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Fluorescence Study of a Mutant Cytochrome b_5 with a Single Tryptophan in the Membrane-Binding Domain†

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

Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, and Department of Biochemistry, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272

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ABSTRACT: Fluorescence studies of cytochrome b_5 are complicated by the presence of three tryptophans, at positions 108, 109, and 112, in the membrane-binding domain. The cDNA for rabbit liver cytochrome b_5, isolated from a λgt11 library, was used to generate a mutated mRNA where the codons for tryptophans-108 and -112 were replaced by codons for leucine. The sequence was expressed in *Escherichia coli* and the mutant protein was isolated. This mutant protein had the expected absorption spectrum, and its amino acid composition was confirmed by amino acid analysis and by DNA sequencing of the construct. The fluorescence emission spectrum of the mutant is blue-shifted and is narrower than that of the native protein. The quantum yield of the mutant protein, per molecule, is only 60% of that of the native protein, and the enhancement when bound to lipid vesicles or detergent micelles is higher for the mutant. Fluorescence anisotropy measurements and quenching studies using brominated lipids suggest that the fluorescence of the native protein is due to tryptophans-109 and -108 while tryptophan-112 does not emit but undergoes nonradiative energy transfer to tryptophan-108. With this mutant, it was shown that incomplete energy transfer from tyrosines-126 and -129 to tryptophan-109 occurs when the membrane binding domain is inserted into lipid vesicles, which suggests that the membrane-binding domain does not exist in a tight hairpin loop.

Cytochrome b_5 (b_5)[1] is an integral membrane protein found in the endoplasmic reticulum of liver cells and other cells. It plays a central role in metabolism by virtue of its involvement in fatty acid desaturation (Holloway, 1983) and elongation (Nagai et al., 1983), cholesterol metabolism (Grinstead & Gaylor, 1982), and the metabolism of xenobiotics (Tamburini & Schenkman, 1988). In addition, because of its relative ease of isolation, it has become a popular model for studies of lipid–protein interactions and for evaluating various physical techniques for the study of membrane proteins in general. It should be noted, however, that it is probably only representative of a subset of membrane proteins. For many of these aforementioned studies, use has been made of the fluorescent

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* To whom correspondence should be addressed.
‡ Permanent address: A. V. Palladin Institute of Biochemistry, Academy of Sciences of the Urkrainian SSR, Kiev 252030, Ukraine, USSR.
§ University of Virginia Health Sciences Center.
‖ Northeastern Ohio Universities College of Medicine.

[1] Abbreviations: b_5, cytochrome b_5 (the complete 133 amino acid residue protein); rabbit b_5, native cytochrome b_5 isolated from rabbit liver; mutant b_5 cytochrome isolated from *E. coli* with Trp-108 and Trp-112 of the native sequence both replaced by Leu; NPP, membrane-binding domain of cytochrome b_5 which is released from cytochrome b_5 by trypsin treatment; BRPC, 1-palmitoyl-2-(dibromostearoyl)phosphatidylcholine; Tris buffer, 5 mM Tris–acetate containing 1 mM EDTA (pH 8.2); OG, octyl β-D-glucoside; TX100H, Triton X-100 hydrogenated; Γ, full width at half-maximal height of a spectral band.

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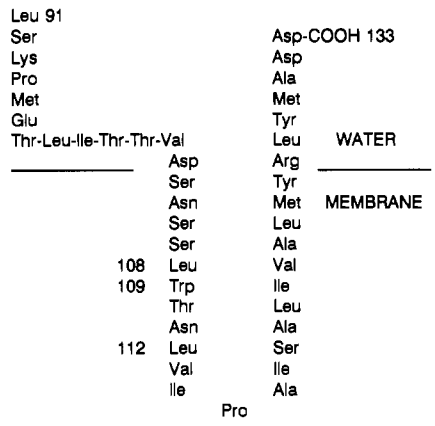
Scheme I



properties of the protein. The whole protein has 133 amino acid residues of which 4 are Trp; 1 of these is located in the hydrophilic heme-containing catalytic domain, and its fluorescence is strongly quenched (Huntley & Strittmatter, 1972). The other 3 are in a 5-residue segment from residues 108 to 112 (Trp-Trp-Thr-Asn-Trp, in rabbit b_5, Scheme I) located in the 36-residue hydrophobic membrane-binding domain (Ozols, 1989). The presence of three Trps complicates fluorescence studies, although for many of these studies it has been convenient to consider the three Trps as so localized as to be equivalent to a single fluorescent center. Indeed, evidence has been produced that the overall emission is solely from Trp-109 (Fleming & Strittmatter, 1978; Fleming et al., 1979).

Many studies of protein structure are taking advantage of bacterial expression and site-directed mutagenesis, and these techniques would be applicable to b_5. Indeed, Beck von Bodman et al. (1986) have already generated b_5, both the whole 133-residue protein and a 98-residue polar fragment, in *Escherichia coli* and they, and others (Funk et al., 1990), have performed extensive studies with mutants of the polar domain. This report describes the isolation of a mutant form of rabbit b_5 which contains a single Trp (Trp-109) in the membrane-binding domain (NPP) and details the first fluorescence studies of this simplified system.

