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Stopped-Flow Fluorescence Studies of the Interaction of a Mutant Form of Cytochrome b_5 with Lipid Vesicles

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Cytochrome b_5 binds spontaneously to lipid vesicles and also self-associates in aqueous solution. Two mutant proteins have been generated, one has a self-association constant which is less than that of the native protein, while the other has a larger self-association constant. All three proteins have Trp in the membrane-binding domain but as aqueous solutions of these proteins contain differing amounts of monomeric protein, the kinetics of fluorescence enhancement, when the proteins are mixed with lipid vesicles, are complex. Similar complex kinetics are seen when the Trp are quenched by the addition of bromolipid vesicles. The mutant which has Trp 108 and 112 both replaced by Leu does not self-associate and shows monoexponential stopped-flow fluorescence kinetics. Identical rate constants are seen with this mutant for fluorescence enhancement by POPC and fluorescence quenching by three bromolipids with bromines at the 6,7-, 9,10-, and 11,12-positions of the *sn*-2 acyl chain. This rate constant is only 1% of the calculated collisional rate constant and it is suggested that the reduced rate is caused by a reduction in the number of productive collisions rather than by a slow rate of penetration of the membrane-binding domain into the bilayer.

KEY WORDS: Cytochrome b_5; site-directed mutagenesis; kinetics; fluorescence; membrane-binding.

INTRODUCTION

The mechanism of incorporation of integral membrane proteins into membranes is under active investigation and, in most cases, appears to involve several ancillary proteins (for a review see Ref. 1). The overall incorporation, rather than being simply a lipid–protein interaction, is much more complex and various protein–protein interactions occur before the final membrane-bound state is achieved. A number of model systems have been generated to examine the thermodynamics and kinetics of membrane–protein interactions but few of these have used complete integral membrane proteins. Cytochrome b_5 (b_5)[5], an integral membrane protein found in the endoplasmic reticulum, is a suitable model for such studies. This protein has been shown to be syn-

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[5] Abbreviations used: b_5, cytochrome b_5 (the complete 133-amino acid residue protein); native b_5, the native cytochrome b_5 isolated from rabbit liver; (108, 112) mutant b_5, the cytochrome isolated from *E. coli* with Trp 108 and Trp 112 of the native sequence both replaced by Leu; (103, 108, 112) mutant b_5, the cytochrome isolated from *E. coli* with Asp 103, Trp 108, and Trp 112 of the native sequence all replaced by Leu; DMPC dimyristoylphosphatidylcholine; DOPC dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; BRPC, 1-palmitoyl-2-(dibromostearoyl)-phosphatidylcholine with bromine atoms at the 6,7-, 9,10-, or 11,12 positions, as indicated.

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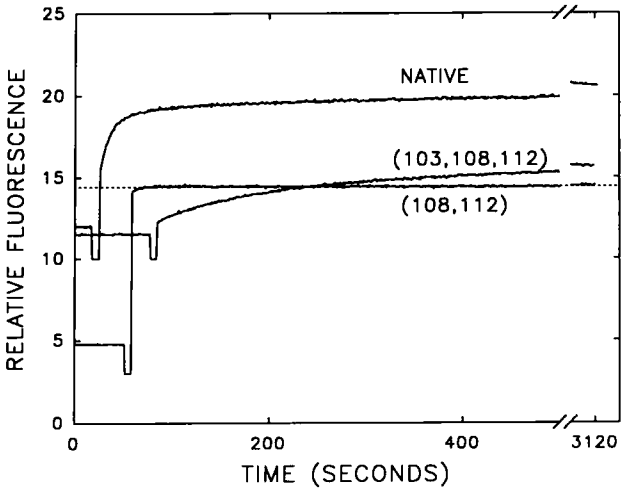

Fig. 1. Rates of fluorescence enhancement upon adding POPC vesicles to native and mutant b_5's. To a solution of each b_5 was added enough POPC vesicles to make the final concentration 2 μM in protein and 400 μM in lipid. The manual mixing in the cuvette was done as rapidly as possible. The horizontal dashed line is drawn through the mean fluorescence signal obtained with (108, 112) mutant b_5 for comparison purposes.

thesized on free cytoplasmic ribosomes [2] and hence the mechanism of *in vivo* incorporation may be similar to that seen *in vitro*. Cytochrome b_5 has been extensively studied by a number of laboratories and preliminary kinetic and thermodynamic studies of membrane binding have been performed [3–5].

