fig. 1. Apomyoglobin was prepared by the method of Rossi-Fanelli et al. (23) and stored in 50% glycerol-0.1 M sodium phosphate, pH 7.5, at −20°C until use. The protein was incubated with an equimolar amount of rhodium(III)-protoporphyrin IX for 10 min at 20°C, and then passed through a column of Sephadex G-25 to separate unbound metalloporphyrin from the protein.

Dimyristoyl-L-lecithin was a product of Fluka AG, (Buchs, Switzerland), and was pure insofar as could be determined by thin-layer chromatography. Unilamellar dimyristoyllecithin vesicles were prepared as described by Huang (24), except that centrifugation was done at 150,000 *g* for 1 h and the gel filtration step was omitted. A typical elution pattern of the resultant liposome preparation on a lipid-equilibrated Sepharose-4B column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) is shown in Fig. 2 A. Rh-cytochrome b_5 was incorporated into the liposomes by incubating the protein and liposome preparation together at the desired lipid:protein ratio at 25°C for 12 h. The incubation buffer was 0.1 M sodium phosphate, pH 7.5. Incorporation of the protein into the liposomes is essentially complete under these conditions (25).

Fig. 3 shows a block diagram of the measurement system. The nitrogen laser (Lambda Physik 100, Lambda Physik, Goettingen, W. Germany) drove a dye laser. The output dye-laser beam was used to excite the triplet state of the rhodium(III)-protoporphyrin IX, and had a pulse duration of 5 ns and a wavelength of 500 nm with >90% vertical polarization.

The unpolarized monitoring beam (wavelength 405 nm, bandwidth 10 nm) passed through the sample at a right angle to the exciting beam and entered a polarizing beam splitter that analyzed the transmitted beam for components of polarization parallel and perpendicular to the exciting-beam polarization. The two orthogonally polarized beams from the beam splitter were focused on two identical photomultipliers (RCA Electro-Optics and Devices, Lancaster, Pa.; model 1P 28A-V1) selected to have similar cathode sensitivities. These photomultipliers were also of the antihysteresis design that is important at high light levels, as in our case. Anode voltage amplifiers of 100-MHz bandwidth drove a differential amplifier whose output was stored in a 20-MHz transient recorder (Data Laboratories Ltd., Mitcham, Surrey, England). Due to the smallness of the signal (1% transmittance change), the digital output of the transient recorder was averaged in a Fabritek signal averager for 4,096 shots (Fabritek Co., Inc., Winchester, Va.).

The anisotropy was calculated from the expression:

$$r(t) = (\Delta OD_{\parallel} - \Delta OD_{\perp})/(\Delta OD_{\parallel} + 2\Delta OD_{\perp}) \quad (1)$$

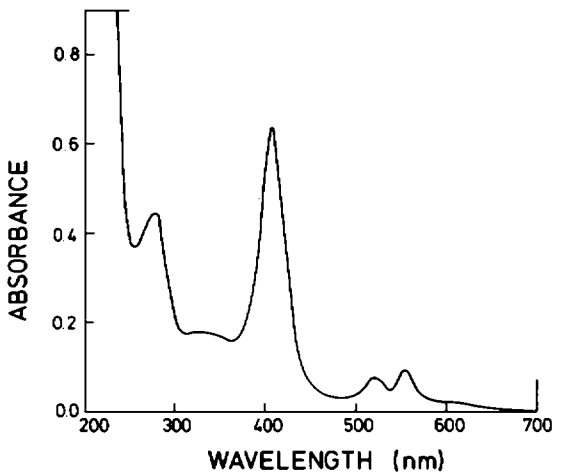

FIGURE 1

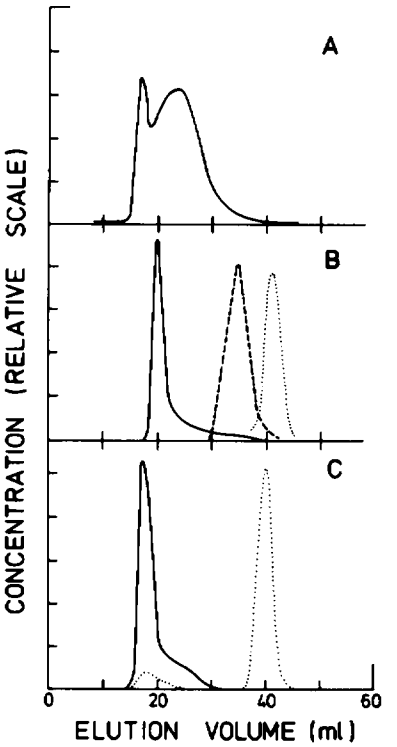

FIGURE 2

FIGURE 1 Absorption spectrum of Rh-cytochrome b_5 in 0.1 M sodium phosphate, pH 7.5, at 20°C.

