Effect of Lipid Unsaturation on the Binding of Native and a Mutant Form of Cytochrome b_5 to Membranes†

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ABSTRACT: The partitioning of native cytochrome b_5 and a mutant form, where Trp-108 and Trp-112 were both replaced by Leu, into small unilamellar lipid vesicles was examined. The vesicles were made from phosphatidylcholines containing mono- and di-unsaturated acyl chains. As these amphipathic proteins self-associate in aqueous solution, the binding was not monitored by a simple lipid titration experiment but by an exchange assay using fluorescence quenching by brominated lipids. Each protein had a greater affinity for lipids containing mono-unsaturated chains than for vesicles containing di-unsaturated chains, and the affinities of both proteins increased in buffers of higher ionic strength. The native protein had a higher affinity than the mutant protein for all vesicles; the ratio of the affinities was relatively constant at approximately 30. This corresponds to a difference in the free energy of partitioning of 2 kcal mol^{-1}. The fluorescence quantum yields of both proteins were much lower in lipids with di-unsaturated chains whereas a similar lowering was not seen with a simple Trp compound. These data suggest that the decreased membrane hydrophobicity seen by the proteins in di-unsaturated membranes is not an inherent property of the bilayer but is induced by the insertion of the protein. Further, the similar behavior of the two proteins suggests this modulation is not sensitive to the amino acid side chains of the inserted domain.

The complexity of the lipid composition of membranes has been appreciated for decades, yet the reasons for this complexity have yet to be established. Model systems have been used to probe the interrelationship between the structure and function of membrane proteins and lipid structure. We have examined the interaction of cytochrome b_5[1] (b_5), itself a member of the enzyme system which introduces unsaturation in membranes (*1*), with lipid vesicles. Cytochrome b_5 is the founding member of a class of "tail-anchored" membrane proteins which are inserted into membranes posttranslationally (*2*). These proteins are targeted to specific locations within the cell by as-yet unknown mechanisms (*3*). Lipid composition may play a role in this targeting as we earlier showed that b_5 has a greater affinity for lipid vesicles made from DMPC than for vesicles made from POPC (*4*). As part of this ongoing project, we generated a number of mutant forms of b_5 with amino acid substitutions in the membrane-binding domain and found that one mutant, where both Trp-108 and Trp-112 were replaced by Leu, had, unexpectedly, a much lower affinity for membranes than did the native protein (*5*).

In this report, both the native (native b_5) and mutant (mutant b_5) forms of b_5 are used to examine the effect of alterations in the acyl chains of the lipid vesicles on the affinities of the two proteins for the vesicles. If the relative affinities of the two proteins varied from vesicle to vesicle, this would suggest that the different acyl chains interact differently with the two proteins. This would lend credence to the suggestion that the complex lipid composition seen in membranes is the result, at least in part, of the protein complexity. Perhaps the various acyl chains pack preferentially around membrane proteins which expose different amino acid side chains. While this present investigation was underway, another study of b_5 was published. Taylor and Roseman confirmed our earlier observation on the variation of the affinity of the native protein for different lipids and also extended it to a large number of PC species, to the effect of vesicle curvature, and to the effect of inclusion of cholesterol, on b_5 binding (*6*). They concluded that b_5 binding correlated with bilayer free volume and that the inhibition of binding caused by cholesterol correlated with the condensing effect on the phospholipid.

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[1] Abbreviations: b_5, rabbit endoplasmic reticulum cytochrome b_5 isolated from *E. coli* by detergent extraction; native b_5, native rabbit endoplasmic reticulum cytochrome b_5 isolated from *E. coli* by detergent extraction; mutant b_5, rabbit endoplasmic reticulum cytochrome b_5 where Trp-108 and Trp-112 are both replaced by Leu, isolated from *E. coli* by detergent extraction; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; OSPC, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; LSPC, 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicle.

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The determination of the affinity of a protein for a membrane is not trivial. We previously used the method of Hille et al. (*7*) to analyze the binding isotherms while the report of Taylor and Roseman employed a Scatchard analysis (*6*). Both these analyses used a "binding-to-sites" model in order to fit the nonhyperbolic isotherms which are typically seen with membrane proteins. We have recently reported that the nonhyperbolic nature of these isotherms can be completely accounted for by the self-association of the protein in the aqueous phase (*8*). The typical initial linear portion seen in these isotherms, rather than being due to a limited number of "sites", is due to a "buffering" of the concentration of the free monomer by dissociation of the aggregated protein. This analysis was applied to previously published data for the binding of native b_5 to POPC vesicles in low ionic strength buffer, where the concentration of monomeric protein could be measured. It was determined that native b_5 had a mole fraction partition coefficient of 1.8×10^7, which corresponds to a ΔG of transfer of b_5 (water to lipid) of -9.9 kcal mol^{-1}. In the present study, both the native form and a mutant form of b_5 were compared, and it was intended to also examine the system at a more physiological ionic strength. Our initial studies of native b_5 aggregation indicated the self-association was enhanced at higher ionic strength such that at physiological ionic strength the concentration of monomeric b_5 would be unmeasurable by our gel-filtration technique (*9*).

Because of these various analytical problems, we chose, in this study, to determine the relative affinities of the two proteins for various types of lipid vesicles by an exchange technique we have used previously (*5*). This technique involves measuring both the rates of exchange of the protein between a donor vesicle population and an acceptor vesicle population and the final equilibrium distribution between the two vesicle populations. Exchange of b_5 can be followed by Trp fluorescence. The protein in the original donor vesicles has enhanced fluorescence whereas the acceptor vesicle is composed of brominated lipid and therefore quenches the protein fluorescence. The measured rate of exchange is a convolution of the rates of binding to and release from the two vesicle populations in each experiment. If the rate of binding of the proteins to the vesicles is very fast and is independent of vesicle composition, and the same brominated lipid is used as acceptor in all experiments, then the relative rates of exchange are reciprocally related to the relative partition coefficients of the proteins for the original donor vesicles.