As with most hydrophobic molecules, b_5 has a tendency not only to bind to membranes but also to self-associate in aqueous solution. This self-association complicates any biophysical study. The membrane-binding domain of native b_5 contains three Trp and the fluorescence of the protein is enhanced when it binds to lipid vesicles. The kinetics of this fluorescence enhancement are complex, due to a large extent to the presence of both monomeric and octameric protein. We were able to show that monomeric protein, isolated by gel filtration, gave rise to an "instantaneous" increase in fluorescence when mixed with lipid vesicles [4]. We have also published a preliminary report of stopped-flow fluorescence studies of the interaction of b_5 with lipid vesicles but these studies were again complicated by the octamer–monomer equilibrium described above [6].

Cytochrome b_5 has now been expressed in *Escherichia coli* [7] and yeast [8] and initial physical studies have been made on mutant forms of b_5. One of these mutants shows a much reduced tendency to self-associate and is therefore more suitable for stopped-flow fluorescence studies [7].

EXPERIMENTAL

Rabbit liver cytochrome b_5 was isolated as described previously and the brominated lipids and small unilamellar sonicated lipid vesicles were prepared as described previously [9]. The two mutants (108, 112) and (103, 108, 112) were isolated from *E. coli* XL-1 cells (Stratagene, La Jolla, CA), which contain the vector pKK223-3 (Pharmacia, Piscataway, NJ) with an insert which carries the prokaryote ribosome-binding site and the rabbit liver b_5 sequence [10] where the codons for Trps 108 and 112 and, in the second mutant Asp 103, have been replaced by codons for Leu, using the procedure of Kunkel [11]. All mutations were confirmed by DNA sequencing. The expression of the protein in *E. coli* and its method of isolation have been described [7] and the methods used were identical for the two mutants. Static fluorescence measurements were made in a stirred cuvette using an SLM 8000c spectrofluorometer (SLM/Aminco, Urbana, IL) with an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Stopped-flow fluorescence measurements were made using the optics from an SLM 4800, an SLM stopped-flow apparatus, and electronics and software from OLIS (Bogart, GA). Excitation was at 280 nm and the emission was observed after passage through a 300-nm cutoff filter. The data were fitted with the NONLIN program of Dr. Mike Johnson [12]. The buffer used throughout was 10 mM Tris-acetate/0.1 mM EDTA, pH 8.1.

RESULTS

The three mutants, although having no apparent differences in secondary structure, have quite different hydrophobicities and these differences are most readily seen in their behavior on gel filtration as described previously [5]. Native b_5 has an elution profile typical of a self-associating system with a maximum concentration of monomeric native b_5 of 0.5 μM, (108, 112) mutant b_5 shows no indication of octamer formation and has a maximum concentration of monomer of at least 2 μM, and (103, 108, 112) mutant b_5 shows only the presence of octamer in the elution profile, with a maximum concentration of monomer of less than 0.05 μM. This aggregation behavior is reflected in the rates of fluorescence enhancement when POPC lipid vesicles are added to a solution of each protein (Fig. 1). The initial instantaneous increase in fluorescence seen is due to monomer binding [(108, 112) > native > (103, 108, 112)], whereas the slower secondary rate is most likely limited by the rate of octamer dissociation to monomer. The

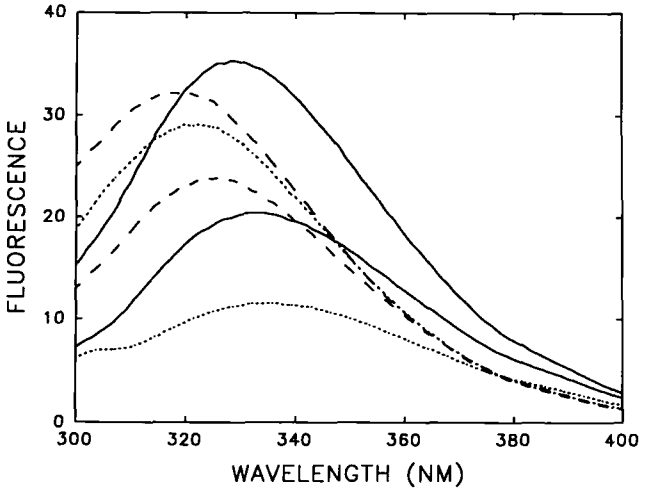

Fig. 2. Fluorescence spectra of native and mutant b_5's before and after the addition of POPC vesicles. Spectra were collected for the samples shown in Fig. 1 before the addition of, and after a 2-h incubation with, POPC vesicles. Native b_5, solid line; (108, 112) mutant b_5, dotted line; (103, 108, 112) mutant b_5, dashed line. For each pair of spectra, the lower spectrum is that of the protein in buffer.