Materials and Methods

Isolation of Rabbit Cytochrome b_5 and Mutant Cytochrome b_5. Rabbit liver cytochrome b_5 was isolated as described previously (Markello et al., 1985). The mutant b_5 was 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 procaryote ribosome-binding site and the rabbit liver b_5 sequence (Dariush et al., 1988) where the codons for Trp-108 and -112 have been replaced by codons for Leu. This construct was made using two oligonucleotides (National Biosciences, Hamel, MN): TCCACAAGCTGGAA (which converts Trp-108 to a Leu) and ATCACCAAGTTGG (which converts Trp-112 to a Leu) and the procedure of Kunkel (1985). One-milliliter stocks of *E. coli*, frozen at −80 °C in 7% DMSO, were used to inoculate 3 × 150 mL of LB medium containing ampicillin (100 μg/mL) which was allowed to grow up at 37 °C overnight and in turn used to inoculate 8.5 L of the same medium in a New Brunswick SF-116 (15-L) fermentor. Growth was followed by OD at 550 nm, and when it reached 1.4, usually in 4 h, the system was induced with 1 mM isopropyl thiogalactoside, and growth was continued for another 3 h. The cells were harvested, washed with Tris buffer, and frozen at −80 °C. For isolation of mutant b_5, the cells were suspended in 140 mL of Tris buffer and broken with a French press. Phenylmethanesulfonyl fluoride was added to 0.6 mM, and hemin to 12 μM, and the mixture was sonicated 5 × 0.5 min at 50% output with a Heat Systems W-350 sonifier. The mixture was heated at 50 °C for 2 min and incubated first with RNase (type III-A, 7 units/mL) for 2 h and then with DNase (type II, 60 units/mL) and 5 mM Mg^{2+} for 2 h at room temperature. These incubations were performed in a dialysis bag while the mixture was dialyzed against 1000 mL of Tris buffer, first without Mg^{2+} and then with 5 mM Mg^{2+}. This removes much of the liberated nucleotides which prevent subsequent binding of the mutant b_5 to columns. After a further 2 h of dialysis at 5 °C, the mixture was removed from the bag, made 10% in glycerol, and was then extracted with 10 volumes of acetone at 0 °C. The pellet was collected by centrifugation and extracted at 5 °C with 1000 mL of Tris buffer containing 0.5% Triton X-100 and 4 μM hemin. After 14 h, the supernatant was isolated by ultracentrifugation, pumped on hydroxylapatite, and eluted with a linear potassium phosphate gradient (1 mM–0.4 M, pH 7.0) containing 27 mM OG. Further purification was obtained by the following sequential procedures: Sephacryl S200 in Tris buffer containing 0.1 M NaCl; DEAE with a linear gradient of Tris buffer containing 27 mM OG to the same with 0.4 M NaSCN; and a final hydroxylapatite column with a linear phosphate gradient from 1 mM to 0.4 M containing 27 mM OG. The final eluate was concentrated, with several dilutions, in an Amicon concentrator with a PM 10 membrane to remove salt and OG.

Lipid vesicles were prepared by sonication as described previously (Markello et al., 1985), and NPPs were isolated by trypsin treatment of the b_5s as described previously (Holloway & Mantsch, 1989). RNase, DNase, and OG were obtained from Sigma Chemical Co. (St. Louis, MO), and Triton X-100, hydrogenated, was from Calbiochem (San Diego, CA).

Fluorescence Measurements. Fluorescence measurements were made with an SLM 8000c spectrofluorometer (SLM/Aminco, Urbana, IL) with a double-grating excitation monochromator and single-grating emission monochromator. The excitation beam was passed through a vertical polarizer, and the emission was monitored through a polarizer at 54.7° from the vertical. To avoid the possible contribution of Tyr, the fluorescence excitation wavelength was 293 nm, unless otherwise indicated. Both excitation and emission slits were 2 nm, unless otherwise indicated. Samples were in 1-cm path-length cuvettes maintained at 20 °C. Fluorescence anisotropy was determined from intensities measured with a vertical (V) and a horizontal (H) position of the polarizer (first subscript) and analyzer (second in subscript):

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where the correction factor $G = I_{HV}/I_{HH}$ is determined by averaging in a series of experiments. The SLM 8000c was used for all measurements except for experiments with excitation at 295 nm when an SLM 4800 with a single-grating excitation monochromator was used. Both spectrofluorometers were in the T-format.

