Fluorescence Study of a Temperature-Induced Conversion from the "Loose" to the "Tight" Binding Form of Membrane-Bound Cytochrome b_5†

Alexey S. Ladokhin,‡,§ L. Wang,‖ A. W. Steggles,‖ H. Malak,⊥ and Peter W. Holloway*,‡

Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, Department of Biochemistry, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272, and Center for Fluorescence Spectroscopy, Department of Biochemistry, University of Maryland, Baltimore, Maryland 21201

Received November 9, 1992; Revised Manuscript Received April 26, 1993

ABSTRACT: Cytochrome b_5 is a liver integral membrane protein that has now been expressed in, and isolated from, *Escherichia coli*. The structure–function relationships of the 43 amino acid membrane-binding domain (nonpolar peptide) have been examined in both native and mutant forms of the protein; in the latter, tryptophan residues at positions 108 and 112 were replaced by leucine. The temperature dependence of the fluorescence quantum yield of the Trp residues in the isolated membrane-binding domain was examined while the domain was bound to lipid vesicles. Both the lipid-bound mutant domain and lipid-bound native domain showed an irreversible increase in fluorescence above 50 °C. When the whole cytochrome b_5 molecule, bound to lipid vesicles, was heated to this temperature, there was a conversion of the metastable, intermembrane-exchangeable ("loosely" bound), conformation to a final, virtually unexchangeable ("tightly" bound), conformation. It has been suggested previously that the protein exists in a "looped back" conformation and a "bilayer penetrating" conformation. Although the present studies are not designed to determine the absolute conformations of the loose and tight forms, the changes observed in steady-state and frequency-modulated fluorescence and the lack of change in depth of Trp 109 in the bilayer are consistent with a movement of the C-terminal segment from a looped back to a bilayer penetrating conformation as the tight form is generated.

Cytochrome b_5 (b_5)[1] is an amphipathic protein of 133 amino acid residues with a polar domain and a hydrophobic membrane-binding domain. The hydrophobic domain is suggested to include residues 90–133, and as pointed out by other authors (Visser et al., 1975), because of its length, it is possible for binding to vesicles to be achieved in two orientations. One of these (a hairpin loop) would have the hydrophobic polypeptide chain loop around to present the carboxyl terminus to the exterior aqueous environment; the other (transbilayer) would have the chain pass through the bilayer so that the carboxyl terminus was in the internal aqueous compartment. The whole domain is not long enough to traverse the bilayer more than once, and the carboxyl terminus is unlikely to be in the hydrophobic portion of the bilayer as it contains a penultimate aspartate residue besides the carboxyl terminal aspartate residue. Although cytochrome b_5 incorporation into phosphatidylcholine vesicles has been extensively studied by a number of laboratories, there is still some controversy as to which conformation is present when the protein is incoporated into membranes.

A second area of uncertainty is in regard to the tightness of the binding of the protein to the vesicles. The binding of b_5 to membranes and the transfer between membranes have been extensively studied by ourselves and others (Leto et al., 1980; Tennyson & Holloway, 1986; Enoch et al., 1979; Dailey & Strittmatter, 1981; Takagaki et al., 1983a,b). It was shown that the binding of cytochrome b_5 to some membranes was in a "loose" form, so that the protein can be exchanged out if fresh vesicles are added, whereas the binding to other membranes was in a "tight" form, so that no such exchange can occur. There were initial suggestions that correlations existed between the tight with transbilayer and loose with hairpin loop, but more recent studies have suggested that even in the tight form the protein is in a hairpin loop conformation (Arinc et al., 1987; Ozols, 1989). Because of these more recent studies there are no indications as to the, presumably subtle, conformational differences between the tight and loose forms. In the previous studies from this laboratory a simple kinetic assay was developed to follow the exchange of b_5 between different membranes (Leto et al., 1980). This assay involves monitoring the fluorescence of the Trp's in the membrane-binding domain while the protein spontaneously exchanges between POPC and BRPC vesicles. In the former vesicles the fluorescence is enhanced, and in the latter vesicles it is quenched. The present paper contains a quantitative implementation of this method to yield the characteristic time constants of the process.

The work described here was initiated to obtain information on the dynamic environment of the membrane-binding domain, by monitoring the temperature dependence of the fluorescence of the single-Trp-containing mutant of b_5 which we have prepared (Ladokhin et al., 1991). During these studies it was

† Supported by NIH GM 23858, NSF DIR-9016177, and funds from the American Heart Association, Ohio Affiliate, and by the Ohio Board of Regents Academic and Research Challenge.