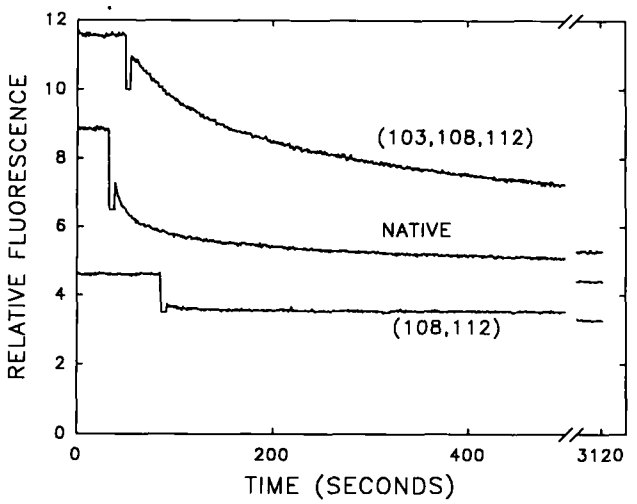

Fig. 3. Rates of fluorescence quenching upon adding 6,7-BRPC vesicles to native and mutant b_5's. To a solution of each b_5 was added enough BRPC vesicles to make the final concentration 2 μM in protein and 400 μM in lipid. The manual mixing in the cuvette was done as rapidly as possible.

magnitudes of the overall fluorescence changes upon binding to lipid are influenced by the emission wavelengths of monomer, octamer, and lipid-bound protein and the choice of monitoring wavelength (320 nm). The spectra of the proteins before and after the addition of POPC vesicle are shown in Fig. 2 and it is apparent that the fluorescence enhancement seen in Fig. 1 with (108,

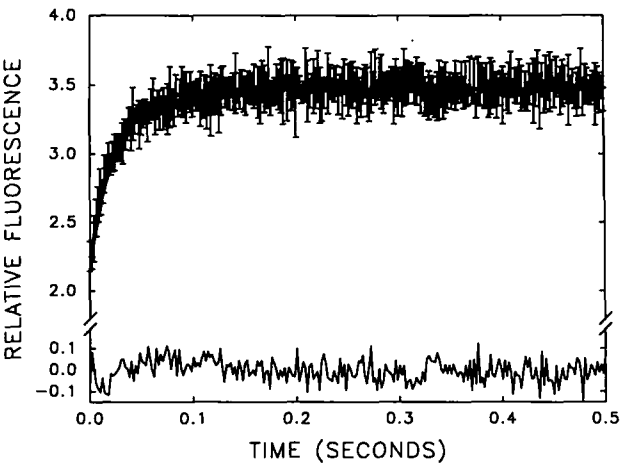

Fig. 4. Stopped-flow fluorescence data from mixing (108, 112) mutant b_5 with POPC vesicles. The mixing syringes contained 4 μM protein and 2 mM POPC. The mean and standard deviations of five consecutive experiments are shown together with the fitted single-exponential line and the residuals.

112) is large because the quantum yield of the monomeric protein is low in aqueous solution and there is a substantial fluorescence enhancement and blue shift upon binding of the monomeric protein to vesicles. In contrast, when the predominantly octameric (103, 108, 112) mutant binds to lipid vesicles, there is little blue shift and only a small enhancement.

Brominated lipids have been used previously to examine the topography of b_5 [9], and the fluorescence quenching data shown in Fig. 3 parallel the data in Fig. 1. The rates of fluorescence quenching are similar to the rates of fluorescence enhancement seen with POPC and the percentages of the "instantaneous" decrease are again in the order (108, 112) > native > (103, 108, 112), reflecting the percentage of monomeric protein in each sample. The relative magnitudes of the quenching are, as expected, in the reverse order from the magnitudes of the overall enhancements seen with POPC. The mutant (108, 112), with an already low quantum yield, undergoes only a small additional quenching, whereas the (103, 108, 112) mutant with a high quantum yield in the octameric form is efficiently quenched when bound to BRPC.