FIGURE 2 Elution profiles on a Sepharose-4B column of: (A) Dimyristoyllecithin liposomes prepared as described in Methods. (B) Proteoliposomes prepared by incubation of native cytochrome b_5 or Rh-cytochrome b_5 with dimyristoyllecithin liposomes at a lipid:protein ratio of 100 (—); intact native cytochrome b_5 or Rh-cytochrome b_5 (- - - -); the hydrophilic head of cytochrome b_5 or Rh-cytochrome b_5 after subtilisin Carlsberg treatment (· · · ·). (C) Proteoliposomes prepared by incubation of native cytochrome b_5 or Rh-cytochrome b_5 with dimyristoyllecithin liposomes at a lipid:protein ratio of 100, after treatment with subtilisin Carlsberg. The lipid (—) was detected by phosphate analysis and the hydrophilic metalloporphyrin-containing head of the protein was detected by the metalloporphyrin absorbance at 413 nm (native cytochrome b_5) or 408 nm (Rh-cytochrome b_5). In all cases the column dimensions were 1.5 × 27 cm. The column was first washed with 10 vol of a 0.1-mM dimyristoyllecithin suspension in 0.1 M sodium phosphate, pH 7.5, followed by 10 vol of 10 mM Tris-HCl, pH 7.5. The eluting buffer was 10 mM Tris-HCl, pH 7.5. The temperature was 25°C.

where $\Delta OD_{\parallel}$ and $\Delta OD_{\perp}$ refer to the absorbance changes parallel and perpendicular to the exciting-beam polarization. The maximum anisotropy possible for a molecule of planar symmetry like the heme group is 0.14 (26). Typically our maximum anisotropies were 0.07. The isotropic absorbance changes were calculated from the expression:

$$\Delta OD = \Delta OD_{\parallel} + 2\Delta OD_{\perp}. \qquad (2)$$

To evaluate whether the anisotropy decay function was independent of the triplet absorption decay function in the system reported upon in this communication, we performed the following experiments: rhodium(III)-protoporphyrin IX was inserted into apomyoglobin and the anisotropy decay of the resulting complex (Rh-myoglobin) was measured at low temperatures in 95% glycerol. The triplet

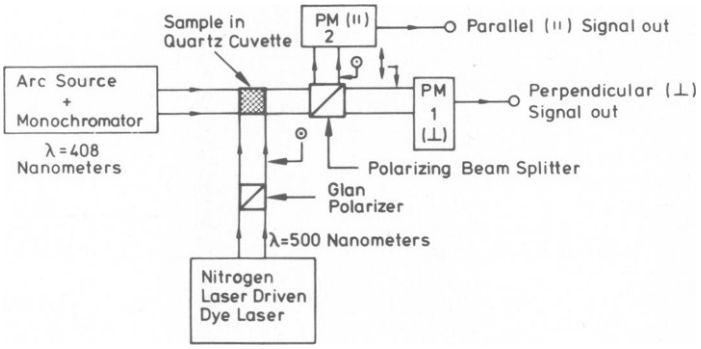

FIGURE 3 A schematic diagram of the optical system for triplet absorption anisotropy measurements. The sample cuvette had a V of 100 μl and an optical depth of 2 mm. Argon gas was continually passed over the sample to maintain an oxygen-free sample. The nitrogen laser produced a 5-ns duration pulse of 337-nm light of 2 mJ energy. The pulse drove a coumarin 307 dye laser with outpulse energy of approximately 100 μJ. The repetition rate was 20 Hz. The photomultipliers (RCA Electro-Optics and Devices; model 1P 28A-V1) had all dynodes connected. 100-MHz DC-coupled amplifiers on the anode load resistor fed the transient digitizer. The measured rise time of the photomultipliers was 5 ns.

absorption decay of Rh-myoglobin under these conditions was found to be multicomponent. Since myoglobin is, to a good approximation, a spherical molecule, the anisotropy decay should be single exponential if it is independent of the multicomponent triplet absorption decay. We verified a good fit to a single exponential for the anisotropy decay in this case. Furthermore, as seen in Fig. 6 (B and C), addition of the detergent deoxycholate to Rh-cytochrome b_5-liposomes changes the anisotropy decay curve from a two-component curve to a single-component curve, although the isotropic absorption decay function remains strongly multicomponent. It is also important in anisotropy measurements that the instrument be properly nulled, i.e., a rapidly spinning chromophore should show a null anisotropy signal. We verified that our instrument was properly nulled by measuring the anisotropy decay of rhodium(III)-protoporphyrin IX and Rh-myoglobin in water. Both these systems have anisotropy decay times that are less than the time response of our present system, and gave no anisotropy signal within an error of ± 0.005.