This study is aimed at a systematic analysis of the effect on protein partitioning of ionic strength, acyl chain unsaturation and placement (*sn*-1 versus *sn*-2), and changes in the hydrophobicity of the membrane-binding domain. By comparing native b_5 and mutant b_5, it should be possible to focus the analysis on the membrane-binding domain. This is because these proteins are dominated by identical large hydrophilic domains and it is known from previous studies that the membrane-binding domains of both proteins are inserted to the same depth in the membrane, at least in brominated lipid vesicles (*10*). It is this deep insertion which makes b_5 a relevant model for other integral membrane proteins.

MATERIALS AND METHODS

Native b_5 and mutant b_5 were isolated from *E. coli* as described previously (*11*). All lipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) except for 6,7-BRPC which was synthesized as described previously (*12*). Small unilamellar vesicles (SUVs) of the donor lipids were prepared from freshly opened vials of commercial lipid. Butylated hydroxytoluene at a 1:500 lipid ratio was added to prevent lipid peroxidation (*13*). The dried lipids were subsequently hydrated with enough 10 mM Hepes−0.1 mM EDTA−50 μM diethylenetriaminepentaacetic acid (pH 8.1 at 25 °C) to produce an approximately 50 mM lipid suspension. This was then sonicated under argon with a probe sonicator until the sample was clear, and contaminating particles were removed by ultracentrifugation (*14*). The size and homogeneity of the lipid vesicles were determined with a quasi-elastic laser light scattering instrument (Nicomp Instruments, Inc., Santa Barbara, CA), and the vesicles were found to have diameters of 30−40 nm. The concentrations of all lipid vesicles were determined by a modification of the Bartlett assay for total phosphorus content (*15*). The extent of peroxidation of the lipids was determined by UV absorbance (*16*) and was always less than 1%.

Gel Filtration of Cytochrome b_5. The self-association of mutant b_5 and native b_5 was examined by gel filtration on a column (1 × 22 cm) of Sephadex G200 Superfine at 20 °C. Three buffers were used in the gel filtration: 50 mM Tris acetate−0.5 mM EDTA (pH 7.5); 50 mM Tris acetate−0.5 mM EDTA (pH 8.1); and 10 mM Hepes−0.1 mM EDTA (pH 7.5). The ionic strengths of these buffers were assessed by measuring their conductivities using an Omega CDB-70 conductivity meter (Omega Engineering, Stamford, CT), and the conductivities were found to be 2050, 1124, and 504 μS, respectively. In each experiment, 6.8 nmol of protein in 40 μL of Hepes buffer was mixed with 10 μL of 5 M KCl, to ensure all the protein was in the aggregated form (*9*), before applying to the column. The flow rate was 2.4 mL/h, and the effluent from the column was monitored at 412 nm with a flow cell in a Gilson Stasar, the output from which was interfaced to a computer via an interface board (Omega Engineering). Data points were taken every 20 s and written in a computer file.

Exchange of Cytochrome b_5 between Normal and Bromolipid Vesicles. The exchange of native and mutant b_5 between the test lipid vesicles and BRPC vesicles was followed by fluorescence (*5*). All measurements were made in a stirred 0.5 cm cuvette thermostated at 20 °C using an SLM-8000c spectrofluorometer (SLM/Aminco, Urbana, IL) with an excitation wavelength of 280 nm (4 nm slit) and an emission wavelength of 335 nm (8 nm slit). The data were exported to an ASCII format and fitted with a nonlinear least-squares program of Dr. Michael Johnson (*17*).

Triplicate samples for each type of lipid vesicle were prepared and contained 1 mM test lipid in 10 mM Hepes−0.1 mM EDTA buffer (pH 7.5). In experiments performed in 150 mM KCl, the vesicles were incubated in 10 mM Hepes−0.1 mM EDTA−150 mM KCl (pH 7.5) overnight to allow the salt to equilibrate across the bilayer (*18*). After a base line fluorescence spectrum of lipid alone was obtained, enough of a protein stock solution was added to produce a 1:500 protein:lipid ratio, and the sample was incubated for

1 h at 25 °C. This time period was extended to 24 h when the samples were examined in buffer containing 150 mM KCl. A sample of BRPC vesicles was then added (final concentration 1 mM), and the fluorescence of the sample was monitored continuously for 1000 s, and then intermittently until the fluorescence stabilized. After each fluorescence study, the lack of protein denaturation was established by measurement of the dithionite-reduced minus oxidized absorbance spectrum. The difference in optical density between 424 and 410 nm was taken as a measure of the b_5 content. The equilibrium partitioning of b_5 was quantitated according to the formula (*19*):

$$F_{EQUIL} = F_{LIPID}X_{LIPID} + F_{BRPC}X_{BRPC} \quad (1)$$

where F_{EQUIL} is the equilibrium fluorescence in the presence of both the test lipid and BRPC vesicles, F_{LIPID} is the fluorescence in the presence of the test lipid alone, and F_{BRPC} is the fluorescence in the presence of BRPC alone. X_{LIPID} and X_{BRPC} are the mole fractions of the protein bound to the two populations of vesicles at equilibrium. The fraction of protein remaining bound to the test lipid at equilibrium is therefore given by

$$X_{LIPID} = (F_{EQUIL} - F_{BRPC})/(F_{LIPID} - F_{BRPC}) \quad (2)$$

and is a measure of the relative affinity of that protein for the test lipid versus BRPC vesicles.