To examine further the mechanism of "binding" a series of stopped-flow fluorescence measurements was made using the (108, 112) mutant b_5, which by all criteria appears to be monomeric in solution. Figure 4 shows the fluorescence signal obtained from mixing (108, 112) mutant b_5 with POPC vesicles. In Figs. 5–7 the data from mixing the protein with the 6,7-, 9,10-, and 11,12 BRPCs are shown. All of these curves showed

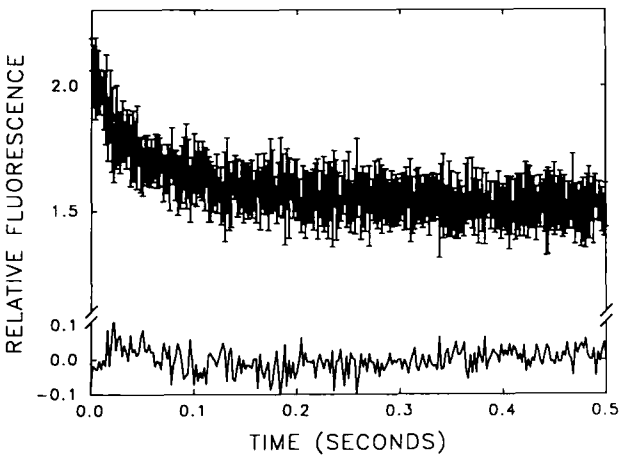

Fig. 5. Stopped-flow fluorescence data from mixing (108, 112) mutant b_5 with 6,7-BRPC vesicles. The mixing syringes contained 4 μM protein and 2 mM 6,7-BRPC. The mean and standard deviations of five consecutive experiments are shown together with the fitted single-exponential line and the residuals.

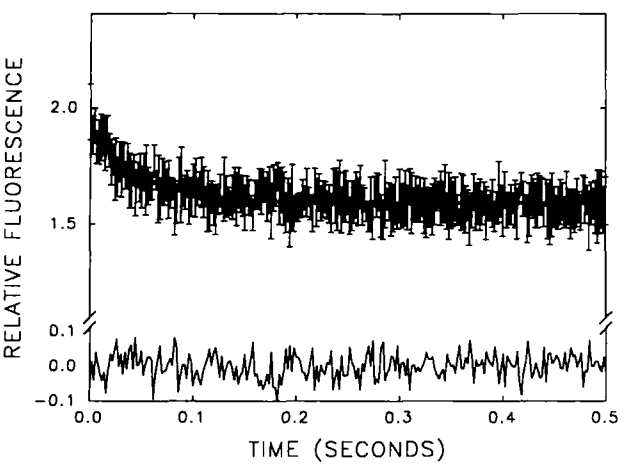

Fig. 6. Stopped-flow fluorescence data from mixing (108, 112) mutant b_5 with 9,10-BRPC vesicles. The mixing syringes contained 4 μM protein and 2 mM 9,10-BRPC. The mean and standard deviations of five consecutive experiments are shown together with the fitted single-exponential line and the residuals.

a rapid phase with a $t_{1/2}$ of approximately 20–50 ms, which was followed by a slow, steady decrease in fluorescence (not shown). As the curves for (108, 112) in Figs. 1 and 3 showed no slow rate of decrease, we assumed that this slower rate seen in the stopped-flow experiments was due to bleaching in the unstirred and more highly irradiated stopped-flow cell. All stopped-flow fluorescence curves were therefore fitted with a single exponential over the first 0.5 s of the transient and these single-exponential curves and the residuals are also