Support for the assumption that the transient absorption changes seen by us are due to the population of the triplet state comes from the following facts: Hanson et al. (20) reported that their rhodium(III)-porphyrin complex emitted a long-lived phosphorescence at −196°C with a quantum yield of 0.23, indicating a good triplet quantum yield for this metalloporphyrin. Fig. 4 shows a difference spectrum we measured for rhodium(III)-protoporphyrin IX in water at 20°C 100 ns after excitation. From this difference spectrum it is evident that bleaching occurs in the Soret absorption maximum, and that a new absorbance with a maximum in the 450 nm region is observed. This difference spectrum is similar to the difference spectra seen when other porphyrin triplets are generated (27). Further evidence for generation of the triplet state in our case is the reversibility of the absorption changes, and the sensitivity of the lifetime to oxygen that is known to quench triplet states with a diffusion-limited rate. We measured the rate of quenching of the triplet lifetime of rhodium(III)-protoporphyrin IX in water at 20°C and found a value of $3 \times 10^9\ M^{-1}\ s^{-1}$, which is a typical diffusion-limited rate for quenching by oxygen in water.

Because the largest absorption change was observed to occur at the Soret maximum (404 nm, see Fig. 4), we used this as the monitoring wavelength to maximize the signal, and hence technically we measured the singlet depletion due to pumping-up of the triplet state. Kinetics measured at the triplet absorption maximum of 450 nm gave the same results as the singlet depletion signal. Measurements were performed in a 0.2 × 1.0-cm rectangular quartz cuvette (Hellma Cells, Inc., Jamaica, N.Y.) that had all four sides polished. The exciting pulsed dye-laser beam was incident on the 0.2-cm end of the cuvette and focused by a 10-cm focal length lens to an area of 0.2 × 0.2 cm. The focused monitoring

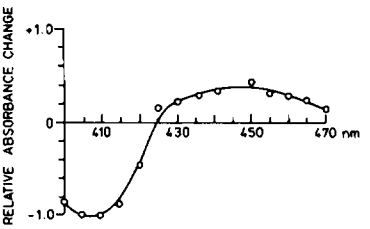

FIGURE 4 Triplet difference absorption spectrum between rhodium(III)-protoporphyrin IX and the triplet excited species. The absorption changes were measured 100 ns after the exciting pulse. Due to the 10-nm bandwidth of the monochromator, some instrumental broadening of the Soret peak at 404 nm is evident. The spectrum is arbitrarily normalized to 1.0 at the Soret peak.

beam from the arc lamp was masked by slits at the cuvette to form a 0.2 × 1.0-cm rectangle through the broad side of the cuvette. The irradiated volume was thus 40×10^{-3} cm^3 and the sample volume was 100×10^{-3} cm^3. Atmospheric oxygen was removed as follows: a small glass-coated magnetic stirring bar agitated the sample slowly while high purity (99.995%) argon was passed through the cuvette via a Teflon (Du Pont Instruments, Wilmington, Del.) tube close to the liquid surface. The stirring continued for 15 min and then the cuvette was placed in the cell holder while argon continued to pass through the cell for the duration of the experiment. In this gentle manner, which involved no bubbling or freezing, very small samples could be rigorously deoxygenated. Tests with long-lived triplet dyes indicated removal of the oxygen to a partial pressure of less than 0.001 bar. Data was evaluated on a PDP-11/20 (Digital Equipment Corp., Maynard, Mass.) via a modified nonlinear Gauss-Newton least-squares regression analysis.[1] The fitting routine essentially minimized the variance between the data and the fitting function. The convergence criterion was that successive iterations have their parameters differing by <0.1% from the next to the last iteration. Although the program assigned error values for the fitted parameters, in our experience the systematic errors are far larger than the statistical errors, and we believe that all values for the time constants should be assigned a ±5% variability. The isotropic absorbance and the anisotropy function were computed from two files created for each set of data: the absorption signal with polarization parallel to the exciting beam; and the difference signal between the parallel and perpendicular signals, which was measured by the difference amplifier.

RESULTS

In preliminary experiments we observed that apocytochrome b_5 could bind protoporphyrin IX. However, although protoporphyrin IX has a high triplet yield and thus should be an ideal candidate as a probe, it was also found that when the apocytochrome b_5-protoporphyrin IX complex associated with lipid membranes, the protoporphyrin IX was transferred to the lipid and was no longer associated with the protein. In further experiments it became evident that a metalloporphyrin with a six-coordinate metal was necessary for firm binding to apocytochrome b_5. Rhodium(III)-protoporphyrin IX is a metalloporphyrin that satisfies this requirement and also has a finite triplet yield, as discussed earlier. To prove that the metalloporphyrin remained bound to the protein in the presence of lipid, a series of experiments was done in which Rh-cytochrome b_5 and native cytochrome b_5 (as a control) were bound to dimyristoyllecithin unilamellar vesicles, and the resulting proteoliposomes were treated with the proteolytic enzyme subtilisin Carlsberg. This proteinase does not permeate the proteoliposomes and cleaves the cytochrome b_5 between the hydrophilic metalloporphyrin-containing "head" and

[1]Avery, L. Work to be published.

the hydrophobic "tail" that is embedded in the lipid. Subsequent gel filtration of the proteinase-treated proteoliposomes quantitatively separated the hydrophilic head groups of the protein from the proteoliposomes made up of lipid bilayer and the hydrophobic tail groups of the protein. The results of these experiments are shown in Fig. 2, B and C. From Fig. 2 C it is evident that the metalloporphyrin is always associated with the protein and not with the lipid in the proteoliposomes formed from cytochrome b_5 or Rh-cytochrome b_5.