To compare the relative affinities of both the native and mutant proteins for the various vesicles, the rates of approach to equilibrium in the above experiment were determined. The process of exchange involves at least four rate processes: the rates of binding to each of the two vesicle populations and the rates of release from each of the two vesicle populations. If equal concentrations of vesicles are used in all experiments and it is assumed that the rates of binding of the two proteins to all types of vesicles are identical (k_{ON}), presumably near the diffusion limit (*20*), then the equilibrium distribution of protein between the two vesicle populations is given by

$$K_{EQUIL} = X_{LIPID}/X_{BRPC} = k_{BRPC}/k_{LIPID} \quad (3)$$

where k_{BRPC} is the rate of release of the protein from the BRPC vesicle and k_{LIPID} is the rate of release from the test vesicle to the BRPC vesicle. The observed rate of exchange monitored by fluorescence quenching (k_{EXCH}) is the sum of k_{BRPC} and k_{LIPID}. Hence, $k_{LIPID} = k_{EXCH}/(1 + K_{EQUIL})$.

In the presence of a single population of vesicles, the equilibrium partitioning of protein between the aqueous solution and the test vesicle is given by: $K_X = k_{ON}/k_{LIPID}$, if the overall process of protein interaction with the lipid vesicle can be described by a single binding process (see Results). Hence, K_X is inversely proportional to k_{LIPID}

RESULTS

Effect of Ionic Strength on Cytochrome b_5 Self-Association. It has been previously noted that native b_5 self-associates in aqueous buffers and that the concentration of monomer in equilibrium with the aggregate decreases as the ionic strength of the buffer was increased (*9*). In contrast, mutant b_5 has been shown to behave on gel-filtration in low ionic strength buffers as a monomer (*5*). When gel-filtration experiments were repeated with both mutant b_5 and native b_5, using a variety of buffers, there appeared to be little correlation between buffer concentration or buffer pH and the extent of self-association. However, when the ionic strengths of the buffers were estimated by measurement of their conductivity, there was found to be a clearer correlation. As shown in Figure 1, the elution profiles of both mutant b_5 and native b_5 were very sensitive to the conductivity of the eluting buffers. We have suggested that b_5 behaves on gel filtration like other self-associating systems, such as detergents (*21*). Such systems are characterized by an aggregate, eluting near the void volume, followed by a trailing "plateau" region of monomer. This behavior is seen with mutant b_5 in a buffer of 1124 μS conductivity (Figure 1B). The aggregate elutes between 10 000 and 15 000 s, and a "plateau" of monomer extends out to 25 000 s. A "plateau" is difficult to discern with native b_5 (Figure 1A) but can be seen on an expanded scale (*8*). The behavior of both proteins in the lowest ionic strength buffer (504 μS) is anomalous. We have previously reported that native b_5 is excluded from gel-filtration matrixes in low ionic strength buffers because of the presence of a small number of carboxyl groups on the matrix (*22*), and these profiles may be influenced by this. In addition, the Stokes' radius of the aggregate would likely increase in buffers of low ionic strength due to increased electrostatic repulsion of the polar domains. In the buffer of lowest ionic strength, if the peak closest to the void volume represents native b_5 aggregate, then its concentration is 27% of that of the more included peak at 16 000 s. With mutant b_5, the peak at 15 000 s has a concentration of 14% of the more included peak at 20 000 s, which again indicates that the concentration of monomeric mutant b_5 is higher than that of native b_5 under these buffer conditions. It can be seen from Figure 1 that at conductivities greater than 2050 μS the concentration of

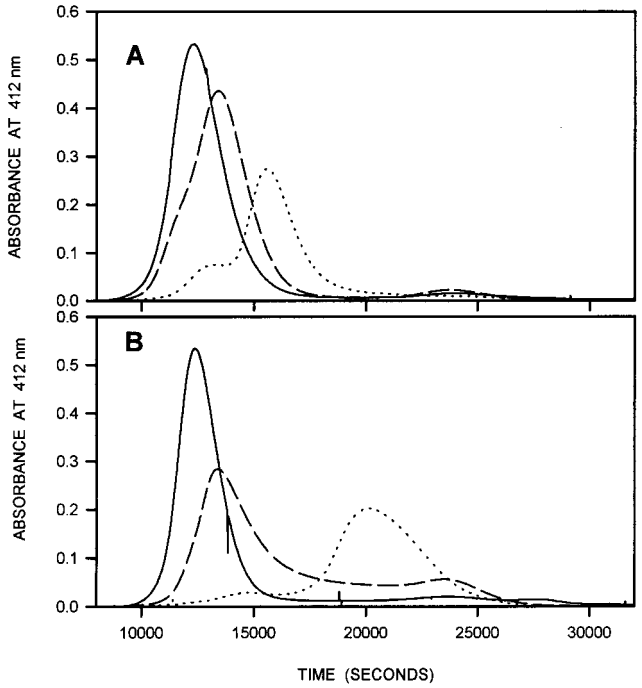

FIGURE 1: Effect of increasing ionic strength upon the gel-filtration profiles of native and mutant cytochrome b_5. Samples of protein (6.8 nmol) were applied to a Sephadex G200 column equilibrated in buffers with conductivities of 2050 μS (solid lines), 1124 μS (dashed lines), or 504 μS (dotted lines), and the absorbance was monitored at 412 nm. (A) Native protein. (B) Mutant protein.