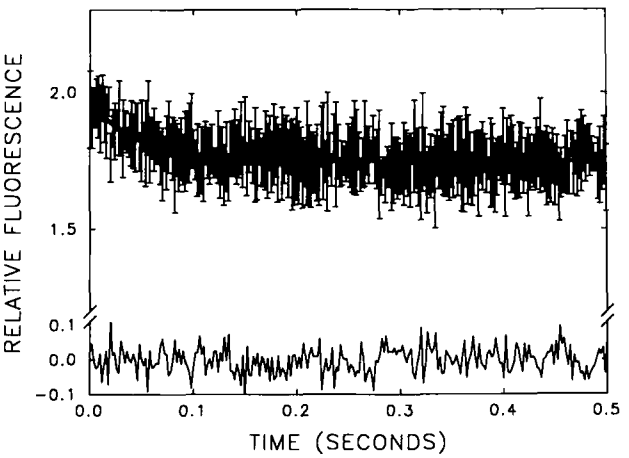

Fig. 7. Stopped-flow fluorescence data from mixing (108, 112) mutant b_5 with 11,12-BRPC vesicles. The mixing syringes contained 4 μM protein and 2 mM 11,12-BRPC. The mean and standard deviations of five consecutive experiments are shown together with the fitted single-exponential line and the residuals.

Table I. Stopped-Flow Fluorescence Analysis of the Interaction of Mutant Cytochrome b_5 with Lipid Vesicles[a]

Lipid	ΔF	k (s^{-1})	F_∞	$\Delta F + F_\infty$
POPC	-1.28 ± 0.03	41.1 ± 2.2	3.47 ± 0.01	2.19 ± 0.04
6,7-BRPC	0.51 ± 0.02	17.3 ± 1.5	1.53 ± 0.01	2.04 ± 0.03
9,10-BRPC	0.32 ± 0.02	20.4 ± 3.0	1.59 ± 0.01	1.91 ± 0.03
11,12-BRPC	0.21 ± 0.02	19.8 ± 4.3	1.75 ± 0.01	1.96 ± 0.03

[a] The stopped-flow fluorescence analysis was performed as described under Experimental and the amplitudes (ΔF), rates (k), and values at infinite time (F_∞) were obtained from single-exponential fits of the data.

shown in Figs. 4–7. The parameters of the single-exponential fits are shown in Table I. Some confidence that most of the transient is being recorded is presented in the last column in Table I, where it is seen that the sum of the ΔF plus the value at infinite time is 2.03 ± 0.11 U over all experiments. This value should represent the fluorescence of the sample at 0 time when all the protein is unbound. The rates of quenching by the BRPCs are all quite similar and appear to be 50% of the rate of enhancement seen with POPC. Although (108, 112) mutant b_5 has the advantage of being monomeric under these solution conditions, it has the disadvantage that the quantum yield in the aqueous state is quite low and hence the fluorescence signal does not fall appreciably upon binding to BRPC vesicles (see Fig. 3). This results in a rather poor signal-to-noise ratio in the quenching data. When the quenching with 6,7-BRPC was repeated

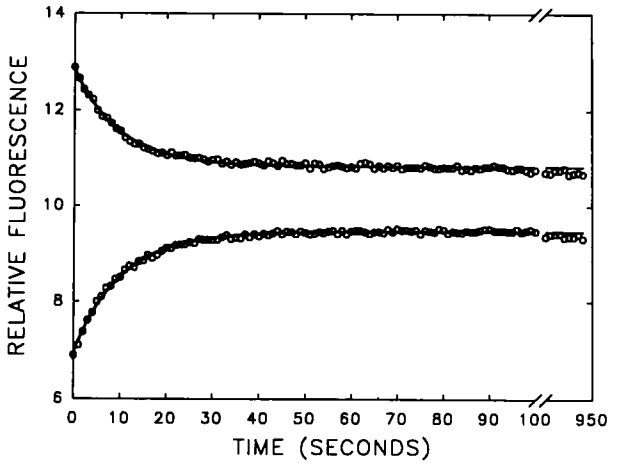

Fig. 8. Analysis of the rates of exchange of (108, 112) mutant b_5 between vesicles. The upper curve indicates the change in fluorescence when 6,7-BRPC vesicles (400 μM) were added to the cuvette at the end of the experiment shown in Fig. 1; the lower curve, when POPC vesicles (400 μM) were added to the cuvette at the end of the experiment shown in Fig. 3. The solid lines are single-exponential fits of the data.

with a lipid concentration 50% of that used previously, the rate fell to 7.2 ± 0.7 s^{-1} from the original value of 17.3 ± 1.5 s^{-1}, but with lower levels or higher levels of lipid the data became too noisy to analyze. This decrease is consistent with the reaction which gives rise to the fluorescence quenching being a bimolecular reaction between free protein and a lipid vesicle.