* Author to whom correspondence should be addressed.

‡ University of Virginia.

§ Permanent address: A. V. Palladin Institute of Biochemistry, Ukrainian Academy of Sciences, Kiev 252030, Ukraine.

‖ Northeastern Ohio Universities College of Medicine.

⊥ University of Maryland.

[1] Abbreviations: b_5, cytochrome b_5 (the complete 133 amino acid residue protein); native b_5, the native cytochrome b_5 isolated from rabbit liver; mutant b_5, the cytochrome isolated from *E. coli* with Trp 108 and Trp 112 of the native sequence both replaced by Leu; NPP, the membrane-binding domain of the cytochrome b_5 which is released from cytochrome b_5 by trypsin treatment; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; 4,5-, 6,7-, 9,10-, or 11,12-BRPC, 1-palmitoyl-2-(dibromostearoyl)-phosphatidylcholine with bromine atoms at the 4,5-, 6,7-, 9,10-, or 11,-12-positions, respectively; TOE, DL-tryptophan octyl ester; preheated sample, sample incubated at 50 °C for 20 min after the protein and membrane components were mixed; unheated sample, sample incubated only at room temperature.

0006-2960/93/0432-6951$04.00/0

observed that there was an irreversible increase in quantum yield of the single Trp (Trp 109) in the membrane-binding domain of this mutant and that, after heating, the cytochrome was no longer in the initial loose state but was now tightly bound. These results indicate that binding of cytochrome b_5 to membranes is not a one-step reaction and involves a prebinding step. The transition from this prebound loose state to a final tight state is promoted by higher temperatures. The increase in Trp fluorescence quantum yield, seen with this transition, is consistent with transition of the polypeptide comprising the membrane-binding domain from a hairpin loop to membrane spanning conformation. A preliminary report of this work has been presented (Ladokhin et al., 1993).

MATERIALS AND METHODS

Rabbit liver cytochrome b_5 (b_5) was isolated, and small unilamellar lipid vesicles were prepared by probe sonication, as described previously (Markello et al., 1985). The mutant rabbit b_5 with Trp 108 and Trp 112 both replaced with Leu was isolated from *Escherichia coli* as described previously (Ladokhin et al., 1991). NPP's were isolated by trypsin treatment of the b_5's as described previously (Holloway & Mantsch, 1989). DL-Tryptophan octyl ester (TOE) was from Sigma Chemical Co. (St. Louis, MO). POPC was from Avanti Polar Lipids, Inc. (Pelham, AL), and BRPC's were synthesized as described previously (Markello et al., 1985; Tennyson & Holloway, 1986). Spectral subtraction and curve fitting was performed with Spectra Calc (Galactic Industries Corp., Salem, NH).

Samples containing 20 μM protein or 5 μM TOE in 10 mM Hepes buffer containing 0.1 mM EDTA (pH 7.1) were mixed with enough of a 30 mM stock solution of sonicated POPC vesicles to achieve a lipid to protein molar ratio of 400 or a lipid to TOE ratio of 1000. The samples were then incubated for 2 h at room temperature before any fluorescence measurements were taken. When exchange of b_5 was studied, samples of protein and POPC vesicles were incubated together, under the conditions specified under Results, and to this mixture was then added sonicated 9,10-BRPC vesicles to achieve an equimolar ratio of POPC to BRPC.

Steady-state fluorescence measurements were made with an SLM 8000c spectrofluorometer (SLM/Aminco, Urbana, IL) in the configuration described previously (Ladokhin et al., 1991). An excitation wavelength of 293 nm was used to avoid possible contribution of Tyr; both excitation and emission slits were 4 nm. Spectra were recorded from 300 to 500 nm. Normally five spectra were averaged to obtain an adequate signal-to-noise ratio. Spectra were then transferred to DOS format, and after subtraction of background, the spectra were fitted with a log-normal distribution

$$I(\lambda) = I_0 \exp -\{\ln 2(\ln \rho)^{-2}[\ln(1 + (\lambda - \lambda_{max})(\rho^2 - 1)/(\rho\Gamma))]^2\} \quad \text{for } \lambda > \lambda_{max} - [(\rho\Gamma)/(\rho^2 - 1)] \quad (1)$$

$$I(\lambda) = 0 \quad \text{for } \lambda < \lambda_{max} - [(\rho\Gamma)/(\rho^2 - 1)]$$

where λ_{max} is the wavelength of maximum intensity of the spectrum, I_0 is the maximum intensity observed at λ_{max}, Γ is the width of the spectrum at half of I_0, and ρ is the parameter of asymmetry.