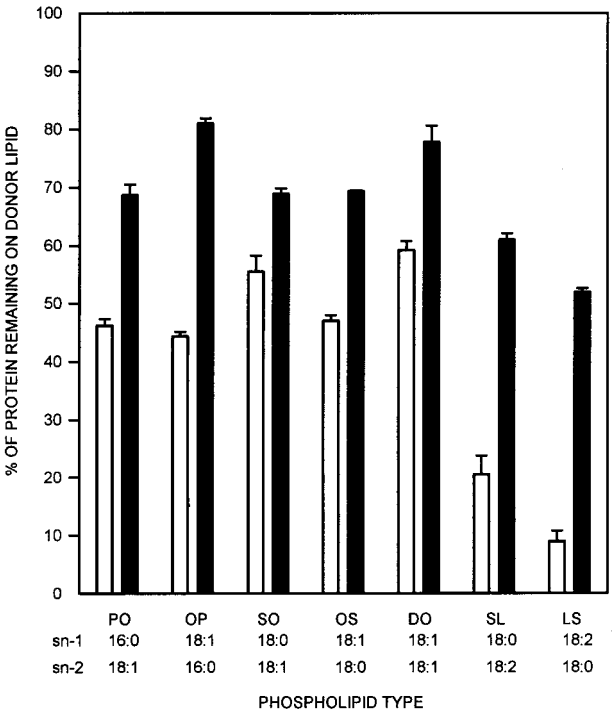

FIGURE 2: Percentage of mutant protein remaining bound to donor lipid after equilibration with BRPC vesicles. The protein was incubated with the donor lipid shown on the abscissa and then allowed to exchange to an equal concentration of BRPC. The distribution of protein between the two populations of lipids was estimated as described under Materials and Methods. Vesicles in low ionic strength buffer (open bars); vesicles in buffer with 150 mM KCl (solid bars).

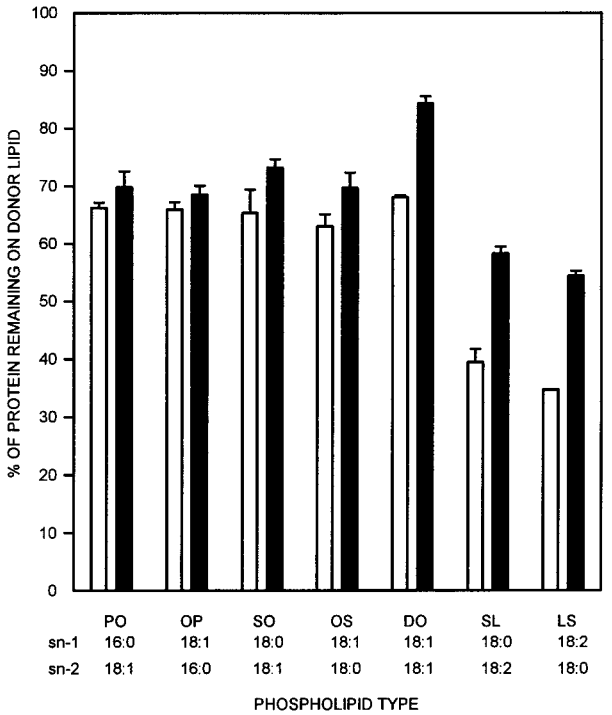

FIGURE 3: Percentage of native protein remaining bound to donor lipid after equilibration with BRPC vesicles. The protein was incubated with the donor lipid shown on the abscissa and then allowed to exchange to an equal concentration of BRPC. The distribution of protein between the two populations of lipids was estimated as described under Materials and Methods. Vesicles in low ionic strength buffer (open bars); vesicles in buffer with 150 mM KCl (solid bars).

monomeric protein in solutions of mutant b_5 and native b_5 would be very small, and virtually all of the protein would be in the aggregated form. Thus, at physiological salt concentrations, much higher salt concentrations than those used here, the concentration of monomeric mutant b_5 or native b_5 would be too low to determine using gel filtration and would thus preclude the use of a simple binding isotherm for measurement of the membrane partition coefficient.

Estimation of the Membrane Affinity of Cytochrome b_5 by an Exchange Assay. The relative partition coefficients of the two proteins can be determined from the rates of release of the protein from a vesicle (*20*). In addition, the relative partition coefficient of a single protein for two different lipids can be estimated from the equilibrium distribution of the molecule between the two lipids. Both of these techniques have been used previously with b_5 (*5*, *23*), and both are used here.

In Figure 2 are shown the amounts of mutant b_5 remaining bound to the different lipids at equilibrium after BRPC vesicles were added, calculated using eq 2. The error bars represent the standard deviations of triplicate assays. In Figure 3 are the data for native b_5. As reported previously (*4*, *6*), the affinity of native b_5 for the vesicles decreases as the level of unsaturation increases; a similar observation is made here for mutant b_5. The inclusion of 150 mM KCl in the samples increases the affinity of both mutant b_5 and native b_5 for the test lipid, especially with lipids containing di-unsaturated chains.

To determine the relative partition coefficients of both mutant b_5 and native b_5 into the various test lipids, the rates of approach to equilibrium in the above exchange assays

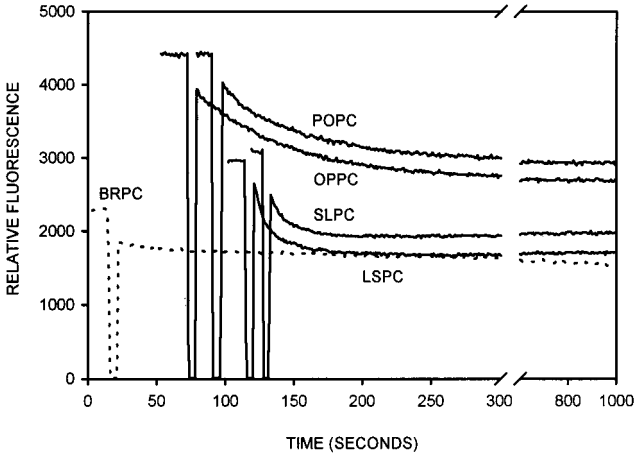

FIGURE 4: Time course of exchange of mutant protein from the donor vesicles after addition of BRPC vesicles in low ionic strength buffer. The fluorescence was observed just before and after addition of BRPC vesicles, shown by the sudden drop and return of signal (solid lines). The time course of fluorescence change upon addition of BRPC vesicles to protein alone is shown by the dotted line.