Analysis of Spectra. Sample and background spectra were transferred into the MSDOS format for further manipulation with Spectra Calc software (Galactic Industries Corp., Salem, NH). After subtraction of background, the spectra were fitted with a log-normal distribution:

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

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

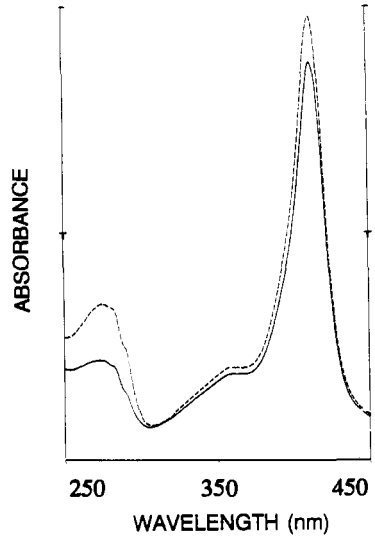


FIGURE 1: Absorption spectra of rabbit b_5 and mutant b_5. The samples were in 10 mM Tris–acetate/0.1 mM EDTA, pH 8.2. The spectra of rabbit b_5 (---) and mutant b_5 (—) are shown.

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. Minor deviations from the log-normal distribution which were observed at 400 nm due to the transmission properties of the monochromator (Lakowicz, 1983) do not affect the results.

Quantum Yield Measurements. Quantum yields were calculated by comparing the total fluorescence (the area under the spectrum) of the protein to that of aqueous tryptophan. Several values for the quantum yield of tryptophan have been reported, but as this uncertainty does not affect our result, a value of 0.13 (Tatischeff & Klein, 1975) was used in the calculations. More critical, as the quantum yields of rabbit and mutant b_5 were to be compared, were the experimental conditions chosen for the determinations, and in order to avoid artifacts, two series of experiments were conducted. In the first, both protein solutions were of the same optical density at the excitation wavelength (293 nm); in the second, equal concentrations of protein were used so that they each had equal absorbance due to heme at the emission wavelength. Under both experimental situations, the optical density at 293 nm was less than 0.07. The true tryptophanyl absorption was calculated by subtraction of that due to heme (Huntley & Strittmatter, 1972) from the total absorbance. Both series of experiments gave the same result for the relative quantum yield of the rabbit and mutant b_5, within experimental precision. To eliminate scattering artifacts caused by the lipid vesicles, the samples were subjected to the dilution technique of Eisinger and Flores (1985). The difference between the corrected and uncorrected intensities was 10–20%.

RESULTS

Characterization of the Mutant Cytochrome b_5. The purity of cytochrome b_5 is usually estimated from the absorbance ratio at 412 nm to that at 280 nm. From Figure 1, it can be seen that this ratio for the mutant is greater than that seen for the rabbit b_5. For rabbit b_5 (each molecule contains one heme, four Trps, and five Tyrs), this ratio is 2.8. For the water-soluble domain of b_5 (one heme, one Trp, and three Tyrs), it is 6.3. Knowing that the molar extinction coefficient of Trp at 280 nm is 5 times that of Tyr, it can be calculated that this mutant should have a ratio of 4.2. Exactly this ratio

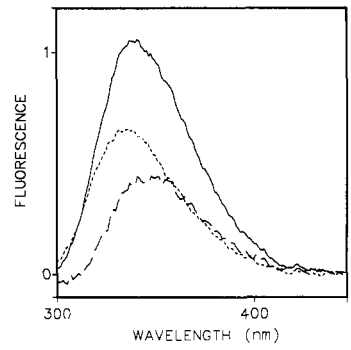


FIGURE 2: Fluorescence emission spectra of rabbit b_5 and mutant b_5. The emission spectra were normalized to protein concentration. The spectra of rabbit b_5 (—), mutant b_5 (---), and the difference (rabbit − mutant) (--) are shown.

was observed. Amino acid analysis of the membrane-binding domain (NPP) of the rabbit and mutant b_5 was performed. Since Trp is destroyed on hydrolysis, Leu was chosen as the reference amino acid to characterize the mutation. The ratio Leu/(Ile + Val + Ser) was found to be 0.43 for rabbit NPP (0.45 predicted from sequence) and 0.58 for the mutant NPP (0.58 predicted).