Another point evident from Fig. 2 is that our method of preparation of proteoliposomes in this work resulted in an asymmetric distribution of the protein in the bilayer. Because subtilisin does not permeate the proteoliposomes, the fact that almost no hydrophilic head groups of the protein appeared associated with the lipid peak indicates that all of the protein was located in the outer monolayer of the bilayer vesicles that was accessible to the proteinase. This result is similar to the observation made earlier by Rogers and Strittmatter (28). Another interesting observation from Fig. 2 is that the proteoliposomes (lipid:protein = 100), when passed through Sepharose 4B, elute as an almost homogenous peak at a volume intermediate between the elution volumes for large multilamellar liposomes and small (250-Å diameter) unilamellar lipid vesicles. An increase in the size of unilamellar lipid vesicles after binding to cytochrome b_5 was also reported by Dufourcq and co-workers (29). From the preceding information, it is evident that while the lipid:protein ratio is 100:1 when the system is taken as a whole, it is actually about 50:1 when only the outer monolayer of the proteoliposomes is considered, for there is no protein in the inner monolayer. We have examined the phase transition behavior of the lipid in homogenously mixed cytochrome b_5-dimyristoyllecithin liposomes prepared by a cholate-dialysis procedure similar to that of Kagawa and Racker (30). These proteoliposomes having a lipid:protein ratio of about 50:1 show a phase transition that occurs between 18 and 25°C with a transition midpoint at 21°C.[2]

Fig. 5 shows the triplet decay of rhodium(III)-protoporphyrin IX alone in water and also inserted into cytochrome b_5 and myoglobin. The data have been plotted on a log absorption versus log time plot because of the long time-range over which significant triplet decay occurs. In Fig. 5 the crosses are the experimental data for the triplet decay of rhodium(III)-protoporphyrin IX alone in water. The dashed line is the computer-derived single exponential fit to the triplet decay of the metalloporphyrin in water. On the log-log plot, the dashed curve can simply be translated along the time axis without change in shape to compare qualitatively the fit of other data points for Rh-cytochrome b_5 and Rh-myoglobin with a single exponential. It is evident that the decay function for Rh-cytochrome b_5 and Rh-myoglobin are multiexponential. In both cytochrome b_5 and myoglobin, the metal in the metalloporphyrin (heme in the case of the native proteins) is liganded to the imidazole side chains of histidine residues in the protein. We attempted to determine whether liganding of imidazole to the metalloporphyrin was responsible for the multiexponential behavior of the metalloporphyrin triplet decay in the proteins. The triplet decay of 10^{-5} M rhodium(III)-protoporphyrin IX in the presence of 10^{-2} M imidazole in 0.1 M sodium carbonate, pH 9, was measured. It was found (data not shown) that the triplet lifetime was not changed from the value for the free metalloporphyrin. It was also observed that the presence of imidazole markedly enhanced the resistance of the

[2]Vaz, W. L. C., and H. H. Fueldner. Work in preparation.

