Preparation of a Fluorescent Derivative of Cytochrome b_5 and Its Interaction with Phospholipids†

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ABSTRACT: A fluorescent derivative of bovine cytochrome b_5 was prepared by using 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride) in deoxycholate. Reaction conditions were established to specifically label the hydrophobic membrane-binding domain of the protein at a ratio of 0.9 ± 0.1 dansyl group per cytochrome b_5. Fluorescence measurements on the dansyl-labeled protein reflected the state of aggregation of the protein and its binding to lipids. The cytochrome b_5 derivative was a sensitive probe for the detection of phospholipid phase transitions in reconstituted phospholipid vesicles. The rotational relaxation time of the labeled protein was strongly influenced by the phospholipid composition and the cholesterol content of the lipid bilayer, but it was largely insensitive to the integrity of the hydrophilic domain of the protein. When the membrane-binding domain of cytochrome b_5 was bound to phospholipid vesicles, a preferential association with either the gel or the liquid-crystalline phase was not observed. The results suggest that the two domains of cytochrome b_5 undergo predominantly independent motion and that the motion of the dansyl-labeled membrane-binding domain directly reflects the properties of the bulk lipids in the bilayer.

Fluorescence spectroscopy provides a very sensitive and convenient method for studying the dynamic properties of proteins and lipids. For comparison of the fluidity of two membrane preparations, for example, it is generally necessary to introduce a fluorophore with properties amenable to spectroscopic measurements. These fluorophores have usually been small lipophilic molecules such as 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] that report information about the properties of the lipid bilayer (Shinitzky & Barenholz, 1974). Since the motion of membrane proteins may be quite different from the motion of small lipophilic molecules, it is desirable to have a protein with a fluorescent label that could serve to compare the properties of different membrane systems.

In addition to being an important protein in its own right, cytochrome b_5 has a number of properties that make it a useful general membrane probe. The structure and properties of cytochrome b_5 have been extensively studied by several groups. It is composed of two domains that can be separated by digestion with proteases (Ito & Sato, 1968; Spatz & Strittmatter, 1971). One domain is approximately 11 000 daltons and is water soluble. This part contains the heme group and retains the catalytic activity after protease digestion. The other domain is approximately 5000 daltons, and it is hydrophobic in nature. This part is responsible for the binding of the cytochrome to phospholipids or membranes. The intact cytochrome or the isolated membrane-binding fragment aggregates in the absence of detergents or lipids. Isolated intact cytochrome b_5 that is free of detergents will rebind to the endoplasmic reticulum, and the added cytochrome b_5 functions in a similar manner to the endogenous cytochrome b_5 (Strittmatter et al., 1972). Enzymatic studies on reconstituted systems suggest that the cytochrome b_5 is randomly distributed in the bilayer and undergoes lateral diffusion in order to interact with other proteins (Strittmatter & Rogers, 1975).

In this study, a method was developed to specifically label the membrane-binding domain of cytochrome b_5 with dansyl chloride. The behavior of DNS-cytochrome b_5 was determined in sodium deoxycholate and synthetic phospholipid vesicles. The results show that the labeled cytochrome b_5 can be used to obtain information about the motion of the protein in lipids and to compare the properties of different lipid systems.

Materials and Methods

Materials. Sodium deoxycholate, DMPC, DSPC, DOPC, and egg phosphatidylethanolamine were all obtained from Sigma Chemical Co. Sodium deoxycholate was recrystallized by using the method of Strittmatter & Enoch (1978) prior to use for fluorescence experiments. Dansyl chloride was obtained from Pierce Chemical Co. DNSHA was obtained from Molecular Probes, Inc. TPCK-treated trypsin was obtained from Worthington Biochemical Corp. ^{3}H-Labeled egg PC was prepared by methylation of egg yolk phosphatidylethanolamine with [^{3}H]CH_3I (New England Nuclear Corp.) by using the method of Stockton & Smith (1974). Egg PC was prepared by the method described by Clark & Switzer (1977).

Protein Purification. Cytochrome b_5 was isolated from bovine liver by using a method similar to that described by previous investigators (Spatz & Strittmatter, 1971; Strittmatter et al., 1978). Cytochrome b_5 concentrations were determined by using an extinction coefficient of 118 cm^{-1} mM^{-1} at 413 nm for the oxidized cytochrome (Rogers & Strittmatter, 1973). Apocytochrome b_5 was prepared by the HCl–acetone extraction method described by Strittmatter (1960). The hydrophobic membrane-binding fragment of cytochrome b_5 was prepared by a method similar to that described by Fleming & Strittmatter (1978). Cytochrome b_5 in 10 mM Tris–acetate, pH 8.1, was incubated at 4 °C for

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[1] Abbreviations: dansyl chloride, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; DMPC, dimyristoylphosphatidylcholine; DNS-cytochrome b_5, dansyl-labeled cytochrome b_5; DNSHA, *N*-[5-(dimethylamino)naphthyl-1-sulfonyl]hexadecylamine; DOC, sodium deoxycholate; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSPC, distearoylphosphatidylcholine; PB, phosphate buffer (12.2 mM Na_2HPO_4–1.5 mM KH_2PO_4, pH 7.2); PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2); PC, phosphatidylcholine; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; TPCK, tosylphenylalanyl chloromethyl ketone.

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72 h with Worthington TPCK-treated trypsin at a cytochrome b_5 to trypsin molar ratio of 100 to 1. The membrane-binding fragment was then isolated by chromatography on a 1.2 × 35 cm column of Sephadex G-100 equilibrated in PB.