Fluorescence Spectroscopic Study of the Mutant Cytochrome b_5. The mutant protein differs from the native protein in several of its spectroscopic properties. The quantum yields of the mutant and rabbit b_5 are 0.048 and 0.040, respectively, relative to the value of 0.13 used for aqueous tryptophan. Since it is known that some of the Trps are not fluorescent, at least the one in the polar domain, it is more convenient to consider the fluorescence yield per molecule (the quantum yield multiplied by the numbers of Trps in the molecule): this has a value of 0.096 for mutant and 0.16 for rabbit b_5. Since the Trp in the polar domain is known to be silent, 0.096 is the quantum yield of Trp-109 in the mutant. If only one Trp (109) is fluorescent in the rabbit b_5, its quantum yield should be 0.16. This discrepancy, as well as the fact that the mutant has a blue-shifted emission spectrum and a narrower width, compared to the native form (Figure 2), suggests that Trp-109 is not the sole fluorescent Trp in the native protein. This is contrary to the conclusions of Fleming and Strittmatter (1978). Assuming complete additivity of fluorophores the spectrum of "this other chromophore(s)", Trp-X, could be obtained by subtracting the spectrum of the mutant from that of the native protein. When this was done, a spectrum with a maximum at 346–347 nm and Γ of 52–53 nm was obtained, although this spectrum did contain a small negative component at the blue edge of the major band (Figure 2). This small negative component indicates that the assumed spectral additivity is not absolute. With this very small reservation, the percentage contributions of these two chromophores to the spectrum of the native protein are 60% from Trp-109 and 40% from the Trp-X. Information as to the nature of this Trp-X came from fluorescence anisotropy measurements. Table I shows that the native protein has a greater degree of depolarization when examined both in aqueous solution and in detergents, which is characteristic of energy transfer between Trps. Rabbit b_5 is known to aggregate in aqueous solution (Calabro et al., 1976), although at these concentrations of protein the solution should be largely monomeric, and the mutant is presumed to have similar properties. There is, therefore, the possibility that the energy transfer seen in aqueous solution could be heteromolecular between adjacent molecules in the aggregate. It has been shown that rabbit b_5 is monomolecular in Triton X-100 (Robinson & Tanford, 1975) and is likely to be so in octyl glucoside; hence, the larger depolarization seen with the

Table I: Anisotropy of the Fluorescence of Rabbit b_5 and Mutant b_5 in Detergents[a]

conditions	λ_{ex} (nm)	r[b] rabbit b_5	r[b] mutant b_5
in buffer	280	0.04 ± 0.01	0.06 ± 0.01
	295	0.05 ± 0.01	0.08 ± 0.01
+OG (27 mM)	280	0.062 ± 0.005	0.095 ± 0.005
+TX100H (2 mM)	295	0.058 ± 0.004	0.084 ± 0.003
	300	0.088 ± 0.004	0.158 ± 0.005

[a] Emission was measured at 350 nm (slit 16 nm) with an SLM 4800 spectrofluorometer (for excitation at 295 nm) or with an SLM 8000c spectrofluorometer (for excitation at 280 and 300 nm with a 4-nm slit). The protein concentration was 4 μM. [b] The fluorescence anisotropy, r, was calculated as described under Materials and Methods.

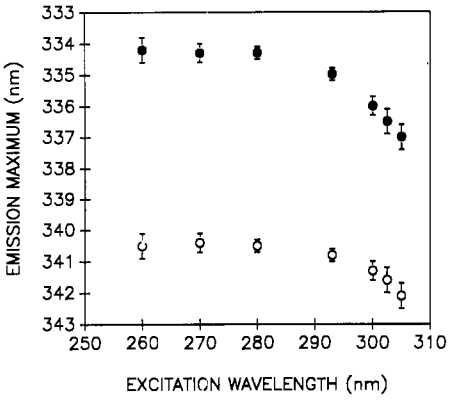


FIGURE 3: Dependence of the wavelength of the emission maxima on the excitation wavelength. Spectra of rabbit b_5 (○) and mutant b_5 (●) at a concentration of 10 μM in buffer were measured.

rabbit b_5 compared to the mutant b_5 in these two detergents is likely due to homomolecular energy transfer between adjacent Trps in the membrane-binding domain of the same molecule.

Information on the microconformational heterogeneity and on the dynamics of the environment of Trp can be obtained by red-edge excitation (Demchenko, 1986; Demchenko & Ladokhin, 1988). Both proteins show a red shift of the emission spectrum when excited at the long-wavelength wing of the absorption spectrum, $\lambda_{ex} > 293$ nm (Figure 3).

Interaction of the Proteins with Lipids and Hydrogenated Triton. Both proteins are able to bind to lipids and detergents, and both show an increase in fluorescence intensity and a blue shift. Assuming complete additivity of Trp-109 and Trp-X contributions to the total intensity, the spectrum of Trp-X can be obtained by subtracting the spectrum of the mutant b_5 from that of rabbit b_5, with weights that correspond to their fluorescent yields under different conditions (for protein in membrane or in micelle). As shown in Table II, in going from buffer to POPC, Trp-109 shows a 2-nm blue shift and a 1.96-fold increase in intensity, and Trp-X shows no blue shift and only a 1.61-fold increase in intensity. In hydrogenated Triton X-100, the corresponding values for Trp-109 are 2 nm and 2.70-fold and for Trp-X no shift and 1.20-fold. These different responses of Trp-109 and Trp-X suggest that Trp-109 is entering a more hydrophobic environment than that seen by Trp-X. This would place the latter closer to the vesicle or micelle surface. These locations were confirmed by use of brominated lipids. As shown in Table III, both rabbit b_5 and mutant b_5 are quenched, relative to the value in POPC or hydrogenated Triton, by bromolipids. Maximal quenching is obtained by 6,7-BRPC with both proteins. If the subtraction procedure described above is used with these data, the quenching of Trp-109 and Trp-X by the different bromolipids