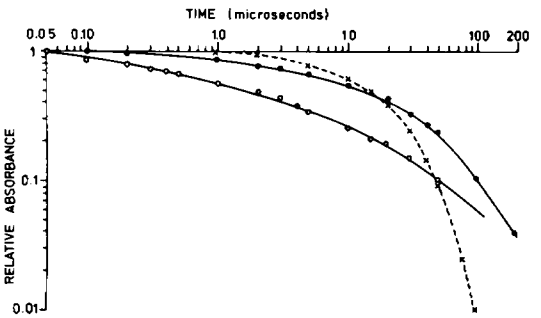

FIGURE 5

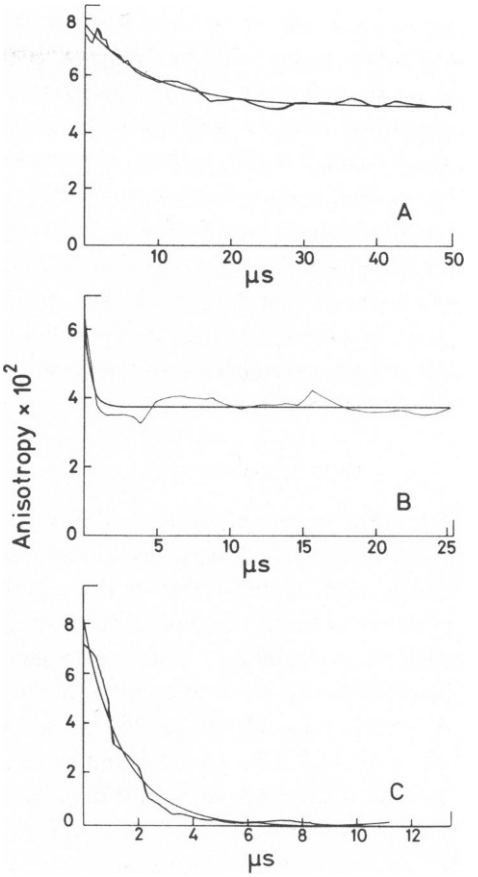

FIGURE 6

FIGURE 5 Triplet isotropic decay functions of rhodium(III)-protoporphyrin IX (x); Rh-cytochrome b_5 (○); and Rh-myoglobin (●). The decay function of the rhodium(III)-protoporphyrin IX is an excellent fit to an exponential function (- - - -), which has a time constant of 22 μs. The broken line can be visually translated along the time axis to compare with the other decay functions. Computer fits to the Rh-cytochrome b_5 triplet decay function indicate at least three exponential components of equal amplitudes having time constants of 0.26, 3.2, and 31 μs.

FIGURE 6 (A) The anisotropy decay of Rh-cytochrome b_5 on dimyristoyllecithin liposomes at 10°C. The heavy line is a computer fit to the decay curve. $r(o)_{fast} = 0.03$, $\tau_s = 9$ μs. The ratio of the fast phase to the total induced anisotropy is 0.4. (B) The anisotropy decay of Rh-cytochrome b_5 on dimyristoyllecithin liposomes at 35°C. The computer fit shows $f(o)_{fast} = 0.03$, $\tau_s = 0.4$ μs. The ratio of the fast phase to the total induced anisotropy is 0.4. (C) The anisotropy decay of Rh-cytochrome b_5 on dimyristoyllecithin liposomes in the presence of 10 mM sodium desoxycholate. The decay is fit by a single exponential, $r(o) = 0.08$, $\tau = 1.4$ μs.

metalloporphyrin to photobleaching. In the absence of imidazole, the triplet signal decreased in size by 70% over 4,096 laser shots, while no photobleaching within an error of ± 5% could be seen in the presence of imidazole. Rh-cytochrome b_5 shows a 10% loss of signal over 4,096 laser shots. The primary bleaching was observed to be due to the exciting laser pulse and not due to the monitoring beam.

Fig. 6, A and B, shows the anisotropy decay curves of Rh-cytochrome b_5 incorporated into

unilamellar bilayer vesicles of dimyristoyllecithin at temperatures above (35°) and below (10°C) the phase transition range of the lipid. The anisotropy decay of the lipid-bound protein is a strong function of the temperature of the sample. As seen in Fig. 6 A, at 10°C the anisotropy decay is biphasic with two components of relative amplitudes 0.4 and 0.6, and decay times of 9 and >20 μs, respectively. The long-lived component decay constant can not be resolved due to faster decay of the triplet state. At 35°C (Fig. 6 B), the anisotropy decay remains biphasic with two components of relative amplitudes 0.4 and 0.6, but the decay times are now 0.4 and >20 μs, respectively. These numbers are subject to some variation due to the small signal:noise ratio even after extensive averaging. When the bilayer structure of the proteoliposomes is broken to a micellar structure by the addition of the detergent deoxycholate, the anisotropy decay of the system is changed from biphasic to single exponential with a time constant of 1.4 μs.

DISCUSSION

The perturbation of the triplet decay of rhodium(III)-protoporphyrin IX upon insertion into apocytochrome b_5 and apomyoglobin is surprisingly large. The perturbation is clearly not due to liganding of the metal in the metalloporphyrin with the imidazole side chains in the proteins, because the imidazole-rhodium(III)-protoporphyrin IX complex shows the same triplet decay kinetics in water as the metalloporphyrin alone. Also, in Fig. 5 it is seen that the perturbation appears to be greater in the case of Rh-cytochrome b_5 than it is in Rh-myoglobin. A possible cause for this effect may be a structural inhomogeneity in the protein preparations. The processes of purification and heme removal from the native proteins may possibly result in some irreversible structural damage to the proteins, giving rise to several populations of protein structures, each of which upon recombination with rhodium (III)-protoporphyrin IX shows its own particular single exponential decay kinetics. It must be pointed out, however, that we could not detect any structural heterogeneity in the protein preparations when these were examined by gel filtration and ion-exchange chromatography. Also, recombination of apocytochrome b_5 with heme resulted in a preparation whose spectral characteristics were indistinguishable from those of the native untreated protein. A more subtle case of structural heterogeneity is the possibility of having a distribution of conformers in equilibrium with each other, where this conformational distribution is innate to a protein of a given amino acid sequence (31). If, under the conditions we used in this work, the relaxation rate between the conformers is very much faster than the triplet state lifetime of rhodium(III)-protoporphyrin IX in the proteins, then the triplet state decay observed would be expected to be single exponential. If, on the other hand, the relaxation between the conformational states is slower than the metalloporphyrin triplet state, the decay would be expected to be multiexponential. It is interesting to observe in this connection that the triplet decay of Rh-myoglobin is closer to a single exponential than the triplet decay of Rh-cytochrome b_5 (see Fig. 5).