Preparation of DNS-cytochrome b_5. Cytochrome b_5 was labeled with dansyl chloride by the addition of the protein to a mixed micelle of deoxycholate and dansyl chloride. The micelle system was prepared by adding 3.1 μmol of dansyl chloride in acetone to 18 μmol of sodium deoxycholate in methanol and evaporating the solvents under a stream of nitrogen to obtain a thin film. The detergent–dansyl chloride dispersion was prepared by the addition of 2.0 mL of 0.1 M $NaHCO_3$, pH 9.0, followed by vortexing. The cytochrome b_5 (1.25 μmol) was then added in enough buffer to adjust the final detergent concentration to 6 mM. After 10 min of reaction at 23 °C, the labeled cytochrome b_5 was separated from unreacted dansyl chloride by chromatography on a 20-mL Sephadex G-25 column equilibrated in 0.01 M Tris–acetate–0.1 mM EDTA, pH 8.1. The labeled cytochrome was applied to a 1.5 × 10 cm DEAE-cellulose column equilibrated in 0.01 M Tris–acetate–0.1 mM EDTA, pH 8.1. Residual noncovalently bound dye was eluted with a 50-mL linear 0.0–0.25 M NaCl gradient in the column buffer. The labeled cytochrome was eluted with 0.25 M NaSCN–0.25% DOC in the starting column buffer. The detergent was removed by dialysis followed by chromatography on a Sephadex G-25 column both also using the column buffer. The labeled protein was stored at −20 °C. Before use, a sodium dodecyl sulfate denatured sample of the protein was chromatographed on a Sephadex G-25 column in the presence of 0.1% sodium dodecyl sulfate to ensure that the preparation did not contain noncovalently bound fluorophore that might arise from spontaneous hydrolysis.

General Assays. Phospholipid phosphate was determined by the ashing procedure of Ames (1966). The cholesterol content of DMPC–cholesterol mixtures was determined as described previously (Gilmore et al., 1979).

Preparation of Cytochrome b_5–Lipid Complexes. Unilamellar phospholipid vesicles were prepared by sonication of the phospholipid at a concentration between 5 and 15 mM in phosphate buffer (12.2 mM Na_2HPO_4–1.5 mM KH_2PO_4, pH 7.2). Phospholipids were sonicated under nitrogen by using the semimicro probe of a Heat Systems-Ultrasonics W-375 sonicator for 20 min. The temperature of the sonication tube was maintained a few degrees above the phase transition of the synthetic phospholipid. Multilamellar phospholipid liposomes were removed by centrifugation at 100000*g* for 1 h.

DNS-cytochrome b_5 was bound to the phospholipid vesicles in the cuvettes used for the fluorescence studies. A cuvette contained 6 nmol of DNS-cytochrome b_5 and 0.6 μmol of phospholipid in 2.0 mL of PB, and it was incubated at 30 °C for 8 h prior to the fluorescence measurements. The phospholipid to DNS-cytochrome b_5 ratio of 100 to 1 was chosen to ensure that all the DNS-cytochrome b_5 was bound in all cases.

Experiments to determine the rate of cytochrome b_5 binding to phospholipids were conducted by observing the enhancement of the tryptophan fluorescence as described previously by Dufourcq et al. (1975) and Leto & Holloway (1979). Samples were excited at 275 nm, and the emission at 330 nm was continuously monitored after the addition of phospholipid vesicles to a solution of cytochrome b_5 or DNS-cytochrome b_5. The binding kinetics were examined at 30 °C by using egg phosphatidylcholine or DMPC vesicles at protein: phospholipid molar ratios between 1:50 and 1:200. The buffer used for these experiments was 20 mM Tris–acetate, pH 8.1.

Preparation of Unilamellar DNSHA–Phospholipid Vesicles. Phospholipid vesicles were labeled with DNSHA by cosonication of the fluorophore with the phospholipids at a phospholipid:DNSHA ratio of 500:1 in PBS. Multilamellar phospholipid vesicles were removed by centrifugation of the solution for 1 h at 100000*g*.

Fluorescence Instrumentation. Fluorescence emission spectra were recorded on a spectrofluorometer described by Wehrly et al. (1976) and Jameson et al. (1977). The fluorescence emission of DNS-cytochrome b_5 was monitored between 420 and 660 nm by using an excitation wavelength of 340 nm. The reported fluorescence intensity represents the integrated fluorescence emission corrected for the background fluorescence of an identical sample containing cytochrome b_5 instead of DNS-cytochrome b_5. Fluorescence polarization measurements of DNS-cytochrome b_5 were made by using a photon-counting polarization instrument described by Jameson et al. (1978). Samples were excited at 380 nm with a 7-54 excitation filter, and scattered light was eliminated with either a Corning 3-72 or a Corning 3-70 filter.

Fluorescence lifetimes were measured by using a cross-correlation phase fluorometer described by Spencer & Weber (1969) with updated electronics by SLM Instruments, Inc. Samples were excited at 380 nm with light modulated at 18 MHz. Scattered light was eliminated with a Corning 3-70 filter. The average of τ_ϕ and τ_{mod} values was used for all calculations.

Rotational relaxation times were calculated by using the Perrin equation (Weber, 1953)

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where p is the polarization, τ is the fluorescence lifetime, and $\bar{\rho}$ is the rotational relaxation time. The p_0 for DNS-cytochrome b_5 was determined as described in the text.

Results

Preparation and Characterization of Labeled Cytochrome b_5. Various procedures were tried to specifically label cytochrome b_5 in the membrane-binding domain with dansyl chloride, a reagent that has been widely used for the preparation of fluorescently labeled proteins since it was introduced by Weber (1952). The most effective procedure involved adding the purified protein to a mixed micelle of deoxycholate and dansyl chloride as described under Materials and Methods. Noncovalently bound fluorophore was removed by washing the cytochrome b_5 on a DEAE-cellulose column. Difference absorption spectroscopy was used to determine the amount of fluorophore incorporated into the protein by using an extinction coefficient of 3.36 cm^{-1} mM^{-1} at 340 nm for the dansyl–protein conjugate (Hartley & Massey, 1956). The labeling procedure used here resulted in the incorporation of 0.9 ± 0.1 dansyl residue per cytochrome b_5.