When the temperature dependence of fluorescence was studied, the sample was thermostated for 15 min at each temperature before any measurements were taken. The temperature in the cell holder, as measured by a thermocouple, was stable to within 0.1 °C during the data acquisition. For each temperature point the area under the spectrum of NPP bound to POPC was divided by the area under the spectrum of the model chromophore, TOE, also in POPC. The relative intensities were then separately normalized to be equal to 1 at 25 °C. This procedure serves to emphasize any differences between the temperature-dependent dynamics of the environment of the indole ring in the protein–membrane complex compared to the indole ring of the TOE, which is in a predominantly lipid environment.

To eliminate scattering artifacts caused by the lipid vesicles, while determining quenching of fluorescence with different BRPCs, the samples were subjected to the dilution technique of Eisinger and Flores (1985). The difference between the corrected and uncorrected intensities was 10–20%.

When the intervesicle exchange of b_5 was studied, an excitation wavelength of 270 nm (8 nm slit) was used and emission was monitored at 340 nm (16-nm slit) every 3 s. The kinetics of the fluorescence intensity decrease observed when 9,10-BRPC vesicles were added to b_5 bound to POPC vesicles was described by fitting to a sum of exponentials

$$I(t) = \sum A_i \exp(-t/\theta_i) + I(\infty) \quad (2)$$

where θ_i is a decay time, A_i is the amplitude of the ith component, and $I(\infty)$ is the intensity at infinite time.

The frequency-domain fluorescence data were obtained on a fluorometer described previously (Lakowicz at al., 1986). The cavity-dumped output of a synchronously pumped rhodamine 6G dye laser was used to generate a laser pulse width of about 5 ps, which was then frequency-doubled to 292 nm and put through a polarizer oriented at the magic angle. Fluorescence was detected by a Hamamatsu R1564U microchannel plate photomultiplier tube placed after a 345-nm cutoff filter. Intensity decay was described either as a sum of exponentials or as continuous distributions of exponentials. The parameters of decay were estimated from the experimental and calculated values of the phase shift and the modulation, at multiple frequencies (Lakowicz, 1987).

All parameters in the frequency domain and kinetic parameters from eq 2 were estimated by using a nonlinear least-squares procedure, and confidence intervals were calculated for 0.67 confidence probability by scanning in multiparametrial space (Johnson & Frasier, 1985).

RESULTS

The temperature dependences of the relative quantum yields of the fluorescence of native and mutant NPP in POPC are presented in Figure 1. The first heating scan (open symbols) was found to pass through an irreversible transition. However, once the sample has been heated, the temperature dependence of its relative fluorescence fell on a straight line (solid symbols) irrespective of whether the sample was being heated or cooled. These data demonstrate that the interaction of these two peptides with the membrane cannot be described simply as a one-step process; rather, before reaching the final conformation, the protein goes through a metastable prebound state. The transition from this pre-bound state to the more stable conformation is achieved only after heating above 50 °C and is irreversible. It should be emphasized that the temperature dependence of the fluorescence of TOE showed no hysteresis, so that the significant increase in the fluorescence ratio of NPP to TOE observed after heating can be solely attributed to an increase in the quantum yield of Trp in the NPP.

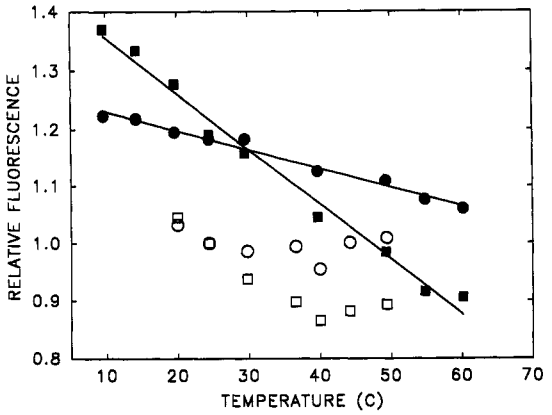

FIGURE 1: Temperature dependences of the relative quantum yield of the fluorescence of native (squares) and mutant (circles) NPP in POPC vs TOE in POPC at the same temperature. The data set for each protein was independently normalized to 1 for the value at 25 °C. Open symbols represent first heating scan. See text for details.