We have built a model to explain the biphasic anisotropy decay of Rh-cytochrome b_5 incorporated into liposomes. The model is depicted in Fig. 7. We assume that the protein consists of two spherical parts rigidly connected to each other at the point of contact of the two spheres. The upper sphere is the hydrophilic metalloporphyrin-containing head of the protein and the lower sphere is the hydrophobic tail of the protein, and is embedded in the lipid bilayer. We assume that it is energetically unfavorable for the head to penetrate the

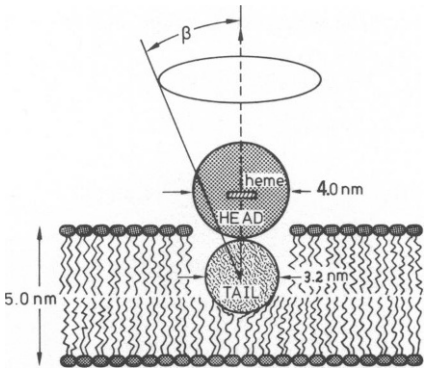

FIGURE 7 Schematic diagram, drawn to scale, of cytochrome b_5 on a lipid bilayer. The angle β shown is the angle over which the vector normal moves to the heme plane. The actual orientation of the heme group relative to the lipid surface is not known, although x-ray crystallographic studies on the heme-containing head of the protein indicate an orientation parallel to the plane of the lipid bilayer (35).

hydrophobic lipid bilayer; hence a restricted cone of allowed angles over which Brownian motion of the head can occur is seen. We further assume, for computational purposes, that the axis of symmetry of this cone of allowed angles is also the axis of symmetry of the protein molecule and passes normal to the plane of the heme (or rhodium(III)-protoporphyrin IX) residue in the protein. An objection to these assumptions could be that the head and tail of the protein are not rigidly joined to each other and thus the head can wobble independently of the tail. It is, however, difficult to understand why this sort of wobbling should be sensitive to the lipid phase transition and consequently the lipid physical state.

Aware of the limitations of the above model, we proceed with an analysis of the anisotropy decay. We follow closely the work of Kinosita et al. (32), which treats the problem of restricted motion in depth. A spherical molecule in an isotropic medium has an exponential anisotropy decay (26)

$$r(t) = r(o)e^{-6D_r t}, \tag{3}$$

where $r(t)$ is the anisotropy at time, t, and D_r is the Stokes-Einstein diffusion coefficient:

$$D_r = kT/8\pi\eta R^3, \tag{4}$$

where η is the microviscosity of the medium in poise, R is the Stokes radius of the protein in cm, T is the temperature in °K, and k is Boltzmann's constant (1.38×10^{-16} ergs/°K). In the case of restricted motion, the anisotropy decay will become multiexponential, with both the amplitudes of the components as well as the decay constants being functions of the allowed cone of motion, whose angle we denote by β. In general, it can be shown that the anisotropy decay function becomes (34):

$$r(t) = r(o) \sum_{i=1}^{\infty} A_i e^{-D_r t/\sigma_i}, \tag{5}$$

where A_i and σ_i are functions of the allowed cone angle β, but D_r retains its interpretation from Eq. 4. In our case, where the accuracy of the data does not allow analysis into the component

exponentials, we use the expression where an average over all σ_i has been made:

$$r(t) = r(o)[A_\infty + (1 - A_\infty)e^{-D_r t/\langle\sigma\rangle}], \tag{6}$$

where

$$\langle\sigma\rangle = \frac{\sum_{i=1}^{\infty} A_i\sigma_i}{\sum_{i=1}^{\infty} A_i}, \tag{7}$$

and

$$A_\infty = [r(\infty)/r(o)] = [(1/2)\cos\beta\,(1 + \cos\beta)]^2. \tag{8}$$

We should note that the heme (or rhodium[III]-protoporphyrin IX) group has uniform absorption for any vector lying within the heme plane and, therefore, the formula for the case of a linear molecule with absorption and emission vectors parallel to the axis of symmetry applies. The maximum value of $r(o)$ becomes 0.14 for the planar molecule (26) rather than 0.40 for the case of the linear one, but this plays no role becaus the ratio $r(t)$:$r(o)$ is used. If the heme does not lie parallel to the lipid membrane surface in the equilibrium position, as we have assumed in our model, then the problem becomes a mixture of the "spinning in a band" model (32) and the simpler model stated, with the result that the factor $\langle\sigma\rangle$ does not go to 0 as β goes to 0, due to the wobbling of the heme plane as the protein rotates around its axis of symmetry. From Eqs. 4, 5, and 6, it is possible to make a rough estimate of the microviscosity of the lipid bilayer. Our measured value of $r(o)$ is 0.07. The radius of the cytochrome b_5 tail can be estimated by assuming that it has the same density as that of a well-described protein like myoglobin. Then we can obtain the Stokes radius of the cytochrome b_5 tail from

$$R_{b_5\text{tail}} = R_{Mb}\left[\frac{4.6 \times 10^3}{1.7 \times 10^4}\right]^{1/3}, \tag{9}$$