For determination of the location of the fluorophore on the protein, a 50-nmol sample of the DNS-cytochrome b_5 was digested with trypsin to cleave the protein into its hydrophilic and membrane-binding fragments. An elution profile of the trypsin-digested protein on a Sephadex G-100 column is shown in Figure 1. The only fluorescent peak eluted at the column void volume with the hydrophobic membrane-binding fragment of the protein which aggregates in the absence of detergents or lipids. The small amount of heme absorbance at 413 nm in the void volume fractions was due to the presence of residual intact cytochrome b_5 that was not cleaved by the trypsin. A

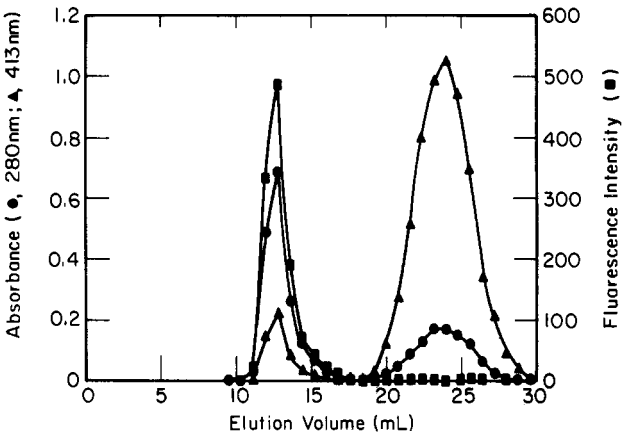

FIGURE 1: Elution profile of a trypsin-digested sample of DNS-cytochrome b_5 on a Sephadex G-100 column equilibrated in PB. DNS-cytochrome b_5 (50 nmol) was incubated with TPCK-treated trypsin at a molar ratio of 100 to 1 for 72 h at 4 °C. The sample was applied to a 1.2 × 35 cm column, and 0.8-mL fractions were collected. The absorbance at 280 (●) and 413 nm (▲) and the fluorescence intensity (■) were determined. The fluorescence emission was monitored between 420 and 660 nm with excitation at 340 nm. The column void volume corresponds to approximately 13 mL.

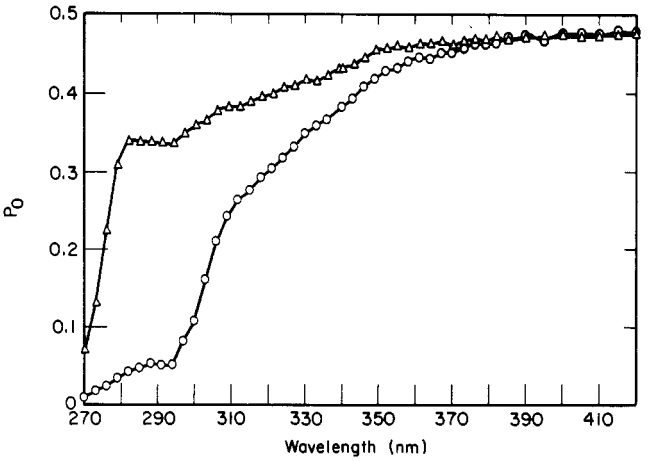

FIGURE 2: Limiting polarization (p_0) for *N*-[5-(dimethylamino)-naphthyl-1-sulfonyl]hexadecylamine (DNSHA) (△) and DNS-cytochrome b_5 (○). The concentrations of DNSHA and DNS-cytochrome b_5 were 10 and 8 μM, respectively. Both fluorophores were dissolved in propylene glycol, and the polarization was determined at −60 °C.

small amount of uncleaved protein was also observed when unlabeled cytochrome b_5 was digested with trypsin under similar conditions as previously reported by Fleming & Strittmatter (1978). Aliquots of the excluded and included fractions from the G-100 column as well as intact DNS-cytochrome b_5 were further characterized by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis. Since the cytochrome b_5 used for the preparation of DNS-cytochrome b_5 was essentially homogeneous, the only fluorescent product observed when the intact protein was run on the gel corresponded to the cytochrome b_5 band (data not shown). The fluorescent material obtained from the void volume fractions of the G-100 column comigrated with the membrane-binding fragment on the gel. No dansyl fluorescence was observed in the lanes containing the hydrophilic fragment.

Previous studies on cytochrome b_5 have shown that the hydrophilic domain contains the heme group and the catalytic activity of cytochrome b_5 (Ito & Sato, 1968; Spatz & Strittmatter, 1971). Labeling the membrane-binding domain with dansyl chloride had no effect on the activity of cytochrome b_5 as would be predicted if the two domains are independent units. A sample of the labeled protein in 0.02 M Tris–acetate, 0.1 mM EDTA, pH 8.1, and 0.1% Triton X-100 could be completely reduced by the addition of NADH and purified cytochrome b_5 reductase (data not shown). The reduction of cytochrome *c* by DNS-cytochrome b_5 also was not inhibited. When the kinetics of binding of DNS-cytochrome b_5 and cytochrome b_5 to phospholipid vesicles were compared, no significant difference was observed (data not shown).

Fluorescence Emission of DNS-cytochrome b_5. The fluorescence emission of DNS-cytochrome b_5 was compared to that of DNS-apocytochrome b_5 to determine whether dansyl–heme energy transfer was significantly quenching the fluorescence. The DNS-apocytochrome b_5 had a 1.9-fold higher fluorescence intensity than the DNS-cytochrome b_5 in PBS where both existed as aggregates. The fluorescence intensity was comparable, however, when the two protein samples were dispersed in PB containing 0.4% deoxycholate. That is, the addition of deoxycholate enhanced the fluorescence of DNS-cytochrome b_5 1.9-fold, but it did not enhance the fluorescence of the apoprotein sample. Visser et al. (1975) have shown that the deoxycholate–cytochrome b_5 complexes are very small and that they contain a single protein molecule in which the protein is the dominant component. Consequently, when DNS-cytochrome b_5 was aggregated in PBS, the fluorescence was reduced by intermolecular energy transfer. When cytochrome b_5 was dispersed by deoxycholate, however, there was no intermolecular or intramolecular quenching of the dansyl fluorescence by the heme.

Limiting Polarization of DNS-cytochrome b_5. The limiting polarization (p_0) of DNS-cytochrome b_5 and DNSHA, the dansyl derivative of hexadecylamine, was determined between 270 and 420 nm in propylene glycol at −60 °C (Figure 2). Lower p_0 values were observed for DNS-cytochrome b_5 than for DNSHA at low wavelengths, particularly between 270 and 310 nm. A comparison of the fluorescence emission spectra of cytochrome b_5 and DNS-cytochrome b_5 between 290 and 730 nm when excited at 275 nm indicated that fluorescence energy transfer was occurring between a tryptophan residue in the membrane-binding domain of the protein and the dansyl fluorophore. This resulted in a depolarization of th fluorescence and accounted for the lower p_0 of DNS-cytochrome b_5. A p_0 value of 0.465 was obtained for both DNS-cytochrome b_5 and DNSHA at 380 nm. Consequently, so that tryptophan–dansyl energy transfer could be avoided, all fluorescence polarization experiments were conducted at an exciting wavelength of 380 nm.