Table I: Spectral Properties of the Steady-State Fluorescence of Native and Mutant NPP Bound to POPC Vesicles Measured at 25 °C[a]

	preheated sample	unheated sample
	Native NPP	
λ_{max} (nm)	340.5	340.7
Γ (nm)	52.9	52.9
ρ	1.343	1.343
	Mutant NPP	
λ_{max} (nm)	335.7	335.6
Γ (nm)	51.2	50.8
ρ	1.304	1.304

[a] The parameters were obtained by fitting spectra to a log-normal distribution as described under Materials and Methods. Γ is the bandwidth, and ρ is the parameter of asymmetry.

It is remarkable that this increase is not associated with any changes in the spectral properties of the NPP. Parameters presented in Table I were precisely obtained by fitting the spectra to a log-normal distribution. As one can see, the position of the maximum of the spectral distribution of intensities (λ_{max}), the bandwidth (Γ), and the parameter of asymmetry (ρ) remain the same for both the unheated and preheated samples of native and mutant NPP bound to POPC. This implies that neither the polarity nor the mobility of the Trp environment are changed, thus ruling out the possibility of thermal denaturation of the peptide or even of any significant displacement of the indole ring in the structure of the protein–membrane complex.

Additional support for a lack of movement of the Trp moiety as well as for lack of a large conformational change in the vicinity of the Trp moiety comes from the data on quenching of fluorescence with lipids brominated at different positions of the *sn*-2-acyl chain (Markello et al., 1985). Figure 2 presents the relative quenching of the fluorescence of mutant NPP with 4,5-, 6,7-, 9,10-, and 11,12-BRPC (the ratio of the area under the spectrum of NPP in BRPC to that in POPC) vs the distance of the bromines from the head group-hydrocarbon boundary (McIntosh & Holloway, 1987). Both curves, for preheated and unheated samples, were normalized separately and are almost overlapping. The absence of thermal denaturation was also demonstrated by Fourier transform infrared spectroscopy of both the native and mutant NPP. We have previously shown that the secondary structures of native and mutant NPP are very similar (Ladokhin et al., 1992b) and have recently found that heating does not appreciably alter the secondary structure. The contributions

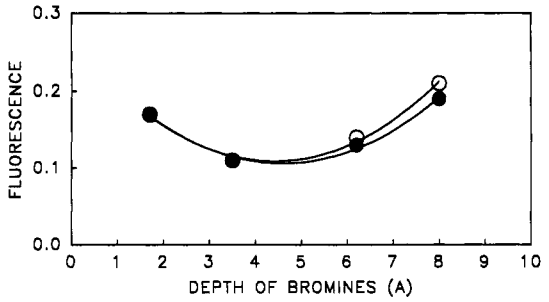

FIGURE 2: Relative fluorescence of the mutant NPP in different brominated lipids. The relative fluorescence is plotted vs the average depth of bromine atoms below the head-group-hydrocarbon boundary (McIntosh & Holloway, 1987). The fluorescence of unheated (○) and preheated (●) is normalized to the fluorescence in POPC for each curve separately.

Table II: Effect of Preheating on Parameters of the Fluorescence Decay of Mutant NPP Bound to POPC[a]

	preheated sample	unheated sample
	Biexponential Decay	
τ_1 (ns)	5.91 [5.72; 6.31]	6.57 [6.13; 7.11]
τ_2 (ns)	2.81 [2.53; 3.30]	3.49 [3.33; 3.69]
α_1[b]	0.71 [0.58; 0.78]	0.45 [0.36; 0.56]
χ^2	1.79	2.01
$\langle\tau\rangle$[c]	5.40 [5.35; 5.49]	5.32 [5.20; 5.44]
	Gaussian Distribution	
τ_{mean} (ns)	5.02 [4.93; 5.08]	4.67 [4.54; 4.77]
half-width (ns)	3.62 [3.37; 3.86]	4.21 [3.91; 4.60]
χ^2	1.98	2.12

[a] Confidence intervals are presented in square brackets. [b] $\alpha_2 = 1 - \alpha_1$. [c] $\langle\tau\rangle$ the average lifetime, as defined under Discussion.

of α helix to the secondary structure of the native NPP in DMPC and POPC vesicles were 58 and 61%, respectively, in the unheated samples and 51 and 48% in the preheated samples. Corresponding values for the mutant NPP were 62 and 62% in the unheated samples and 51 and 50% in the preheated samples (P. W. Holloway, unpublished data). In addition, it should be noted that the NPP contains only 44 amino acid residues, and its isolation involves gel filtration in 50% acetic acid–water, which would be denaturing, yet, the secondary structure of this isolated domain is virtually indistinguishable from the secondary structure of the domain in the whole b_5 (Holloway & Mantsch, 1989).