where $R_{b_5\text{tail}}$ and 4.6×10^3 are the Stokes radius of the cytochrome b_5 tail and its molecular weight (21), respectively, and R_{Mb} and 1.7×10^4 are the Stokes radius of myoglobin and its molecular weight, respectively. The Stokes radius of myoglobin has been measured to be 2.4 nm (33). Because of the large viscosity of the lipid bilayer (in which the cytochrome b_5 tail is buried), compared to water (in which the cytochrome b_5 head is located), we may ignore the head of the protein in these calculations.[3] The results of the calculations on the basis of the model presented are summarized in Table I. It is interesting to note that if Eqs. 3 and 4 are used naïvely in an evaluation of the microviscosity, the result is only a factor of two, different from the value obtained by the more sophisticated treatment presented above. This is so primarily because our value for the allowed cone angle is reasonably large and does not change through the phase transition of the lipid.

An interesting feature of our results is that the cone of allowed motion appears to be the

[3]A referee has correctly pointed out that the head of the cytochrome b_5 is actually at least partially located in a region of "bound" water whose viscosity is probably quite different from that of "bulk" water. However, no estimates of the viscosity of the bound water exist and we have therefore used the value of viscosity of bulk water in the preceding argument.

TABLE I
ROTATIONAL DIFFUSION PARAMETERS FOR Rh-CYTOCHROME b_5 IN DIMYRISTOYLLECITHIN BILAYER MEMBRANES

Parameter	T = 35°C	T = 10°C
β	33°	33°
σ	0.095	0.095
τ	0.4 μs	9 μs
D_r	$2.4 \times 10^5\ s^{-1}$	$1.1 \times 10^4\ s^{-1}$
η	1.7 poise	34 poise
$r(\infty):r(o)$	0.6	0.6

same above and below the lipid phase transition. This contrasts the results obtained with small hydrophobic lipid probes (32, 34). A possible reason is that the angle of the cone in our case is limited by the steric interactions of the hydrophilic head of the protein with the surface of the lipid membrane.

We have not attempted an analysis of the anisotropy decay curve obtained when the protein is included in a mixed micelle of lipid and deoxycholate (see Fig. 6 C), because there are some uncertainties at this stage about the shape and size of the protein-containing micelles. The anisotropy decay in this case could arise from a tumbling of the entire protein-containing micellar structure, rather than from the rotation of the protein alone within the micelle.

In summary, it has been the purpose of this paper to characterize the structural and physical integrity of our protein-lipid system, and to perform some basic measurements on the rotational diffusion of cytochrome b_5 on lipid bilayers. Many questions remain to be answered before the motion of this protein on lipid membranes is fully understood. Improvements in the time and amplitude resolution of our equipment should allow us to provide fuller answers.

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REFERENCES

1. SINGER, S. J., and G. L. NICOLSON. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D.C.).* **175**:720–731.
2. EDIDIN, M. 1974. Rotational and translational diffusion in membranes. *Annu. Rev. Biophys. Bioeng.* **3**:179–201.
3. CHERRY, R. J. 1976. Protein and lipid mobility in biological and model membranes. *In* Biological Membranes. D. Chapman and D. F. H. Wallach, editors. Academic Press Inc. Ltd., London. 47–102.
4. JUNGE, W. 1972. Brownian rotation of the cytochrome oxidase in the mitochondrial inner membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **25**:109–112.
5. BROWN, P. K. 1972. Rhodopsin rotates in the visual receptor membrane. *Nat. New Biol.* **236**:35–38.
6. CONE, R. A. 1972. Rotational diffusion of rhodopsin in the visual receptor membrane. *Nat. New Biol.* **236**:39–43.
7. POO, M., and R. A. CONE. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature (Lond.).* **247**:438–441.
8. LIEBMAN, P. A., and G. ENTINE. 1974. Lateral diffusion of visual pigment in the photoreceptor disk membranes. *Science (Wash. D.C.).* **185**:457–459.
9. EDIDIN, M., and D. FAMBROUGH. 1973. Fluidity of the surface of cultured muscle fibers. Rapid lateral diffusion of marked surface antigens. *J. Cell Biol.* **57**:27–37.