The limiting polarization of DNS-cytochrome b_5 at 380 nm was also determined from Perrin plots of the fluorescence polarization vs. temperature for the protein dissolved in PBS or in a complex with deoxycholate (Figure 3). The Perrin plots were corrected for the temperature dependence of the fluorescence lifetime of the protein. The p_0 value determined for DNS-cytochrome b_5 in PBS (0.446) was higher than the value obtained for the DNS-cytochrome b_5–deoxycholate complex (0.432). This difference in p_0 may be due to slight motion of the protein within the small detergent micelle. The effect of altering the aqueous solvent viscosity upon the fluorescence polarization of DNS-cytochrome b_5 was examined as well (Figure 3). The addition of recrystallized sucrose to the DNS-cytochrome b_5–deoxycholate complex gave a Perrin plot similar to that obtained by varying the temperature (data not shown). In contrast, no change in the polarization of the DNS-cytochrome b_5 bound to egg phosphatidylcholine vesicles was observed after increasing the aqueous solvent viscosity by adjusting the solution to 35% (w/w) sucrose (Figure 3). These data suggested that the motion of the dansyl-labeled mem-

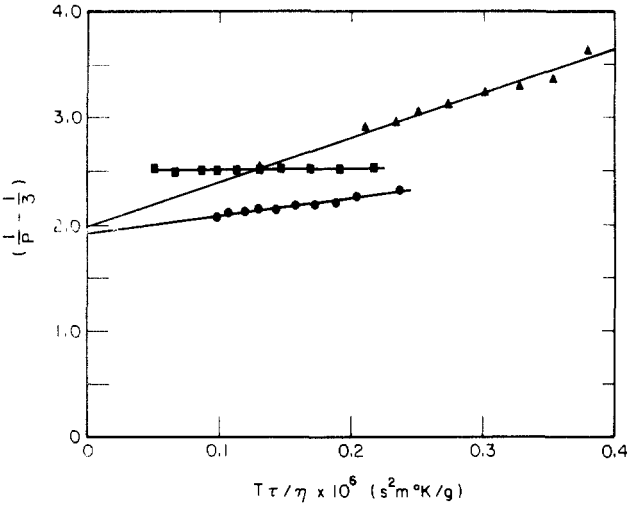

FIGURE 3: Perrin plots of $1/p - 1/3$ vs. temperature for DNS-cytochrome b_5 in PBS (●) and in PB containing 0.4% DOC (▲). The fluorescence polarization was determined between 2 and 40 °C. The fluorescence polarization of DNS-cytochrome b_5 bound to egg phosphatidylcholine vesicles (■) was measured at 25 °C. The viscosity of the aqueous medium in this sample was altered by the addition of recrystallized sucrose. The Perrin plots for DNS-cytochrome b_5 in PBS and in deoxycholate micelles were corrected for the temperature dependence of the fluorescence lifetime. The DNS-cytochrome b_5 concentration was 3 μM.

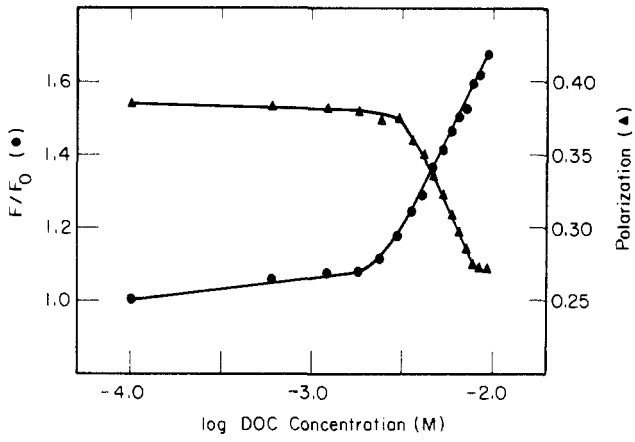

FIGURE 4: Effect of sodium deoxycholate concentration on the fluorescence intensity and polarization of DNS-cytochrome b_5. A 2 μM solution of DNS-cytochrome b_5 in PB was titrated with successive additions of 250 mM sodium deoxycholate. The fluorescence polarization (▲) and the fluorescence intensity (F) were recorded after each addition. F_0 is the fluorescence intensity of DNS-cytochrome b_5 in the absence of detergent. The fluorescence intensity data are expressed as F/F_0 (●).

brane-binding domain in egg phosphatidylcholine vesicles was not altered by the decrease in motion of either the vesicle itself or the hydrophilic domain of the protein that occurred when the viscosity was increased.

The presence of residual unbound DNS-cytochrome b_5 in the samples of DNS-cytochrome b_5 bound to phospholipid vesicles could result in errors in the observed fluorescence polarization. For determination of whether unbound DNS-cytochrome was present in the samples used for fluorescence experiments, DNS-cytochrome b_5 was bound to ^{3}H-labeled egg PC vesicles and subjected to chromatography on Sepharose 4-B. All the DNS-cytochrome b_5 eluted with the ^{3}H-labeled egg PC (data not shown). No free DNS-cytochrome b_5 could be detected by fluorescence spectroscopy.