Table II: Fluorescence Parameters of Rabbit b_5 and Mutant b_5 in Lipid and Detergent[a]

conditions		rabbit b_5	mutant b_5 (Trp-109)	rabbit − mutant[b] (Trp-X)
in buffer	I[c]	0.160 (100%)	0.096 (60%)	0.064 (40%)
	λ[d] (nm)	341	335	346–347
+POPC[e]	I	0.291 (100%)	0.188 (65%)	0.103 (35%)
	λ (nm)	339	333	346–347
+TX100H (2 mM)	I	0.336 (100%)	0.259 (77%)	0.077 (23%)
	λ (nm)	337	333	346–347

[a] Fluorescence spectra were measured at 20 °C with an SLM 8000c spectrofluorometer under the following conditions: excitation, 293 nm; emission and excitation slits, 2 nm. Protein concentrations varied from 2 to 5 μM (see Materials and Methods). [b] Fluorescence parameters for rabbit b_5 and mutant b_5 were calculated from the measured spectra as described under Materials and Methods. The parameters of the spectrum obtained by subtraction of the spectrum of the mutant b_5 from that of rabbit b_5 (rabbit − mutant) (see Figure 2) are presented. [c] The fluorescence yield of the molecule (I) was calculated from the quantum yield [using a value of 0.13 for tryptophan in water (Tatischeff & Klein, 1975)] normalized to the number of Trps in the molecule. For each condition, the percentages in parentheses indicate the contribution of the different Trps to the total fluorescence. [d] The emission maxima of the spectra (λ) were calculated as described under Materials and Methods. [e] The lipid to protein ratio was 200.

Table III: Relative Fluorescence of Rabbit b_5 and Mutant b_5 in Bromolipids[a]

rabbit b_5 F (%)	conditions	mutant b_5 F (%)
55 ± 1	in buffer	33
100	+POPC	65
33	+4,5-BRPC	21
29	+6,7-BRPC	17
37	+9,10-BRPC	18
47	+11,12-BRPC	24
116	+TX100H	90

[a] The relative fluorescence, F (fluorescence normalized to protein concentration), was corrected for scattering artifacts by the method of Eisinger and Flores (1985) as described under Materials and Methods. Standard deviations do not exceed 1 for any value. Protein concentratino was 3 μM, lipid to protein molar ratio was 200, and concentration of hydrogenated Triton X-100 was 2 mM.

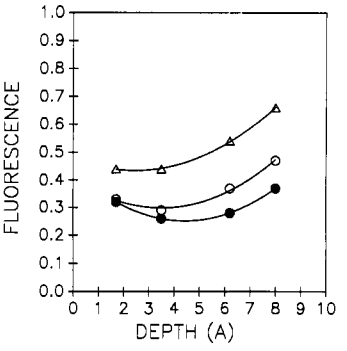


FIGURE 4: Relative fluorescence of b_5 in different brominated lipids. The relative fluorescence is plotted versus the average depth of bromine atoms below the head-group–hydrocarbon boundary (McIntosh & Holloway, 1987). The fluorescence of rabbit b_5 (○), mutant b_5 (●), and the difference (rabbit − mutant) (△) is normalized to the fluorescence in POPC for each curve separately.

is as shown in Figure 4. The minima of the parabolas plotted through the data points are the positions of Trp-X (2.5 Å) and Trp-109 (4.5 Å) below the head-group–hydrocarbon interface.

Estimation of Tyr–Trp Energy Transfer in the Mutant Membrane-Binding Domain. A number of previous studies have shown that the interaction of the isolated NPP with lipids is the same as the interaction of the NPP when it is part of the whole molecule (Fleming & Strittmatter, 1975; Kleinfeld

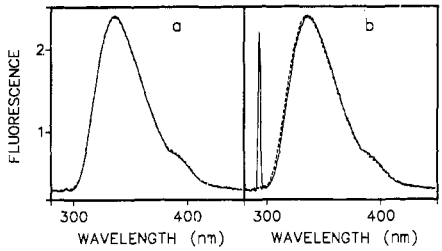


FIGURE 5: Fluorescence emission spectrum of the nonpolar peptide of mutant b_5 in buffer and in lipid vesicles. The emission spectrum of mutant NPP (7 μM) (a) in buffer and (b) in 1 mM POPC was measured at 20 °C with an SLM 8000c spectrofluorometer. The excitation wavelengths were 278 (---) and 293 nm (—), with a 2-nm slit, using vertical polarization. Emission was monitored with a 2-nm slit using horizontal polarization.

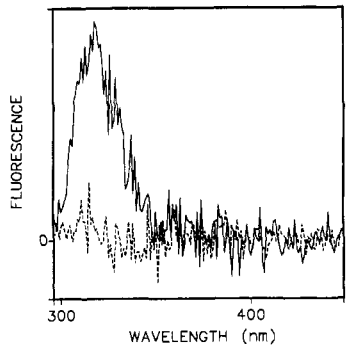


FIGURE 6: Difference between the fluorescence spectra measured with an excitation of 278 nm and with an excitation of 293 nm for the mutant NPP in POPC (—) and in buffer (---) (see Figure 5).