were determined. The rates of exchange of mutant b_5 and native b_5 from the donor lipid vesicle to BRPC vesicles were followed for each of the test lipid species, by monitoring the quenching of Trp fluorescence (*5*). Figures 4 and 5 show examples of these processes for mutant b_5 and native b_5 and contain a large amount of useful qualitative information. In these figures, the traces have been separated along the "Time" axis for visibility, but the "Relative Fluorescence" of each experiment is all on the same scale. First consider the addition of BRPC to protein alone (dotted lines). In low

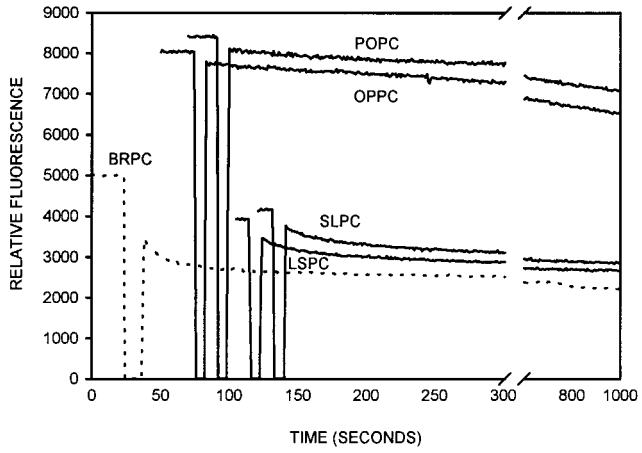

FIGURE 5: Time course of exchange of native protein from the donor vesicles after addition of BRPC vesicles in low ionic strength buffer. The fluorescence was observed just before and after addition of BRPC vesicles, shown by the sudden drop and return of signal (solid lines). The time course of fluorescence change upon addition of BRPC vesicles to protein alone is shown by the dotted line.

ionic strength, almost all of the decrease in fluorescence obtained with mutant b_5 when BRPC is added (Figure 4) occurs within the mixing time (~10 s) whereas with native b_5 (Figure 5) approximately 30% of the decrease occurs after the 10 s mixing time. In high ionic strength buffer, there is no sudden decrease in fluorescence with either protein. This behavior is consistent with the observations in Figure 1 that, in low ionic strength buffers, more mutant b_5 exists as monomer than does native b_5, and that an increase in ionic strength markedly decreases the concentration of monomer with both proteins. The sudden large decrease in fluorescence seen when both proteins were in low ionic strength buffer suggests that monomeric forms of both proteins bind rapidly to BRPC vesicles. Our previous studies have shown that the interaction of b_5 with vesicles occurs via the monomer, and this process is very rapid, $t_{1/2}$ of 15 ms, with mutant b_5 (*23*, *24*).

In the exchange reactions at low ionic strength, in contrast, the addition of BRPC to mutant b_5 (Figure 4) or native b_5 (Figure 5) mixtures, preincubated with the test lipid, produces no large immediate drop in fluorescence. The small immediate decreases are attributable to dilution by the added BRPC, and this indicates that there was no appreciable amount of free protein in these incubations and that the rates of fluorescence decrease observed are the result of exchange. In addition, these figures illustrate that the rates of exchange of mutant b_5 are larger than those with native b_5 and that the rates are faster with lipids which contain di-unsaturated acyl chains. When the exchange process was studied in high ionic strength buffer, the rates of all exchange reactions decreased.

Attempts were made to fit the progress curves from all exchange assays to single exponentials, but although most of the progress curves could be fit by single exponentials, some, especially those obtained with lipids which contained di-unsaturated chains, could only be fit with two exponentials. The reason for the two exponentials is not known. One logical explanation: the presence of unbound b_5 is inconsistent with the qualitative analysis above concerning the fluorescence changes immediately upon addition of lipid. In addition, neither of the two rates seen in double-exponential

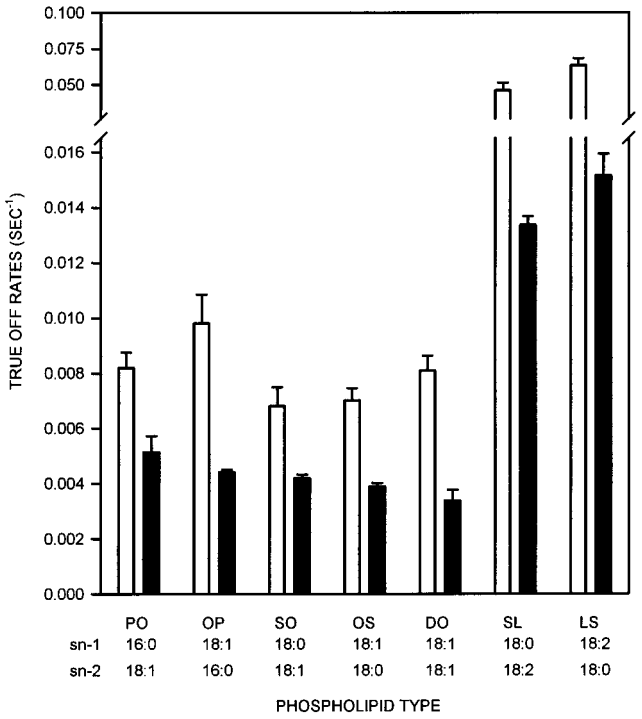

FIGURE 6: Rates of transfer of mutant protein from the donor vesicles to the aqueous phase. The rates of transfer from the donor vesicles (shown on the abscissa) were calculated from the rates of approach to equilibrium, as in Figure 4, and the percentage bound to the donor vesicle shown in Figure 2, as described under Materials and Methods. Low ionic strength buffer (open bars); buffer with 150 mM KCl (solid bars).