To further study the effect of preheating on protein conformation, the fluorescence demodulation and phase shift of the fluorescence lifetimes of the mutant NPP bound to POPC vesicles were measured. The best fit parameters of fluorescence decay are presented in Table II. The fluorescence decay is not monoexponential, and at least three parameters (two exponents) are required to get a reasonable fit, which is consistent with our early results (Ladokhin et al., 1992a). An alternative fit with a continuous Gaussian distribution of lifetimes (two parameters are fitted) is of the same quality and is also presented. It appears that the fluorescence decay of the preheated sample is prolonged as compared to that of the unheated; however, the difference is almost marginal. These data will be discussed later.

The existence of two, or more, states of membrane interaction is reminiscent of earlier observations on the two types of interaction of b_5 with membranes. It was therefore logical to compare the exchangeability of the preheated and unheated states of b_5. The experimental protocol was as follows. The b_5 (native or mutant) was mixed with POPC vesicles in a molar ratio of 1:400 (Ladokhin et al., 1992b) which ensured all protein was bound. After incubation for

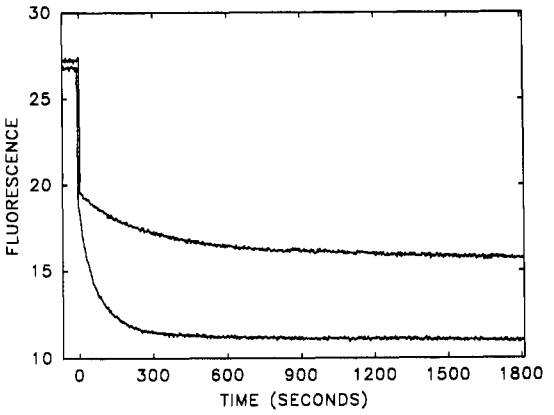

FIGURE 3: Kinetics of the fluorescence decrease of preheated (upper curve) and unheated (lower curve) samples of mutant b_5 in POPC vesicles due to the protein exchange to 9,10-BRPC vesicles. The intensity drop at zero time is caused by the dilution of the sample when the 9,10-BRPC vesicles were added. See text for details.

3 h at 25 °C, the sample was divided into two parts; one portion was incubated further at 25 °C for 4 h, the other was kept for 20 min at 50 °C to convert the membrane-binding domain into its final conformation (it was feared that further heating might cause significant loss of heme). The samples were then brought to 25 °C, and to each sample were then added equal amounts of 9,10-BRPC vesicles, so an overall protein:lipid molar ratio of 1:800 was achieved. The loss of fluorescence intensity due to exchange of protein from POPC to 9,10-BRPC vesicles was monitored (Figure 3). As can be seen in Figure 3, after the initial drop in fluorescence intensity due to dilution, further decreases occurred with rates depending on the thermal history of the sample. The rate of exchange of the mutant b_5 between POPC vesicles and 9,10-BRPC vesicles, revealed by this fluorescence decrease, is slower with the preheated sample.

Figure 4 shows the same data as in Figure 3 but presented in a semilogarithmic plot, together with the multiexponential fits. The solid line in Figure 4A (preheated sample) represents the best fit with a sum of three independently decaying exponential components, and the dashed line shows the fit with only two components. It should be noted that the three-exponential fit is not at all altered if the last two, long time point, data are deleted. The goodness of fit of the data in Figure 4A is very similar with three or two exponentials, and the latter gives the same distribution of amplitudes as for the three-exponential fit, except that the third decay time is ∞. Similarly, in Figure 4B (unheated sample) the solid line is for two components and the dashed line is for one. Both preheated and unheated samples show a behavior which is more complex than can be described by a single-exponential decay. However, as shown in Table III, the amplitude of the slow component ($\theta \approx 10^3$ s) seen with the unheated sample only accounts for 10% of the total intensity loss and a component with approximately the same amplitude, and decay time was also found in the decay of the preheated sample. We suggest that this, minor, slow component is due to some event unrelated to protein exchange (perhaps lipid exchange among POPC and BRPC vesicles, vesicle fusion, or bleaching), and it is ignored in the further analysis. With this simplification, with the unheated sample the exchange process proceeds rapidly with a single decay time, as is expected from the loosely bound protein. In contrast, when the sample was preheated, the intensity loss could only be accounted for by including two components which contribute almost equally to the intensity loss but have significantly different decay times. The very