Interaction of DNS-cytochrome b_5 with Deoxycholate. The effect of deoxycholate on the fluorescence polarization and

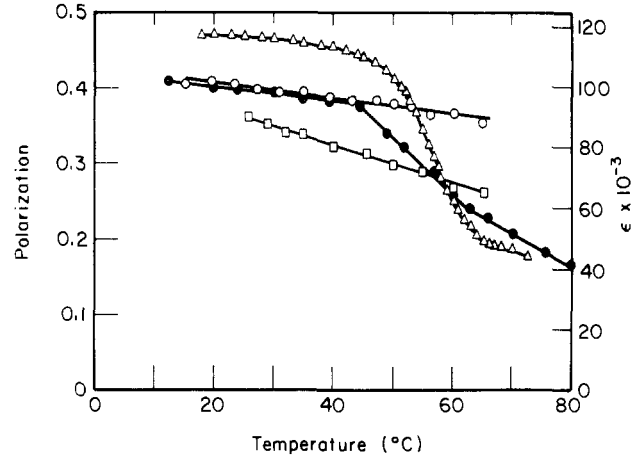

FIGURE 5: Temperature dependence of the fluorescence polarization of DNS-cytochrome b_5 in PBS (●), DNS-cytochrome b_5 in DMPC vesicles (□), and the dansyl-labeled membrane-binding fragment in PBS (○). The temperature dependence of the molar extinction coefficient at 413 nm of cytochrome b_5 in PBS (△) is also shown. The concentrations of DNS-cytochrome b_5 and the dansyl-labeled membrane-binding fragment were approximately 3 μM. The absorbance at 413 nm was determined at a protein concentration of 10.3 μM.

intensity of DNS-cytochrome b_5 is shown in Figure 4. The increase in the fluorescence intensity and the decrease in the fluorescence polarization were due to the interaction of the protein with deoxycholate micelles. The value of 4.5 mM for the midpoint of the change in polarization and intensity is a reasonable value for the critical micelle concentration of the detergent in a buffer of this ionic strength (I = 0.034). Previous investigators have shown that the critical micelle concentration of deoxycholate is dependent upon ionic strength. At an ionic strength of I = 0.1, a value of approximately 3.0 mM has been reported, while at I = 0.01, values of 4.0–6.0 mM have been reported (Benzonana, 1969; Makino et al., 1973; Robinson & Tanford, 1975). The change in the aggregation state of DNS-cytochrome b_5 from an aggregate to a detergent–protein complex decreased the polarization from 0.384 to 0.273.

Denaturation of DNS-cytochrome b_5. The fluorescence polarization of DNS-cytochrome b_5 in DMPC and the dansyl-labeled membrane-binding fragment in PBS showed a steady decrease in polarization with increasing temperature (Figure 5). The membrane-binding fragment was extensively aggregated in PBS, and consequently it had a higher polarization value. The intact DNS-cytochrome b_5 in PBS, which was also aggregated, showed a steady decrease in polarization until approximately 45 °C, and then the polarization decreased abruptly. This coincided with an abrupt decrease in the heme absorbance at 413 nm in the intact protein. This indicated that above 45 °C the hydrophilic domain was denaturing and was responsible for the abrupt decrease in the polarization that was observed for the intact protein in PBS. The membrane-binding fragment, on the other hand, appeared not to denature and remained aggregated within the temperature range that was studied.

Fluorescence Lifetime of DNS-cytochrome b_5. The temperature dependences of the fluorescence lifetimes of DNS-cytochrome b_5 in PBS, deoxycholate micelles, and DMPC vesicles are shown in Figure 6. The fluorescence lifetime of the dansylated membrane-binding fragment bound to DMPC vesicles is also shown. The fluorescence lifetime of the labeled protein was greatly affected by the solvent environment and the aggregation state of the protein. An increase in the fluorescence lifetime of the labeled protein was observed upon

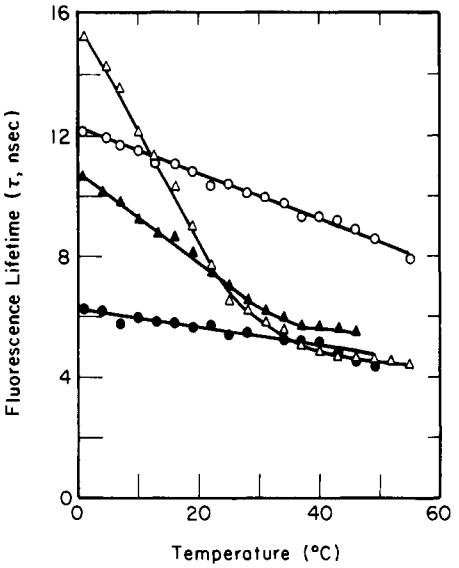

FIGURE 6: Fluorescence lifetimes of DNS-cytochrome b_5 in PBS (●), in PB containing 0.4% deoxycholate (○), and when bound to DMPC vesicles (▲) between 1 and 50 °C. The fluorescence lifetimes of the dansyl-labeled membrane-binding fragment in DMPC are also shown (△). The values shown here are the average of τ_ϕ and τ_m with the excitation light modulated at 18 MHz. The DNS-cytochrome b_5 concentration was 3 μM in all samples.

binding to DMPC vesicles. The increase in lifetime was particularly apparent below the phase transition of the phospholipid. Because of the large lifetime differences for the protein in different samples, polarization measurements alone were not sufficient to give an indication of the motion of the protein.

Rotational Relaxation Times of DNS-cytochrome b_5 in Phospholipid Vesicles. The rotational relaxation times of DNS-cytochrome b_5 in unilamellar egg PC and DMPC vesicles were calculated from the measured values of the fluorescence polarization and the lifetimes. In Figure 7, the rotational relaxation times of the protein in DMPC and DOPC vesicles are compared to the rotational relaxation times of DNSHA, the dansylated derivative of hexadecylamine. Both DNS-cytochrome b_5 and DNSHA showed a similar phase transition for DMPC centered at 21 °C. The observed rotational relaxation times for the dansyl-labeled protein were much longer in these vesicles than for the corresponding dansyl-labeled alkylamine.

The phase transition of the DMPC vesicles that was detected by DNS-cytochrome b_5 can be abolished by the inclusion of cholesterol in the bilayer. No distinct phase transition was observed when the vesicles contained more than 20 mol % cholesterol (Figure 8).