& Lukacovic, 1985). We confirmed this conclusion with the mutant NPP by measuring fluorescence changes upon interaction with membrane. When the isolated mutant NPP was added to vesicles composed of 6,7-BRPC, the residual fluorescence was only 26% of that seen when the same mutant NPP was added to POPC vesicles. This is the same degree of quenching as seen with the whole mutant b_5 and indicates that isolated NPP is an adequate model for further study. One of the unresolved problems of b_5 topology is the tertiary structure of the NPP in the membrane. The question is whether the peptide spans the bilayer or makes a tight hairpin loop structure. This latter structure, for the mutant form of the NPP, is shown in Scheme I, on the basis of a structure proposed by Ozols (1989). In this structure, it can be seen (Scheme I) that Trp-109 is in the descending arm of the loop and Tyr-126 and Tyr-129 are relatively close by in the ascending arm of the loop. Tyr–Trp residues are suitable fluorescence donor–acceptor pairs for distance measurements and could give information on the distance between the two arms of the NPP.

Fluorescent spectra of the mutant NPP with excitation at 278 nm, where both Tyr and Trp absorb strongly, and at 293 nm, where only Trp has appreciable absorption, were obtained to estimate Tyr → Trp energy transfer. As shown in Figure 5, the spectra obtained at the two excitation wavelengths, when normalized to equal maximum intensity, were identical when the NPP was in buffer but were different when the NPP was bound to POPC vesicles. In Figure 6 are shown the spectra, on an expanded scale, which result from a subtraction of the fluorescence spectra measured with an excitation of 293 nm from that measured with an excitation of 278 nm for the mutant NPP both bound to POPC vesicles and in buffer. The peak observed in the former case is suggestive of Tyr emission and shows that there is not complete Tyr → Trp energy transfer when the NPP is bound to POPC vesicles. As the R_0 of this donor–acceptor pair is 15 Å, this suggests that the simple model shown in Scheme I is not absolutely correct. The complete absence of Tyr emission when the NPP is examined in buffer could be due to energy transfer to Trps in adjacent molecules in the NPP aggregate which exists in aqueous solution.

Discussion

The fluorescence properties of cytochrome b_5 have been extensively investigated in order to obtain information as to the topography of the membrane-binding domain. All of these studies have had the ambiguity that this domain has three Trp residues. The closeness of these residues allows them to be treated as a, more or less, localized fluorescent unit, and several groups, including our own, have tested the efficacy of various types of "depth dependent" quenching molecules at quenching this unit. Evidence has also been presented (Fleming & Strittmatter, 1975; Fleming et al., 1979) that only Trp-109 is fluorescent; this would simplify the analysis, but other data do not support the idea of a single fluorescent Trp (Markello et al., 1985). For more sophisticated topographical and dynamic studies of the NPP of b_5, including time-resolving investigations, there is a need for a single-Trp mutant of b_5. In addition, it was hoped that an investigation of the fluorescence properties of such a single-Trp mutant would help in the understanding of the large amount of data already generated with the native three-Trp protein.

Cytochrome b_5 was first expressed in *E. coli* by Beck von Bodman et al. (1987), who constructed two synthetic double-stranded DNA molecules based on the published amino acid sequence (Ozols & Heinemann, 1982) for rat liver b_5. One coded for 134 amino acids and 1 for a 99 amino acid fragment. Upon expression and removal of the initiator codon, these 2 should produce the whole rat b_5 (133 residue) and the erythrocyte (98 residue) forms of the protein. They showed that these DNA molecules could be incorporated into a vector and introduced into *E. coli*. The proteins were expressed, and on SDS gels, the liver form (133 residues) was larger than the polar fragment (98 residues). They also noted that the polar fragment was expressed at a much higher level. There are other reports where the polar domain was expressed and mutant forms of the polar domain were isolated for various biophysical and enzymological studies (Rodgers et al., 1988; Funk et al., 1990); however, as far as we are aware, no further studies have been reported on the intact b_5. As part of our studies on the regulation and control of cytochrome b_5 expression, the cDNA for rabbit b_5 was isolated from a λgt11 library and sequenced. These aspects have already been reported (Dariush et al., 1988; Wang & Steggles, 1991). To generate the single-Trp mutant, the procedure of Kunkel (1985) was used to replace Trp-108 and -112 with Leus, and the identity of the constructs was confirmed by DNA sequencing.