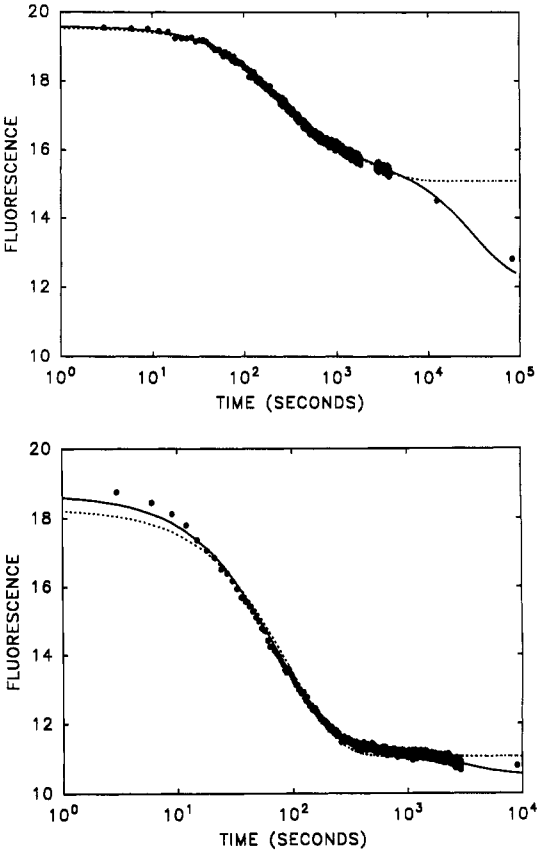

FIGURE 4: Semilogarithmic plot of the fluorescence kinetics when 9,10-BRPC vesicles were added to the mutant b_5 bound to POPC vesicles. (A, top) Preheated sample. The solid line represents a three-exponential decay using the parameters given in Table III. The broken line is for a two-exponential fit and is given for comparison. (B, bottom) Unheated sample. The solid line represents a two-exponential decay using the parameters given in Table III. The broken line is for a one-exponential fit and is given for comparison. See text for details.

Table III: Analysis of the Rate of Decrease of Fluorescence when 9,10-BRPC Vesicles Are Added to Mutant b_5 Bound to POPC Vesicles[a]

	preheated sample	unheated sample[b]
A_1	2820	7260 (±70)
θ_1 (s)	220	73.4 (±1)
A_2[c]	1020	890 (±110)
θ_2[c] (s)	1150	2750 (±800)
A_3	3550	
θ_3 (s)	31070	
$I(\infty)$	12170	10050 (±20)

[a] The parameters describing the fitted solid lines in Figure 4 were obtained as described under Results. The time dependences of the fluorescence intensity were fitted to the following equation: $I(t) = \sum A_i \exp(-t/\theta_i) + I(\infty)$, where θ_i is a decay time, A_i is the amplitude of the ith component, and $I(\infty)$ is the intensity at infinite time. [b] Confidence intervals, shown in parentheses, were calculated only for the unheated sample, since too many parameters were required to fit the kinetics of the preheated sample. [c] This component is very similar in both amplitude and time in both preheated and unheated samples and is ignored in further analysis, as described under Results.

slowly exchanging component ($\theta = 3 \times 10^4$ s) may be interpreted as a virtually unexchanging tightly bound protein. The interpretation of the nature of the other component ($\theta =$ 220 s) cannot be categorized as simply a loose or a tight form and requires further examination. It should be strongly emphasized that these present studies used a mutant of b_5

which has quite different hydrophobic properties from native b_5 and its loosely bound form exchanges approximately 100 times faster than that of the native b_5 (Tretyachenko-Ladokhina et al., 1992). If it is assumed that native b_5 also contains this intermediate component and the same 100-fold ratio exists between the decay times for mutant and native b_5, then it would not be expected to be observable with native b_5. In fact, no exchange at all was observed after samples of native b_5 were heated with POPC vesicles whereas unheated native b_5 gave the same result as reported earlier (Leto et al., 1980).

As noted above, there was a significant difference between the fluorescence intensities of the preheated and unheated samples when these were compared after prolonged times when protein exchange is completed. This is to be expected as the preheated sample has a higher quantum yield than the unheated protein, as shown in Figure 1.