fits corresponded to the rates seen when BRPC was added to protein alone. This latter rate would correspond to the rate of dissociation of the aggregated protein, which would be identical no matter to what lipid the protein were binding. Despite the requirement for two exponentials in some cases, it was considered that the rate which accounted for the majority of the amplitude of the fluorescence decrease was the true rate of approach to equilibrium, k_{EXCH}. This rate, together with equilibrium partitioning data shown in Figures 2 and 3, was used to calculate k_{LIPID}. These values are shown in Figures 6 and 7. It can also be seen that the addition of 150 mM KCl decreases the off-rate in all cases. When the ratio of the off-rates is calculated from these two sets of data, the errors propagate, but it appears that there is a relatively constant ratio of affinities of the two proteins for any given lipid of approximately 30, as shown in Figure 8. An approximate ratio of 45 was previously reported with POPC vesicles (*5*).

Fluorescence Emission Spectra of Mutant b_5 and Tryptophan Octyl Ester. The fluorescence emission spectra of mutant b_5 (2 μM) and tryptophan octyl ester (2 μM) in 1 mM lipid vesicles are shown in Figures 9 and 10. As seen in the earlier time-course experiments, the enhancement of fluorescence of mutant b_5 in SLPC is much lower than in POPC or DOPC. In contrast, tryptophan octyl ester has similar fluorescence intensities in all three lipids.

DISCUSSION

It has been estimated that 20−30% of all genomes encode for membrane-bound proteins, with larger genomes encoding larger percentages (*25*). The lipid matrix which accom-

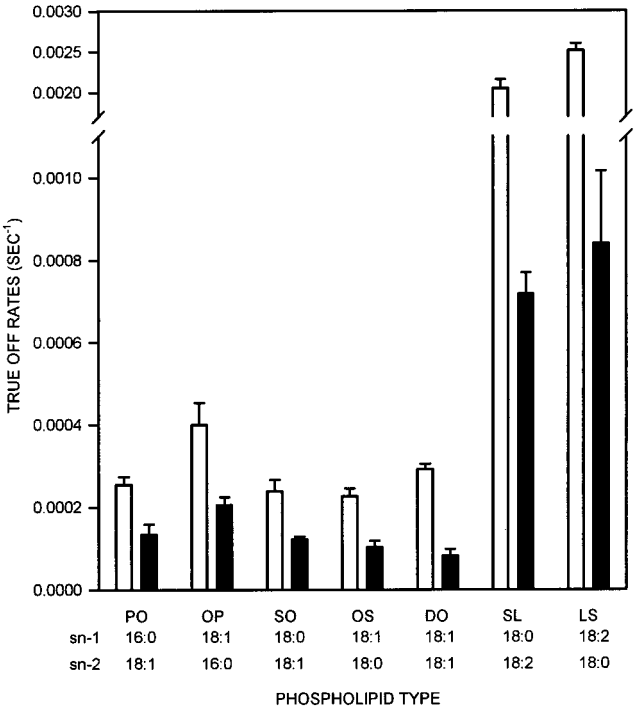

FIGURE 7: Rates of transfer of native protein from the donor vesicles to the aqueous phase. The rates of transfer from the donor vesicles (shown on the abscissa) were calculated from the rates of approach to equilibrium, as in Figure 5, and the percentage bound to the donor vesicle shown in Figure 3, as described under Materials and Methods. Low ionic strength buffer (open bars); buffer with 150 mM KCl (solid bars).

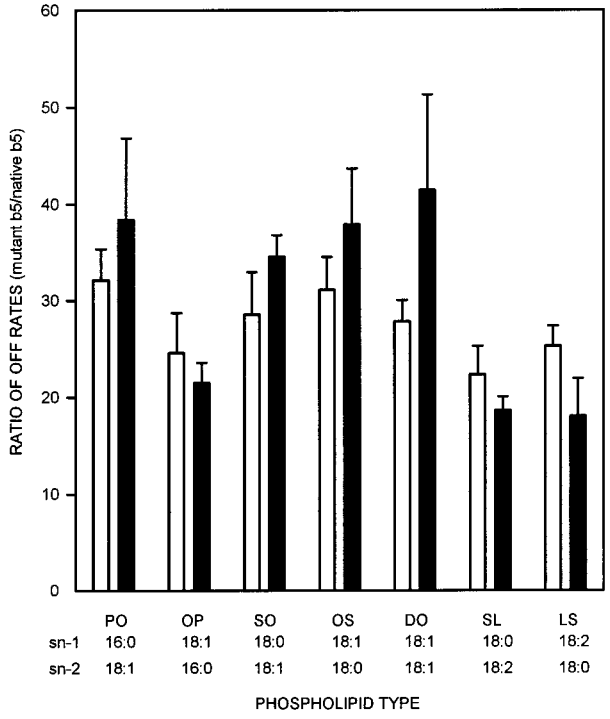

FIGURE 8: Ratio of the rates of transfer of mutant protein and native protein from donor vesicles to the aqueous phase. The ratios were calculated from Figures 6 and 7. Vesicles in low ionic strength buffer (open bars); vesicles in buffer with 150 mM KCl (solid bars).