10. PETERS, R., J. PETERS, K. H. TEWS, and W. BAEHR. 1974. A microfluorometric study of translational diffusion in erythrocyte membranes. *Biochim. Biophys. Acta.* **367**:282–294.
11. EDIDIN, M., Y. ZAGYANSKY, and T. J. LARDNER. 1976. Measurement of membrane protein lateral diffusion in single cells. *Science (Wash. D.C.).* **191**:466–468.
12. ZAGYANSKY, Y., and M. EDIDIN. 1976. Lateral diffusion of concanavalin A receptors in the plasma membrane of mouse fibroblasts. *Biochim. Biophys. Acta.* **433**:209–214.
13. SCHLESSINGER, J., W. W. WEBB, E. L. ELSON, and H. METZGER. 1976. Lateral motion and valence of Fc receptors on rat peritoneal mast cells. *Nature (Lond.).* **264**:550–552.
14. SCHLESSINGER, J., D. E. KOPPEL, D. AXELROD, K. JACOBSON, W. W. WEBB, and E. L. ELSON. 1976. Lateral transport on cell membranes: mobility of concanavalin A receptors on myoblasts. *Proc. Natl. Acad. Sci. U.S.A.* **73**:2409–2413.
15. AXELROD, D., P. RAVDIN, D. E. KOPPEL, J. SCHLESSINGER, W. W. WEBB, E. L. ELSON, and T. R. PODLESKI. 1976. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **73**:4594–4598.
16. JACOBSON, K., E. S. WU, and G. POSTE. 1976. Measurement of the translational mobility of concanavalin A in glycerol-saline solutions and on the cell surface by fluorescence recovery after photobleaching. *Biochim. Biophys. Acta.* **433**:215–222.
17. JACOBSON, K., Z., DERZKO, E. S. WU, Y. HOU, and G. POSTE. 1976. Measurement of the lateral mobility of cell surface components in single living cells by fluorescence recovery after photobleaching. *J. Supramol. Struct.* **5**:565–576.
18. CHERRY, R. J., A. BUERKLI, M. BUSSLINGER, G. SCHNEIDER, and G. R. PARISH. 1976. Rotational diffusion of band 3 proteins in the human erythrocyte membrane. *Nature (Lond.).* **263**:389–393.
19. CHERRY, R. J., U. MUELLER, and G. SCHNEIDER. 1977. Rotational diffusion of bacteriorhodopsin in lipid membranes. *FEBS (Feb. Eur. Biochem. Soc.) Lett.* **80**:465–469.
20. HANSON, L. K., M. GOUTERMAN, and J. C. HANSON. 1973. Porphyrins. XXIX. The crystal and molecular structure and luminescence of bis(dimethylamine)-etio(I)porphinatorhodium(III) chloride dihydrate. *J. Am. Chem. Soc.* **95**:4822–4829.
21. SPATZ, L., and P. STRITTMATTER. 1971. A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1042–1046.
22. STRITTMATTER, P. 1960. The nature of the heme binding in microsomal cytochrome b_5. *J. Biol. Chem.* **235**:2492–2497.
23. ROSSI-FANELLI, A., E. ANTONINI, and A. CAPUTO. 1958. Studies on the structure of hemoglobin. I. Physicochemical properties of human globin. *Biochim. Biophys. Acta.* **30**:608–615.
24. HUANG, C. 1969. Studies on phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry.* **8**:344–352.
25. VAZ, W. L. C., H. VOGEL, F. JAEHNIG, R. H. AUSTIN, and G. SCHOELLMANN. 1978. Kinetics of the incorporation of cytochrome b_5, an integral membrane protein, into unilamellar dimyristoyl-lecithin liposomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **87**:269–272.
26. STEPANOV, B., and V. GRIBOVSKII. 1968. Theory of Luminescence. Iliffe Books Ltd., London. 1–74.
27. HOPF, F. R., and D. G. WHITTEN. 1975. Photochemistry of porphyrins and metalloporphyrins. *In* Porphyrins and Metalloporphyrins. K. M. Smith, editor. Elsevier Scientific Publishing Company, Amsterdam. 667–700.
28. ROGERS, M. J., and P. STRITTMATTER. 1975. The interaction of NADH-cytochrome b_5 reductase and cytochrome b_5 bound to egg lecithin liposomes. *J. Biol. Chem.* **250**:5713–5718.
29. DUFOURCQ, J., R. BERNON, and C. LUSSAN. 1976. Binding of bovine cytochrome b_5 to phosphatidylcholine liposomes. Characterization of the reconstituted lipid-protein vesicles. *Biochim. Biophys. Acta.* **433**:252–263.
30. KAGAWA, Y., and E. RACKER. 1971. Partial resolution of enzyme catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477–5487.
31. AUSTIN, R. H., K. W. BEESON, L. EISENSTEIN, H. FRAUENFELDER, and I. C. GUNSALUS. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* **14**:5355–5373.
32. KINOSITA, K., S. KAWATO, and A. IKEGAMI. 1977. A theory of fluorescence polarization decay in membranes. *Biophys. J.* **20**:289–305.
33. AUSTIN, R. H., and S. S. CHAN. 1978. The rate of entry of dioxygen and carbon monoxide into myoglobin. *Biophys. J.* **24**:175–186.
34. KAWATO, S., K. KINOSITA, and A. IKEGAMI. 1977. Dynamic structure of lipid bilayers studied by nanosecond fluorescence techniques. *Biochemistry.* **16**:2319–2324.
35. MATHEWS, F. S., M. LEVINE, and P. ARGOS. 1972. Three-dimensional Fourier synthesis of calf-liver cytochrome b_5 at 2.8 A resolution. *J. Mol. Bol.* **64**:449–464.