DISCUSSION

Temperature Dependence of the Transition from Loose to Tight Binding. Several papers have previously shown that membrane-bound b_5 can exist in either loosely or tightly bound forms depending on the nature of the membrane. In addition, a tightly bound form can be generated by incorporating the b_5 in the presence of detergent, with subsequent removal of the detergent (Enoch & Strittmatter, 1979; Tennyson & Holloway, 1986). The data presented here indicate that the transition from loose to tight can also be achieved by heating. The addition of b_5 to vesicles produces a form that can be easily exchanged (loosely bound), while incubation of this form at higher temperatures generates a protein conformation that exchanges very slowly (tightly bound). The exchange process was here monitored by the movement of the protein from POPC vesicles, where it has a high quantum yield, to 9,10-BRPC vesicles, where the fluorescence is quenched. With this sensitive assay, and using a mutant form of the protein which is less hydrophobic, it was found that, after heating, the mutant b_5 actually existed in two conformations, one of which exchanged very slowly ($\tau \approx 3 \times 10^4$ s) and one with an intermediate rate ($\tau \approx 200$ s). It is possible that this latter form may represent an incomplete transition from the initial to the final conformation. It is also possible that, at room temperature, fluctuations might lead to the appearance of a loosely bound conformation even after heating, which may lead to loss of fluorescence intensity. In this case what we see as an intermediate component is a convolution of the transition from a tight to a loose conformation within one membrane with the exchange of the resultant loose form between membranes.

Conformation of the NPP in the Loose and Tight Forms. In support of our suggestion that heating produces a protein with a different conformation, which is the tightly bound form, it was observed that the quantum yield was higher after heating. Although we have no direct evidence that there is a major conformational change, this increase in fluorescence quantum yield is certainly in agreement with the original proposal (Visser et al., 1975; Enoch et al., 1979; Takagaki et al., 1983a,b) that in the tight form of b_5 the NPP traverses the bilayer. This transbilayer form would be expected to have a higher quantum yield now that the Trp in the NPP is only in contact with the lipid acyl chains rather than being in contact with the adjacent peptide, as it would be in the hairpin loop conformation. It should be noted that the position of the Trp in the depth of the bilayer could remain virtually the same in both conformations, as is seen from the quenching profile with various BRPC's (Figure 2).

The frequency domain data on Trp fluorescence provide additional information on protein conformation. Multiple-exponential fluorescence decay is usually considered as evidence for multiple conformations, with each conformation having a single-exponential lifetime while the pre-exponential factors are related (although not necessarily linearly) to the relative concentrations of molecules adopting those conformations. According to such a description, the data in Table II would suggest that two conformations of NPP exist at room temperature both before and after heating. Preheating appears to have a greater effect on the ratio of the two species than on their lifetimes, with an increase in the abundance of the long-lived component.

In our previous study we suggested that the lifetime heterogeneity in NPP is not necessarily indicative of the existence of two conformations but is rather a way to account for the nonexponentiality of decay, itself due to a number of possible reasons (such as dipolar relaxation quenching) (Ladokhin et al., 1992a). Thus, the assignment of lifetimes obtained during the multiexponential fit to individual species could be misleading. We suggested two ways of further analysis (Ladokhin et al., 1992a): (1) to compare changes in steady-state intensity with changes in the average fluorescence lifetime, calculated as $\langle \tau \rangle = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$, and (2) to analyze lifetime distributions. As is evident from Table II, the average lifetimes of preheated and unheated mutant NPP are very similar. The small, almost marginal, increase cannot account for the significant increase in the quantum yield (Figure 1). We therefore suggest that the increase in steady-state intensity is mainly due to the reduction in the static quenching. It is possible that in the prebound conformation Trp interacts with some amino acid side chain group, so that a nonfluorescent complex is formed: reaction (quenching) can occur in the ground state or immediately after excitation. Preheating will lead to a conformational change in which the quenching group will no longer be in the immediate environment of the Trp. Another possible explanation would be the difference in natural lifetimes (Toptygin et al., 1992). The argument for the static quenching, from our perspective, comes from the observation that the shape of the fluorescence band is preserved after preheating (Table I).

An alternative way of presenting the fluorescence decay data is to fit them to a continuous lifetime distribution. In our previous study we showed that a unimodal symmetrical Gaussian distribution gives the most adequate fit for the mutant NPP in POPC (Ladokhin et al., 1992a). The results presented in Table II show a very small effect of preheating on the parameters of the Gaussian band. The slight narrowing of the half-width may indicate that, upon reaching the final conformation, the protein adopts fewer substates than before heating. The interpretation of the small but definite shift of the center of the distribution to longer lifetimes is the same as for the average lifetime discussed earlier.