modates these proteins is complex, early analyses indicated in excess of 200 different molecular species, and most membrane lipids have a characteristic *sn*-1 saturated, *sn*-2 unsaturated (often polyunsaturated) pattern (*26*). The requirement for many different proteins is obvious; less obvious is the need for so many different lipids, especially for the large variety of acyl chains. Studies with rhodopsin have shown its function is linked to the composition of the retinal rod outer segment (*27, 28*), but few other examples exist. Although it is likely that many membrane signaling proteins with multispanning helices would have specific lipid requirements to allow for protein−volume fluctuations during the signaling process, there are other membrane proteins where the membrane-inserted domain is merely an anchor, or has some role in targeting (*2, 29*). Cytochrome b_5 belongs to this class, and the amino acid composition of its membrane-binding domain is critical for the targeting (*30*). Perhaps these proteins are targeted to the correct membrane by a specific acyl chain composition in the typical *sn*-1/*sn*-2 asymmetry because one or another of the acyl chains is able to conform to the surface of the membrane-inserted domain. We have shown that small changes in the amino acid composition of the membrane-binding domain of b_5 also have profound changes in the partitioning of the protein into POPC vesicles (*5*).

Numerous studies have been made of model membrane proteins in an effort to determine the factors which account

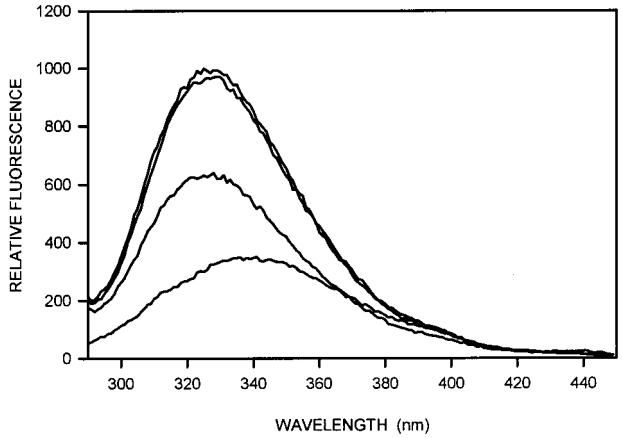

FIGURE 9: Fluorescence emission spectrum of mutant cytochrome b_5 in various lipids. The spectra were obtained as described under Materials and Methods with the protein in, in order of decreasing signal intensity, POPC, DOPC, SLPC, and low ionic strength buffer alone.

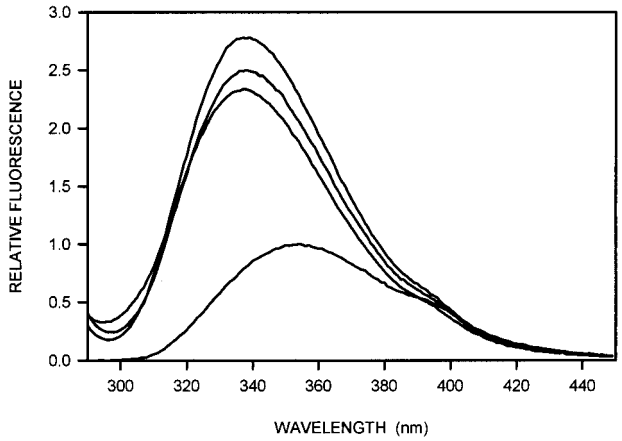

FIGURE 10: Fluorescence emission spectrum of tryptophan octyl ester in various lipids. The spectra were obtained as described under Materials and Methods with the tryptophan octyl ester in, in order of decreasing signal intensity, POPC, DOPC, SLPC, and low ionic strength buffer alone.

for their membrane-binding properties. Several methods have been used to compare the hydrophobicities of the amino acid side chains, and the most complete experimental study is that of Wimley and White (*31*), which builds upon earlier studies (*32*−*34*). They measured the mole fraction partition coefficients between buffer and lipid vesicles of a series of "host−guest" pentapeptides, in which the central residue in the pentapeptide was each of the 20 amino acids taken in turn. These studies were performed with large unilamellar vesicles made from POPC, and the authors mentioned that their hydrophobicity scale, specifically Trp being more hydrophobic than Leu ($\Delta\Delta G$ of transfer = 1.29 kcal mol^{-1}), accounted for the difference in affinity of mutant b_5 and native b_5 for POPC. Wimley and White also commented on differences seen between the partitioning of the pentapeptides between octanol−water and membrane−water. They noticed that Trp appears to be more hydrophobic in the bilayer than in octanol and suggested that the membrane interfacial region, where these guest residues reside, has a higher dielectric constant than the octanol interface because of the glycerol, phosphate, and choline groups.

As mutant b_5 and native b_5 have such very different partition coefficients (*5*), the present study was initiated to examine the roles that acyl chain composition and placement (*sn*-1 versus *sn*-2) had on the membrane partitioning of the two proteins. In our previous report, the partitioning of native b_5 into POPC vesicles in a buffer of low ionic strength was evaluated from a binding isotherm. This analysis was only possible because the concentration of monomeric native b_5 could be estimated in the presence of a large excess of self-aggregated native b_5 (*8*). The mole fraction partition coefficient which was determined corresponded to a value for ΔG of −9.9 kcal mol^{-1}. In the present study, it was intended to examine the binding in both low and high ionic strength buffers; in the latter situation, the concentration of monomeric protein would be too low to measure. Because of this, the membrane affinities were estimated by measuring the off-rates of the proteins from the various membranes. These off-rates can be used to calculate the absolute free energy of partitioning of the two proteins under the various conditions, but instead we have chosen to express the results as differences in free energy between the two proteins, or the difference in free energy of one of the proteins under varying conditions.