Rotational Relaxation Times of the Membrane-Binding Fragment in DMPC. The rotational relaxation time of the dansyl-labeled membrane-binding fragment bound to DMPC vesicles is shown in Figure 8. The observed rotational relaxation times of the membrane-binding fragment were slightly longer than those of the intact molecule at temperatures below the phase transition of the phospholipid and shorter above the phase transition. The higher rotational relaxation times observed below the phase transition temperature were primarily due to a longer fluorescence lifetime of the membrane-binding fragment when bound to the phospholipid vesicles. The similarity between the rotational relaxation times of the membrane-binding fragment and the intact molecule indicated that the motion of the labeled membrane-binding domain in the lipid vesicles was not affected to a great extent by the presence of the hydrophilic domain. Therefore, it is possible to use the

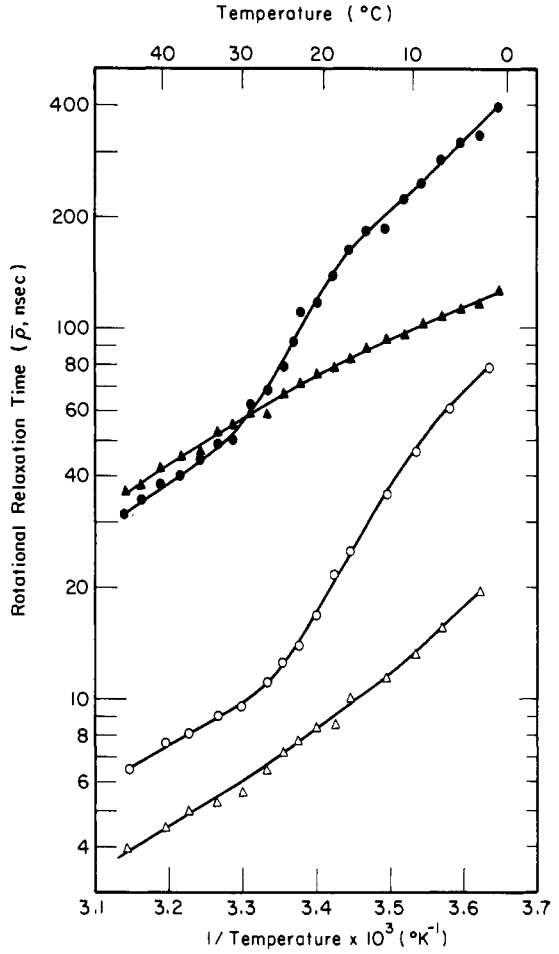

FIGURE 7: Temperature dependence of the rotational relaxation times of DNS-cytochrome b_5 in DMPC vesicles (●), DNS cytochrome b_5 in egg PC vesicles (▲), DNSHA in DMPC vesicles (○), and DNSHA in DOPC vesicles (△). DNS-cytochrome b_5 was bound to unilamellar phospholipid vesicles as described under Materials and Methods. The concentrations of DNS-cytochrome b_5 and DNSHA were 3 and 6 μM, respectively.

dansyl-labeled membrane-binding fragment alone in phospholipid vesicles to understand the properties of the intact molecule.

Partitioning of the Membrane-Binding Fragment between Solid and Fluid Phospholipids. The dansyl-labeled membrane-binding fragment was allowed to bind to phospholipid vesicles prepared from DMPC, DSPC, and a mixture of DMPC and DSPC (44.5 mol % DMPC) at 40 °C. The membrane-binding fragment bound to DMPC vesicles above its phase transition temperature and to DSPC vesicles below its phase transition temperature. The DMPC–DSPC vesicles do not show ideal mixing (Phillips et al., 1970; Shimshick & McConnell, 1973). If the dansyl-labeled protein had an unequal affinity for either the gel or the liquid-crystalline phases, it would not accurately report the bulk melting behavior of a mixture of phospholipids. The unequal partitioning of *trans*-paranaric acid between gel and liquid-crystalline phases has been demonstrated by using mixtures of synthetic phospholipids (Sklar et al., 1977, 1979). The fluorescence polarization of the dansyl-labeled membrane-binding fragment bound to DMPC, DSPC, and DMPC–DSPC mixtures is shown in Figure 9 as a function of temperature. The polarization changes of the dansyl-labeled protein were qualitatively similar to the changes seen with DPH in these phospholipids (Lentz et al., 1976b). Since DPH partitions equally between the gel and liquid-crystalline phases (Lentz et al., 1976b), the data indicated that the dansyl-labeled protein also showed no

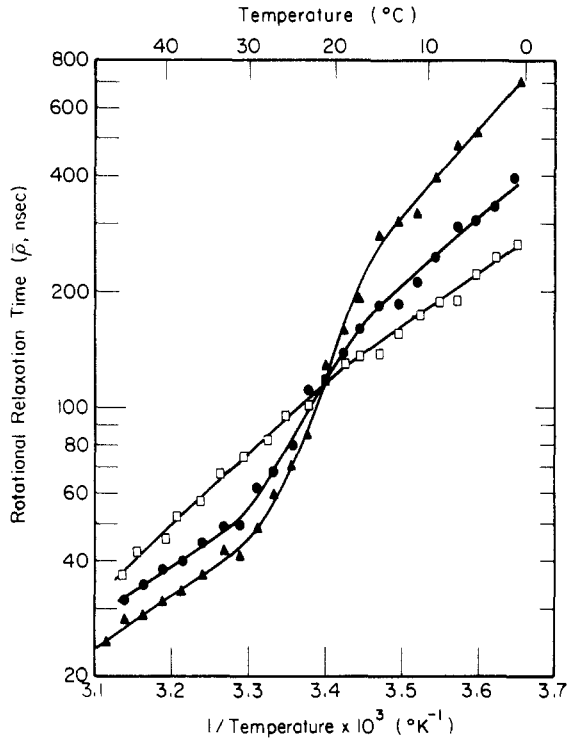

FIGURE 8: Temperature dependence of the rotational relaxation times of DNS-cytochrome b_5 in DMPC vesicles (●), DNS-cytochrome b_5 in DMPC vesicles containing 20 mol % cholesterol (□), and the dansyl-labeled membrane-binding fragment in DMPC vesicles (▲). The dansyl-labeled membrane-binding fragment was obtained by chromatography of a trypsin-digested sample of DNS-cytochrome b_5 as described in Figure 1. Binding of the intact protein and the membrane-binding fragment to unilamellar vesicles was accomplished as described under Materials and Methods. The concentrations of DNS-cytochrome b_5 and the dansyl-labeled membrane-binding fragment were approximately 3 μM.

preferential association with either the gel or the liquid-crystalline phases.

Discussion

The initial objective of this study was to specifically label the membrane-binding domain of cytochrome b_5 with a fluorophore in order to study the motion of the protein in membranes. Reaction conditions were worked out to label the protein with dansyl chloride by using deoxycholate such that approximately one dansyl group was covalently bound per cytochrome b_5. The DNS-cytochrome b_5 retained its activity, and it bound to phospholipid vesicles as did the unmodified protein. The reaction conditions used to label cytochrome b_5 were mild and should be generally applicable for the introduction of hydrophobic reagents into the hydrophobic domains of other membrane proteins.