Conclusions. We suggest the following sequence of events during binding of b_5 to the lipid vesicle. The protein first binds to the membrane in a metastable prebound state. At this stage binding is loose and the protein can be easily exchanged. Transition to the true bound state requires overcoming an energy barrier, but once it is crossed, this new protein conformation is preferred. It may be that the increasing fluctuational dynamics induced by heating assists in crossing this energy barrier, but it is possible that prolonged incubation (perhaps several days) at room temperature would also suffice, providing the lipid vesicles are themselves stable during this time. At this point the protein is tightly bound

and it will exchange only very slowly.

In most of the previous studies, the generation of loose and tight binding of b_5 to membranes was dependent either on the choice of membrane (Enoch et al., 1979) or upon the method of incorporating the protein into the bilayer (Enoch & Strittmatter, 1979; Takagaki et al., 1983a,b; Tennyson & Holloway, 1986). Our study demonstrates that this is an internal feature of the protein itself and that in a single experiment we can manipulate the tightness of binding by varying thermodynamic parameters. This provides a much simpler system with which to examine the transition. Obviously, since living organisms cannot easily manipulate their temperature over a broad range, other ways could be used to control the binding of b_5 to a certain membrane. For example, a variation in acyl chain length could affect the energy barrier between the prebound and final conformations, providing that, in structural terms, this transition is related to the flip-flop of the C terminus of the NPP across the bilayer. Our preliminary data indicate that this prebinding step is virtually undetectable when b_5 is added to dimyristoylphosphatidylcholine vesicles.

ACKNOWLEDGMENT

We thank Dr. Michael L. Johnson for his technical assistance with the computer analysis of the fluorescence kinetics data.

REFERENCES

Arinc, E., Rzepecki, L. M., & Strittmatter, P. (1987) *J. Biol. Chem. 262*, 15563–15567.

Dailey, H. A., & Strittmatter, P. (1981) *J. Biol. Chem. 256*, 3951–3955.

Eisinger, J., & Flores, J. (1985) *Biophys. J. 48*, 77–84.

Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145–149.

Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1979) *J. Biol. Chem. 254*, 6483–6488.

Holloway, P. W., & Mantsch, H. H. (1989) *Biochemistry 28*, 931–935.

Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.

Ladokhin, A. S., Wang, L., Steggles, A. W., & Holloway, P. W. (1991) *Biochemistry 30*, 10200–10206.

Ladokhin, A. S., Malak, H., Johnson, M. L., Lakowicz, J. R., Wang, L., Steggles, A. W., & Holloway, P. W. (1992a) *Proc. SPIE—Int. Soc. Opt. Eng. 1640*, 562–569.

Ladokhin, A. S., Tretyachenko-Ladokhina, V. G., Holloway, P. W., Wang, L., & Steggles, A. W. (1992b) *Biophys. J. 62*, 79–81.

Ladokhin, A. S., Steggles, A. W., & Holloway, P. W. (1993) *Biophys. J. 64*, a59.

Lakowicz, J. R., Laczko, G., & Gryzcynski, I. (1986) *Rev. Sci. Instrum. 57*, 2499–2506.

Lakowicz, J. R., Cherec, H., Gryczynski, I., Joshi, N., & Johnson, M. L. (1987) *Biophys. Chem. 28*, 35–50.

Leto, T. L., Roseman, M. A., & Holloway, P. W. (1980) *Biochemistry 19*, 1911–1916.

Markello, T., Zlotnick, A., Everett, J., Tennyson, J., & Holloway, P. W. (1985) *Biochemistry 24*, 2895–2901.

McIntosh, T. J., & Holloway, P. W. (1987) *Biochemistry 26*, 1783–1788.

Ozols, J. (1989) *Biochim. Biophys. Acta 997*, 121–130.

Takagaki, Y., Radhakrishnan, R., Gupta, C. M., & Khorana, H. G. (1983a) *J. Biol. Chem. 258*, 9128–9135.

Takagaki, Y., Radhakrishnan, R., Wirtz, K. W. A., & Khorana, H. G. (1983b) *J. Biol. Chem. 258*, 9136–9142.

Tennyson, J., & Holloway, P. W. (1986) *J. Biol. Chem. 261*, 14196–14200.

Toptygin, D., Svobodova, J., Konopasek, I., & Brand, L. (1992) *J. Chem. Phys. 96*, 7919–7930.

Tretyachenko-Ladokhina, V. G., Ladokhin, A. S., Wang, L., Steggles, A. W., & Holloway, P. W. (1992) *FASEB J. 6*, A85.

Visser, L., Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 1194–1199.