As discussed in detail by Schwarz *et al.* [13] the overall relaxation curve in Fig. 4 has a rate which is given by

$$k = k_{as} \cdot c_v + k_{dis}$$

where k_{as} and k_{dis} are the rate constants for association and dissociation. Hence the value of k_{dis} can be determined from the rate of fluorescence enhancement at different concentrations of lipid vesicles. Unfortunately, in the present case the range of available vesicle concentrations was limited. At low lipid levels the vesicles become saturated and at high lipid levels the signal-to-noise ratio became unacceptable. The value of k_{dis} can, however, be estimated by measuring the exchange rate of the protein as it moves from an "enhancing" vesicle to a "quenching" vesicle (or vice versa) provided the on-rate is much faster than the off-rate [14]. Figure 8 shows the exchange of (108, 112) mutant b_5 in these two situations. Both curves were fitted by a single exponential and the upper curve gave a value of $k_{dis} = 0.103 \pm 0.004$ s^{-1} and the lower curve $k_{dis} = 0.097 \pm 0.002$ s^{-1}. For the POPC $k = 41$ s^{-1} and $c_v = 10^{-3}/2500$ mM (to allow for the number of lipids per POPC vesicle [15]), then $k_{as} = 1 \times 10^8$ M^{-1} s^{-1} and the overall partition coefficient (k_{as}/k_{dis}) is 1×10^9.

DISCUSSION

A number of studies have made use of fluorescence techniques to examine the kinetics of binding of hydrophobic molecules to membranes. Detailed theoretical treatments have been made by Schwarz and co-workers [13] in studying the binding of alamethicin. They analyzed their data to derive kinetic constants for surface contact, penetration, conformational change, and aggregation of the peptide during interaction with the bilayer.

A comparison can be made between the rate of the fluorescence enhancement seen upon mixing of b_5 with POPC vesicles ($k_{as} = 1 \times 10^8$ M^{-1} s^{-1}) and that expected from a diffusion-limited encounter. From the Smoluchowski equation for collisional frequency,

$$k_{coll} = 4\pi N(D_p + D_v)(R_p + R_v)/1000$$

where p and v refer to protein and vesicle and D and R are the diffusion constants and radii. Values used for D_v and R_v are 1.9×10^{-7} cm^2 s^{-1} and 106 Å [15] and D_p and R_p are 8×10^{-7} cm^2 s^{-1} and 26 Å [16], hence $k_{coll} = 9.9 \times 10^9$ M^{-1} s^{-1}. This produces a ratio of $k_{as}/k_{coll} \approx 0.01$, which is considerably smaller than that seen by Schwarz *et al.* with alamethicin (0.16) [13].

Although the observed enhancement of fluorescence has been equated with binding, the nature of the complex and location of the Trp in the depth of the bilayer are not predictable from these data with POPC vesicles. When the (108, 112) mutant b_5 "binds" to POPC vesicles, there is a blue-shift of the emission spectrum and a twofold enhancement in fluorescence, but this could be induced by the interface region of the vesicle and the actual penetration could be fluorescently "silent" and occur on a much slower time scale. The kinetics of "penetration" can be monitored by the use of a series of brominated lipids where the depth of the bromines varies from 3.5 Å from the hydrocarbon-head group boundary to 8.0 Å from this boundary [17]. As shown in Table I the rates of quenching seen with all three BRPCs were very similar but were only 50% of the rate seen with POPC. Although these brominated lipid vesicles have not been characterized as extensively as those made from POPC, we have noted previously that these brominated vesicles have an approximately 1.3-fold larger radius than those made from POPC [9]. This larger radius will have a minimal effect on the diffusion properties of the vesicles, but because each brom-