Although we have had extensive experience with the isolation of cytochrome b_5 from rabbit liver, these procedures failed completely when applied to *E. coli*. As reported by Beck von Bodman et al. (1987), the protein expressed in *E. coli* appears after cell disruption in both the soluble and membrane fractions. We therefore made no attempt to isolate the cell membranes but instead isolated the protein from disrupted whole cells by detergent extraction. Initial trials showed that extensive proteolysis of the b_5 occurred during extraction, and we found that this could be reduced dramatically if a heat treatment step were included. In addition, the binding of the negatively charged b_5 to either DEAE or hydroxylapatite was prevented by nucleotides formed by the RNase and DNase

steps, and it was found that these nucleotides could be largely removed by performing the enzymic treatments in a dialysis bag which was immersed in a large volume of buffer. Finally, the addition of free hemin at two steps in the isolation appeared to facilitate the isolation of the holoprotein. It has been noted before, when producing the polar domain in *E. coli*, that there is a lag between apoprotein production and heme synthesis (Funk et al., 1990). The purified protein showed a single band on SDS gels with the same mobility as b_5 isolated from rabbit liver and had the appropriate ratio of absorbances at 412 nm verses 280 nm (4.2). Final confirmation of the structure came from amino acid analysis of the NPP which showed that the mutant had the correct increase in Leu content.

The fluorescence properties of the mutant b_5 are very different from those of the native, which immediately suggests that in rabbit b_5 more than Trp-109 is fluorescent. The quantum yield of the mutant, on a molecule basis, is only 60% of that of rabbit b_5; the emission spectrum is blue-shifted and is much narrower. This increased width suggested that it contained the emission spectrum of another chromophore, and an attempt was made to study the properties of this "other chromophore" (Trp-X). If the amino acid sequence of the Trp-containing region of rabbit b_5 is folded into an α helix, then Trp-108 and -112 are seen to stack with their rings parallel; Trp-109 cannot be so oriented. This suggested that energy transfer could occur between Trp-108 and Trp-112. It would also be expected that the fluorescence of Trp-109 in the rabbit b_5 would have the same characteristics as that of Trp-109 in the mutant and the spectrum of the latter could be subtracted from the former to generate the emission spectrum of the Trp-X. This difference spectrum is centered at 346–347 nm and accounts for 40% of the quantum yield of the rabbit b_5. This peak position suggests that the Trp-X is sensing a more polar or more mobile environment than Trp-109, which implies that Trp-X is closer to the membrane interface. According to Scheme I, Trp-X is more likely to be Trp-108 than Trp-112. When the fluorescence anisotropy of the rabbit b_5 was compared to that of the mutant b_5, it was found to be lower in buffer, octyl glucoside, and TX100H micelles which supports the proposal that Trp → Trp energy transfer is occurring in the rabbit b_5. The above analysis of emission spectra and anisotropy data suggests the following model for the fluorescence behavior of rabbit b_5 in aqueous solution, assuming that Trp-109 in rabbit b_5 has the same fluorescent properties as that residue in the mutant. Trp-109 is fluorescent with a λ_{max} of 335 nm, a Γ of 51 nm, and a quantum yield of 0.096; Trp-108 is also fluorescent with a λ_{max} of 346–347 nm, a Γ of 52–53 nm, and a quantum yield of 0.064. Trp-112 does not emit and is responsible for the loss of polarization due to nonradiative energy transfer to another Trp(s) (most probably Trp-108).

Support for this model also comes from a study of the effects of lipid and detergent on the fluorescence of rabbit and mutant b_5. The fluorescence emission from Trp-109 in the mutant b_5 is blue-shifted 2 nm upon binding to POPC and TX100H, and the intensity is enhanced 1.96-fold in POPC and 2.70-fold in TX100H. In contrast, Trp-X shows no blue shift, a 1.61-fold increase in intensity in POPC, and only a 1.20-fold increase in TX100H. In general, these changes again suggest that Trp-X is Trp-108 rather than Trp-112.

Final confirmation of the proposed assignments of the fluorescent Trps of rabbit b_5 came from use of lipids brominated at the 4,5-, 6,7-, and 9,10-positions of the *sn*-2 C_{18} chain. Both rabbit and mutant b_5s gave qualitatively similar quenching profiles; however, the location of Trp-X appears to be somewhat closer to the surface than Trp-109. Trp-109 was maximally quenched by 6,7-BRPC whereas Trp-X was equally quenched by 4,5- and 6,7-BRPC. It is impossible to imagine structure in which Trp-112, located in one loop of an α helix descending from the polar domain (Scheme I), lies closer to the surface than Trp-109. It should be pointed out that after excitation the indole ring changes its dipole moment significantly and therefore is expected to move into a more polar environment. Such a movement of fluorophores, including indole derivatives, toward the membrane surface has been reported (Demchenko & Shcherbatska, 1985; Abrams & London 1991), but if lifetimes are short (as for Trp), then this movement only slightly affects the accuracy of the depth measurements. Nevertheless, the depths reported here will be somewhat smaller than the actual positions of the Trp before absorption of the exciting quantum of light. The existence of such a movement in this sytem is supported by the observation of a small, but detectable (1–3 nm), red shift of emission of the mutant b_5 in BRPCs as compared to POPC.