As seen in Figure 8, the relative off-rates indicate that there is no obvious trend in the relative hydrophobicity of the two proteins in the different lipids. The average value for all these ratios corresponds to a ΔG (mutant b_5 − native b_5) of 2.0 ± 0.2 kcal mol^{-1} for this whole range of lipids. Interestingly, this value is somewhat smaller than would be expected from the Wimley and White peptide studies. It is intermediate between the value calculated from the pentapeptides partitioning into lipid vesicles (2.58 kcal mol^{-1}) and the pentapeptides partitioning into octanol (1.69 kcal mol^{-1}). This is as expected because the Trp and Leu residues of b_5 are deeper in the bilayer than the interfacial region where the pentapeptides are located. There is a distinct preference seen with both proteins for lipids with mono-unsaturated acyl chains. The ΔG for partitioning of native b_5 into lipids with di-unsaturated chains versus mono-unsaturated chains is less favorable, both under low ionic strength, by 1.2 ± 0.4 kcal mol^{-1}, and under high ionic strength, by 1.1 ± 0.4 kcal mol^{-1}. Similar values are seen with mutant b_5 under the same conditions: 1.1 ± 0.2 and 0.8 ± 0.2 kcal mol^{-1}, respectively. Such a preference for mono-unsaturates has been reported previously for native bovine b_5 by Taylor and Roseman (*6*). They found, using a different binding assay, that the ratio of the affinity of bovine native b_5 in 100 mM NaCl for POPC versus SLPC was 3.8. We find a ratio of 5.4 ± 1.1 for native b_5 and 2.6 ± 0.3 for mutant b_5 in these two lipids. The difference between these two values suggests that the less hydrophobic mutant b_5 is less sensitive to changes in membrane hydrophobicity than is the native b_5. This effect is similar to the data summarized by Tanford (*35*) for hydrocarbon solubility in alcohols, suggested to indicate the alcohols had some structural organization. Although the partitioning of both native b_5 and mutant b_5 into all vesicles was increased in high ionic strength buffer ($\Delta G \approx -0.5$ kcal mol^{-1}), there was no statistical difference between the response of the native versus the mutant protein. This increase in partitioning could be a manifestation of "salting out", although Tanford indicates this should not be significant for hydrophobic solutes at 150 mM salt (*35*). In the present situation, the salt may be shielding ionic repulsion between the membrane surface and the highly charged (identical) polar domains of the two bound proteins. However, with these two proteins, the situation is quite complex as this increase in ionic strength causes almost complete protein self-aggregation, presumably mainly by neutralization of charge repulsion by the polar domains. Overall, the similar effect of increased ionic strength on the binding of the two proteins suggests that high ionic strength is not influencing the structure of the two different membrane-binding domains.

The relatively constant difference between the partition free energies for mono- and di-unsaturated lipids, with both mutant b_5 and native b_5 under low and high ionic strength, may be a manifestation of a lower membrane hydrophobicity in di-unsaturated compared to mono-unsaturated lipids. However, it is also possible that the difference is due to poorer packing of the di-unsaturated acyl chains around BOTH mutant b_5 and native b_5. Is the difference inherent in the bilayer or is it induced by the proteins? In an attempt to distinguish between these two possibilities, the fluorescent properties of b_5 and a simple Trp compound were compared.

The fluorescence properties of mutant b_5, which contains a single Trp in the membrane-binding domain, were examined for simplicity, and in low ionic strength, to avoid self-association of the protein. Figure 9 shows that the emission spectrum of mutant b_5 in POPC and DOPC vesicles had a larger intensity than that seen with SLPC, although the position of the emission maximum was similar in all three. The lower fluorescence yield with SLPC is not due to incomplete binding as the exchange experiments in Figure 4 indicate little free protein exists. The lower enhancement is in agreement with SLPC providing a more polar environment, but this is not a general phenomenon of SLPC bilayers as the model fluorophore, tryptophan octyl ester, showed similar fluorescence spectra in all three vesicles (Figure 10). Previous detailed studies of tryptophan octyl ester and mutant b_5 in brominated lipids have shown the single Trp residues in both molecules reside at a similar depth in the bilayer (11.3 and 10.1 Å from the center of the bilayer, respectively) (*10*). This suggests either that tryptophan octyl ester and mutant b_5 are in contact with different acyl chains or that it

is the presence of the protein which is altering the polarity of the bilayer, perhaps by allowing entry of water.

It was noted that the exchange of both mutant b_5 and native b_5 between SLPC and BRPC could not be fit by a single exponential. Although this observation does complicate the precise interpretation of the observed "off-rates", the differences in equilibrium partitioning (Figures 2 and 3) are in complete agreement with the absolute values of the differences in affinity between SLPC (and LSPC) and the other lipids. The multiphasic nature of the exchange process might suggest that additional intermediates exist in SLPC which are not present in the other lipids. The detailed structure of the membrane-binding domain of cytochrome b_5 is not known, but FT-IR experiments indicate there were no gross differences in secondary structure between the membrane-binding domains of the two proteins in any of the membranes; all appeared to be predominantly α-helix, as reported previously (*36*).

From these studies with a simple membrane protein, it would appear that the *sn*-1 versus *sn*-2 acyl chain distribution found in natural membranes does not play a gross role in protein binding. The inclusion of di-unsaturated acyl chains does decrease the partition coefficient of this simple protein, but the mechanism appears complex. Rather than there being a simple decrease in hydrophobicity induced by the di-unsaturated double bonds, it is possible that the binding of the protein decreases the hydrophobicity, perhaps by allowing water penetration into the bilayer. As the same decrease in the free energy of binding was seen with both native b_5 and mutant b_5, it is unlikely that acyl chain "packing" around the amino acid side chains has any thermodynamic consequence. Further suggestions as to the roles of membrane lipid compositional complexity must await more sophisticated evaluations of membrane protein function.

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