olipid vesicle will contain many more lipid molecules, the actual concentration of brominated lipid *vesicles* derived from a given molar concentration of bromolipid will be much lower than the concentration of POPC *vesicles* derived from the same molar concentration of POPC. If the values of Mason and Huang [18] for the dimensions of egg PC vesicles are used as an approximation for POPC vesicles, then these have an outer anhydrous vesicle radius of 99 Å and an inner radius of 62 Å. A 1.3-fold increase in outer radius, with a maintenance of bilayer thickness in brominated lipids as we have shown by X-ray diffraction [17], will produce an increase in the total volume of bilayer in the BRPC vesicles by 1.9-fold relative to that in the POPC vesicles. If the lipid packing is similar in the two types of lipid, then the BRPC vesicles will contain 1.9-fold the number of lipid molecules that are in the POPC vesicles. A more precise analysis with a similar bromolipid gave a ratio of 1.7 [15]. Hence, in the stopped-flow experiments the concentration of *vesicles* in the BRPC experiments was only 50% of the vesicle concentration used in the POPC experiments. If the reaction is bimolecular, as proposed, this would decrease the observed rate of fluorescence change by 50%. That this would occur was shown by repeating the stopped-flow experiments with a 6,7-BRPC lipid concentration of 50% of that used previously, whereupon the rate decreased to 7.2 ± 0.7 from the previous 17.3 ± 1.5 s^{-1}.

Further evidence in support of the equivalence of the rate constants of fluorescence enhancement and quenching comes from examination of the long time points in Fig. 8. Although the two curves do not converge at long times, as they should, it is apparent that the average of the signals at long times (10 U) is midway between the two 0 time points (7 and 13). This indicates that the protein is partitioning equally between the two vesicles, and as the two equilibration rates (off-rates) are identical, the on-rates should also be identical. This suggests that these fluorescence changes are monitoring the same transport phenomena—the deep penetration of the domain into the bilayer. As noted above, the association rate observed is only 1% of the diffusion-limited rate, but with the present information it is not possible to determine the nature of the kinetic barrier for "binding" of b_5 or the mechanism of insertion of the membrane-binding domain. Two possible extreme explanations for this lower rate are that the process involves the approach of the protein to the vesicle, a diffusion-limited surface association and a slow penetration, or, alternatively, that it involves a surface association which is reduced to below the diffusion-limited process by steric requirements or electrostatic effects and is followed by the rapid entry of the membrane-binding domain into a "defect" in the bilayer. For the former scenario to be operating, knowing that all the lipids have identical k_{as} and k_{dis}, it is necessary for the slow diffusion in the bilayers to be the same in all lipids. Unpublished diphenylhexatriene fluorescence depolarization data from our laboratory indicate that the "microviscosity" of BRPC vesicles is over twice that of POPC vesicles and a similar difference was seen between POPC and a similar bromolipid [15]. In support of the actual collision being limited, we note that b_5, unlike alamethicin [13], has a large negatively charged polar domain (90 amino acids) and it is possible that this domain may sterically shield the membrane-binding domain as the b_5 approaches the lipid vesicle. In addition, it is possible that already-bound b_5 may exert an electrostatic repulsion on further approaching b_5 molecules. Although the maximum number of b_5 molecules per vesicle at completion of binding is only 5, our previous studies showed that under these same conditions, saturation of the vesicle occurs at only 15 proteins per vesicle [5]. We make the tentative suggestion that the overall mechanism of binding may occur via insertion of the protein into preexisting "defects" in the membrane.

These results obtained with (108, 112) mutant b_5 can be compared to kinetic studies with other membrane-associating systems. As mentioned earlier the binding of alamethicin, a 20-amino acid peptide, to DOPC vesicles proceeds at 16% of the diffusion-limited rate, but it was not established that it was then in the final "transbilayer" configuration [13]. A recent paper used brominated lipids to examine the incorporation of the colicin A thermolytic fragment into phosphatidylglycerol vesicles and found a $k \approx 0.02$ s^{-1} with 400 μM lipid. This rate was concluded to be due to the slow insertion of a hydrophobic hairpin into the bilayer [19]. An early study was made of the binding of natural b_5 to DMPC vesicles and this was found to have a $t_{1/2}$ of 20 min at 40°C [3]. The latter b_5 study is complicated, as were our earlier ones [6], by the presence of octameric protein. In this paper we emphasize that a prerequisite to performing kinetic analysis of membrane insertion of proteins, using either fluorescence enhancement or fluorescence quenching by brominated lipids, is the establishment of the aggregation state of the protein. We were fortunate that the (108, 112) mutant b_5 which we generated had a much lower hydrophobicity than the native, and we expect to generate other mutants with similar low hydrophobicities. Indeed, we have found that routine gel filtration analysis of our isolated mutants is an extremely sensitive criterion for determining the reproducibility of their purity.

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