Further experiments have shown that the dansyl group was located only on a histidine residue in the membrane-binding domain of the protein (D. M. Satterwhite, R. Gilmore, and M. Glaser, unpublished experiments). Since the membrane-binding domain of bovine cytochrome b_5 contains a single histidine residue (His-127) located seven amino acids from the carboxyl-terminal end (Fleming et al., 1978), the fluorophore must be located at this position. Experiments using carboxypeptidase Y digestions have indicated that the accessibility of the carboxyl terminus of cytochrome b_5 bound to phospholipid vesicles is dependent upon the method of incorporating the protein into the vesicle and upon the phospholipid studied (Enoch et al., 1979; Tajima & Sato, 1980). Recently, Dailey & Strittmatter (1981) have proposed a model for the structure of the "tightly" bound form of cytochrome

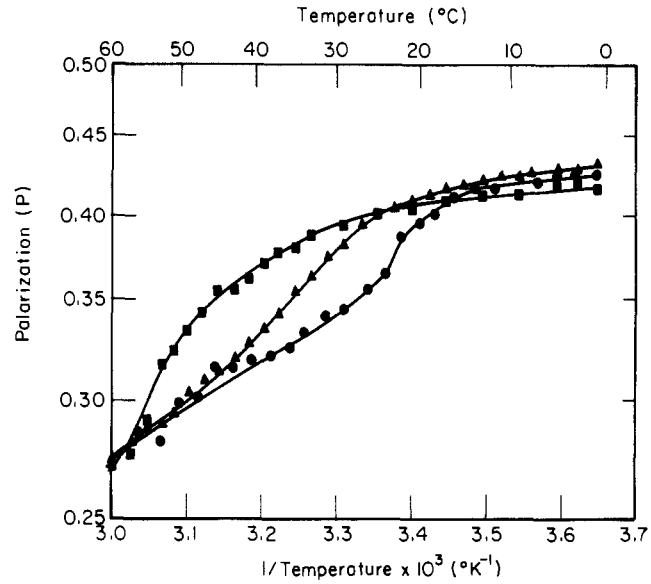

FIGURE 9: Temperature dependence of the fluorescence polarization of the dansyl-labeled membrane-binding fragment of cytochrome b_5 bound to DMPC (●), 44.5 mol % DMPC in DSPC (▲), and DSPC vesicles (■). The concentration of the dansyl-labeled membrane-binding fragment was approximately 3 μM.

b_5 that places tyrosine-126 within the acyl chain region of the phospholipid bilayer while tyrosine-129 is located within the polar head-group region. Several experiments described here showed that the motion of the dansyl-labeled protein is dependent upon the physical state of the phospholipid bilayer. Consequently, the dansyl-labeled histidine residue must also be located within the acyl chain region of the phospholipid bilayer.

The alteration of the aqueous solvent viscosity by the addition of sucrose to a sample of DNS-cytochrome b_5 bound to egg phosphatidylcholine vesicles did not alter the rotational relaxation time of the labeled protein. If the fluorophore was on a part of the protein that was exposed to the aqueous solvent, the addition of sucrose to the sample would result in decreased motion and an increased polarization. This experiment also demonstrated that the change in motion of the hydrophilic domain of the protein which was exposed to the aqueous solvent, and that the change in motion of the vesicle as a whole, did not affect the motion of the membrane-binding domain.

The investigation of the interaction of cytochrome b_5 with deoxycholate micelles as shown in Figure 4 yielded results that were similar to those previously observed by Robinson & Tanford (1975). At concentrations of deoxycholate near the critical micelle concentration, there was a strong interaction with DNS-cytochrome b_5. The DNS-cytochrome b_5 went from an aggregated state to a dispersed state in the deoxycholate micelles. The change in polarization was fairly large and shows that the DNS-cytochrome b_5 molecule is a sensitive probe for changes in the aggregation state and motion of the cytochrome b_5 molecule. The increase in fluorescence intensity observed upon the addition of the detergent was due to a decrease in intermolecular energy transfer that took place between the dansyl fluorophore and the heme residues within the cytochrome b_5 aggregate. No intramolecular quenching of the dansyl fluorescence by heme took place since the fluorescence intensities of DNS-cytochrome b_5 and DNS-apocytochrome b_5 were the same in deoxycholate micelles.

DNS-cytochrome b_5 can be used as a fluorescent probe of membrane structure over a broad temperature range. Above 45 °C, a rapid change in polarization of DNS-cytochrome b_5

in PBS was observed which was due to a change in conformation of the hydrophilic domain of the cytochrome b_5 molecule. This conformational change did not affect the motion of the membrane-binding domain of the protein when DNS-cytochrome b_5 was bound to DMPC vesicles, thereby confirming the independent motion of the two protein domains. A rapid change in polarization was not observed for the membrane-binding fragment of cytochrome b_5 in PBS between 20 and 65 °C, suggesting that the conformation of this domain is not significantly altered within this temperature range. The independent denaturation of the two domains of cytochrome b_5 has been observed previously by Tajima et al. (1976) using guanidine hydrochloride as a denaturing agent.

The broad phase transition centered about 21 °C for the unilamellar DMPC vesicles using DNSHA or DNS-cytochrome b_5 as a fluorescent probe is similar to that observed using the fluorescent probe DPH (Lentz et al., 1976a). The rotational relaxation time observed for DNS-cytochrome b_5 was approximately 5-fold higher than that observed for the dansyl-labeled alkylamine (DNSHA) bound to the DMPC vesicles. This observation is consistent with the much larger molecular weight of the hydrophobic domain of cytochrome b_5 than that of DNSHA and shows that the rotational relaxation time of DNS-cytochrome b_5 reflects the motion of the protein rather than the fluorophore itself.