With the availability of this single-Trp mutant, more critical fluorescence experiments can be performed, and two preliminary ones are described here. Red-edge excitation of both rabbit b_5 and mutant b_5 produces red shifts in the emission spectra (Figure 3). This indicates that the fluorescence from both proteins occurs from a Trp excited state before the fluorophore has reached complete equilibrium with its dipolar environment and that there exists a distribution of microstates with different dipole orientations having rates of dipolar relaxation which are comparable to the fluorescence lifetime of the Trp (Demchenko, 1986; Demchenko & Ladokhin, 1988). A similar red-edge effect was also seen in preliminary studies with both proteins in detergents and POPC which suggests similar conclusions can be drawn for the membrane-bound proteins. These preliminary data were, however, not of sufficient precision to allow the effect of membrane binding on the structural dynamics of the proteins to be evaluated.

The existence of relatively slow dipolar dynamics of the Trp environment is also supported by some preliminary time-resolved measurements (unpublished experiments of Ladokhin, Malak, Lakowicz, and Holloway) which show that the fluorescence lifetime of mutant NPP increases with the increase in the emission wavelength.

With the mutant, it has also been possible to examine the topography of the NPP. The hairpin loop shown in Scheme I places the two Tyrs approximately 12 Å from Trp-109; at such a separation, there should be complete Tyr → Trp energy transfer. When the mutant NPP is examined in buffer, no Tyr spectrum is seen; when it is bound to POPC vesicles, Tyr emission is seen. This suggests that in the POPC the separation is significantly greater than 15 Å [the R_0 for this pair (Steinberg, 1971)]. The lack of Tyr emission seen in aqueous solution can be accounted for by the probable aggregated nature of the NPP. Although the solution properties of the mutant b_5 and its NPP have not been examined, the native NPP would certainly be highly aggregated under these conditions, and so the lack of the Tyr spectrum seen with the mutant NPP could be due to energy transfer of the Tyr to Trp on adjacent molecules in the aggregate. It should be emphasized that the appropriateness of using the membrane-bound NPP as a model for this domain in the intact protein was shown by the identical relative quenching by 6,7-BRPC of the whole mutant b_5 and the mutant NPP.

These initial studies demonstrate that mutant forms of cytochrome b_5 can be isolated from *E. coli* in sufficient quantities for biophysical experiments and that this initial mutant has

similar membrane-binding properties to the native protein. This single-Trp mutant not only offers a simpler system for fluorescence studies but also can be used to help understand the spectroscopic behavior of the native protein itself. Other studies are already underway to further modify the amino acid sequence of the membrane-binding domain to correlate changes in the amino acid sequence with changes in the secondary and tertiary structure of this domain as well as with catalytic activity. Preliminary data obtained with this mutant indicate that its ability to participate in desaturation of long-chain fatty acids is the same as that of rabbit b_5 (unpublished experiments of Ladokhina-Tretyachenko and Holloway).

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Mapping the Binding Domain of a Myosin II Binding Protein†

Gary Walker, Rachel Yabkowitz,‡ and David R. Burgess*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

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ABSTRACT: The way in which actin and myosin II become localized to the contractile ring of dividing cells resulting in cleavage furrow formation and cytokinesis is unknown. While much is known about actin binding proteins and actin localization, little is known about myosin localization. A 53-kDa (53K) polypeptide present in the sea urchin egg binds to myosin II in a nucleotide-dependent manner and mediates its solubility in vitro [Yabkowitz, R., & Burgess, D. R. (1987) *J. Cell Biol. 105*, 927–936]. The binding site of 53K on the myosin molecule was examined in an effort to understand the mechanism of 53K-induced myosin solubility and its potential function in myosin regulation. Blot overlay and chemical cross-linking techniques utilizing myosin proteolytic fragments indicate that 53K binds to fragments proximal to the head–rod junction of myosin. Fragments distal to the head–rod junction do not bind 53K. In addition, the binding of 53K to myosin largely inhibits protease digestion that produces the head and rod fragments. The binding of 53K to the head–rod domain of myosin may be critical in regulation of myosin conformation, localization, assembly, and ATPase activity.

The actin-based cytoskeleton of the sea urchin egg cortex plays a vital role in cytokinesis. After fertilization, changes in the cytoskeleton are induced which mediate contractile ring formation and subsequent contraction. Actin and myosin II, the chemomechanical transducer, become concentrated to the contractile ring which is responsible for cleavage furrow action (Cao et al., 1990; Fujiwara et al., 1976; Schroeder, 1987; Schroeder et al., 1988; Mittal et al., 1987; Nunnally et al., 1980; Aubin et al., 1979). Myosin II, which forms conventional bipolar filaments, has been shown to be critical for contraction of the contractile ring (Mabuchi et al., 1977). Whether myosin exists as bipolar filaments, such as in muscle, and undergoes assembly–disassembly in nonmuscle cells is not known although myosin filaments appear to form in the furrow cortex in *Dictyostelium* during division and can be induced

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* Author to whom correspondence should be addressed.

‡ Present address: Department of Pathology, M7510, University of Michigan Medical School, MRSB 1, 1150 W. Medical Campus Dr., Ann Arbor, MI 48109-0650.

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