The similar shape of the DMPC phase transition measured with DNS-cytochrome b_5, DNSHA, and DPH indicates there is no special annular ring of lipids around cytochrome b_5 with different properties than the bulk lipid in the bilayer. The absence of a nonexchanging layer of boundary lipid is consistent with our previous studies on cytochrome b_5 using deuterium nuclear magnetic resonance spectroscopy (Oldfield et al., 1978). This conclusion is supported also by the changes in the rotational relaxation time of DNS-cytochrome b_5 in DMPC vesicles containing cholesterol. Cholesterol increased the rotational relaxation times above the phase transition of DMPC and decreased them below the transition. This is similar to observations on the effect of cholesterol on pure phospholipid bilayers made by other physical techniques (Oldfield & Chapman, 1972).

A number of spectroscopic studies have been carried out to understand the rotational motion of proteins by using protein-bound triplet probes [e.g., see Nigg et al. (1980)]. These studies were mainly directed at large molecular weight proteins that undergo slow motions in membranes. Recently Vaz et al. (1979) used triplet absorbance spectroscopy to study the rotational diffusion of cytochrome b_5. They prepared cytochrome b_5 derivatives that contained rhodium(III) protoporphyrin IX instead of heme. The relaxation times obtained for this derivative in DMPC vesicles were significantly longer than those obtained here with DNS-cytochrome b_5. This discrepancy may be due to a number of factors including the different domain of the protein labeled and the different time frame examined by these two techniques. Due to the relatively short fluorescence lifetime of DNS-cytochrome b_5, this probe will predominantly be sensitive to relatively rapid motions (approximately 10^{-8} s) of the membrane-binding domain of the protein in the phospholipid bilayer. Conversely, triplet absorption anisotropy will be sensitive to very slow motions of the protein (approximately 10^{-6} s or longer) and possibly reflect the motion of the phospholipid vesicle itself. Further studies are necessary to define the various types of motion cytochrome b_5 undergoes. In any event, the motion of DNS-cytochrome b_5 is sensitive to the lipid composition and characteristics of the bilayer to which it is bound, and it can be used to measure the interaction of the protein with lipids and to compare the properties of different lipid systems.

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Differences in Stability against Thermal Unfolding between Trypsin- and Detergent-Solubilized Cytochromes b_5 and Structural Changes in the Heme Vicinity upon the Transition: Resonance Raman and Absorption Study†

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ABSTRACT: Thermal unfolding of the hydrophilic domain of cytochrome b_5 was investigated with resonance Raman and absorption spectroscopy. The trypsin-solubilized cytochrome b_5 (t-b_5) and detergent-solubilized cytochrome b_5 (d-b_5) exhibited a similar transition at definitely different temperatures (66 ± 2 °C for t-b_5 and 57 ± 2 °C for d-b_5), although such difference was unexpected from the proposed structural independence of the hydrophobic and hydrophilic domains of d-b_5. The Raman spectra of the native and initial high-spin species of t-b_5 were very close to those of the six-coordinate bis(imidazole) and five-coordinate 2-methylimidazole complexes of iron octaethylporphyrin, respectively. Accordingly, the unfolding of t-b_5 was deduced to involve, in the beginning, a change of the heme structure from the six-coordinate low-spin to five-coordinate high-spin form but later a change from the five-coordinate high-spin form to six-coordinate high-spin form presumably with a water molecule at the sixth coordination position. The changes were partially reversible. The irreversibly changed ferric high-spin t-b_5 was converted to the native-like low-spin form by addition of imidazole on the one hand, but on the other hand, by simple reduction without addition of imidazole, this was changed to the ferrous low-spin heme, probably with two histidine residues at the axial positions. The Raman spectra of the two kinds of five-coordinate ferric high-spin forms, produced by the thermal unfolding and acidification, were very alike, but the reversibility of the change was distinctly different. The acid-produced high-spin form was completely recovered to the native low-spin form in contrast with the partial recovery of the thermally produced high-spin form. Consequently, we conclude that the apparently simple thermal transition of cytochrome b_5 around 55–65 °C actually involves the reversible unfolding and the concomitant irreversible changes, and the contribution of the latter depends upon the experimental conditions such as the duration at higher temperatures. Nonetheless, the thermal transition appeared as if it were a two-state transition with an isosbestic point in the visible spectrum, because an additional change in the polypeptide structure, which is probably decisive of the reversibility, is not explicitly reflected in the heme absorption spectrum. A plausible origin for the stability difference between t-b_5 and d-b_5 is also discussed.

Cytochrome b_5 is a well characterized membrane protein involved in the microsomal electron transport system of various tissues. Intact cytochrome b_5 is known to be solubilized by treatment of microsomes with detergent (Ito & Sato, 1968; Spatz & Strittmatter, 1971), while digestion of microsomes with proteases allows the isolation of cytochrome b_5 with a smaller molecular weight (Strittmatter, 1960; Omura et al., 1967; Kajihara & Hagihara, 1968). The former and the latter preparations provide so-called detergent-solubilized cytochrome b_5 (d-b_5)[1] and trypsin-solubilized cytochrome b_5 (t-b_5), respectively, and t-b_5 has been proved to be the heme-containing hydrophilic moiety of d-b_5 (Ito & Sato, 1968; Spatz & Strittmatter, 1971). The amino acid sequences of several t-b_5 molecules from different sources are quite similar (Tsugita et al., 1970; Ozols et al., 1976), and their molecular weights are all about 11 000. The details of the molecular structure of calf liver t-b_5 have been analyzed with X-ray crystallography (Mathews et al., 1972). For rabbit liver t-b_5, the symmetry of the unit cell is found to be the same as that of calf liver t-b_5 (Kretsinger et al., 1970), while d-b_5 has never been crystallized so far. According to the recent NMR study (Keller & Wüthrich, 1980), there are two molecular conformations for t-b_5 in an aqueous solution, one of which corresponds to the

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[1] Abbreviations: t-b_5, trypsin-solubilized cytochrome b_5; d-b_5, detergent-solubilized cytochrome b_5; Im, imidazole; 2MeIm, 2-methylimidazole; Fe(OEP)(ClO_4), (octaethylporphyrinato)iron(III) perchlorate; Fe(OEP)($Im)_2$, (octaethylporphyrinato)iron(III)–bis(imidazole) complex; Fe(OEP)(2MeIm), (octaethylporphyrinato)iron(III)–2-methylimidazole complex; Gdn·HCl, guanidine hydrochloride; cmc, critical micelle concentration; CD, circular dichroism; Mb, myoglobin.

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