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Characterization of Cytochrome b_5 Reconstituted with a Ferric Chlorin and a Ferric Oxochlorin†

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ABSTRACT: The role of the electronic properties of the heme group of rat cytochrome b_5 in biological electron transfer was investigated by substituting chlorin analogues for the native protoporphyrin IX prosthetic group. The resultant purified proteins displayed physical and chemical properties distinct from those of the native enzyme. Optical spectroscopy of the ferric chlorin substituted cytochrome b_5 revealed a blue-shifted Soret at 404 nm and a band at 586 nm characteristically red-shifted from the protohemin absorption band. The reduced, reconstituted protein displayed maxima at 406, 418, 455, 563, and 600 nm. The oxidized cytochrome b_5 containing the oxochlorin analogue produced a red-shifted Soret with maxima at 338, 416, and 602 nm. The reduced species differed only in the visible region with absorption maxima at 508, 554, and 600 nm. Characterization by EPR spectroscopy of the oxochlorin-substituted cytochrome b_5 yielded g values of 2.566, 2.375, and 1.756 and respective axial Δ/λ and rhombic V/λ components of 2.857 and 3.287, indicating significant electronic distortion in the chlorin ring and an increase in electron donation from the axial histidine ligands. A decrease in the reduction potential of 52 ± 5 mV (50 mM KP_i, pH 7.0, 25 °C) for the chlorin-reconstituted cytochrome b_5 was determined with respect to that of native cytochrome b_5. The reduction potential for the oxochlorin-containing cytochrome b_5 was unchanged from that of the native system. Both of the reconstituted proteins were found to be capable of transferring electrons to cytochrome c in a reconstituted system dependent on NADH and cytochrome b_5 reductase, thus simulating the activity of native cytochrome b_5.

Cytochrome b_5, a low molecular weight heme protein, is a native component of liver microsomes (Ito & Sato, 1968) and functions in hepatic electron transfer systems. Specifically, cytochrome b_5 has been found to be active in fatty acid hydroxylation in association with cytochrome P-450 and NADPH-cytochrome P-450 reductase (Morgan & Coon, 1984; Chiang, 1981; Hildebrandt & Estabrook, 1971) and in stearoyl-CoA[1] desaturation involving cytochrome b_5 reductase and desaturase (Strittmatter et al., 1974; Shimakata et al., 1972; Oshino & Sato, 1971). The amphipathic protein is composed of two distinct regions, a hydrophobic tail which is imbedded in the endoplasmic reticulum (Spatz & Strittmatter, 1971; Rogers & Strittmatter, 1974) and a soluble catalytic core. The hydrophobic tail may be proteolytically clipped with trypsin yielding the soluble unit which retains fully activity (Ito & Sato, 1968). A soluble form of cytochrome b_5 lacks the COOH-terminus membrane anchor domain and is active as a methemoglobin reductase in erythrocytes (Hultquist & Passon, 1971).

The prosthetic group, ferric protoporphyrin IX (Figure 1), is liganded by bis(imidazole) coordination to axial histidines-39 and -63. The b-type heme is held rigidly in a hydrophobic crevice in an orientation such that the peripheral vinyl substituents are buried and the propionic acid groups are exposed to the aqueous environment (Mathews et al., 1972). The X-ray crystal structure of ferric cytochrome b_5 has revealed that one propionic acid interacts freely with solution, while the other is partially buried and forms hydrogen bonds with the hydroxyl group of serine-64 and a peptide amide (Mathews & Argos, 1975). It has been proposed that this acidic carboxyl group may form a salt bridge with the iron center of the heme,

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[1] Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A; cytochrome b_5/chl, cytochrome b_5 reconstituted with iron(III) chlorin; cytochrome b_5/ochl, cytochrome b_5 reconstituted with iron(III) oxocholorin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; KP_i, potassium phosphate; NADH, reduced nicotinamide adenine dinucleotide.

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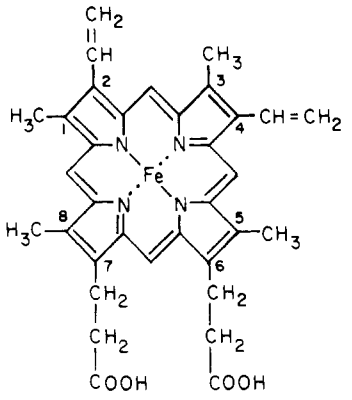

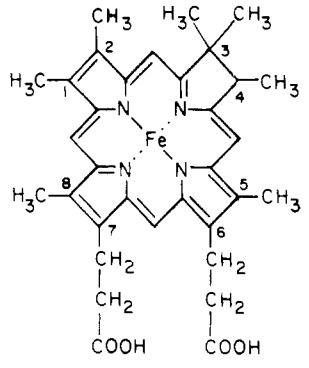

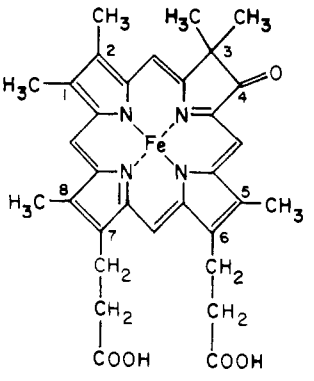

FIGURE 1: Structure of the native iron protoporphyrin IX of cytochrome b_5 and iron(III) oxochlorin and chlorin, which were reconstituted into apocytochrome b_5.

contributing to the stability of the oxidized species.

The prosthetic groups of heme proteins have been extensively studied in order to determine their role in a multitude of diverse biological mechanisms. Numerous modifications to the heme have been designed pursuant to the understanding of their contribution of the protein's physicochemical characteristics, including spectral properties, redox potentials, and enzymatic activity. Focus has been directed toward several components of the heme, including propionate groups (Reid et al., 1984), other peripheral side chains (Chang et al., 1984; Bornheim et al., 1986), and substitution of the chelated transition metal (Morgan & Coon, 1984; Gelb et al., 1982). The ligand environment of cytochrome b_5 has also been actively studied by employing site-directed mutagenesis to replace the amino acid and/or to alter the coordination about the metal center (von Bodman et al., 1986; Sligar et al., 1987). In addition, chemical modifications of the axial ligands have been specifically engineered in order to vary its orientation in relation to the porphyrin, producing a strain in the chelation and coordination of the heme (Walker et al., 1986).

The investigation reported herein was designed to evaluate the pyrrole ring saturation effect upon the heme group of the heme in its biological activity by reconstitution of soluble rat cytochrome b_5 (von Bodman et al., 1986) with chlorin prosthetic groups (Boxer & Wright, 1979; Wright & Boxer, 1981). The chlorins utilized are saturated analogues of protoporphyrin IX in which the ring system is made up of only 10 conjugated double bonds as opposed to the 11 of native heme. In this case, the π-bond between carbons 3 and 4 has been broken by addition of a methyl and a hydrogen to the respective positions (Figure 1). The oxochlorin has a ketone group added to the periphery at carbon 4. The reconstituted proteins, termed cytochrome b_5/chl and cytochrome b_5/ochl, were characterized by optical and electron paramagnetic resonance spectroscopies, reduction potential, and electron transfer velocities in a reconstituted system with NADH-cytochrome b_5 reductase.

Experimental Procedures

Soluble rat cytochrome b_5 was obtained and purified as previously described (von Bodman et al., 1986). The complete synthesis of iron(III) chlorin and oxochlorin has been previously described (Chang & Sotiriou, 1985). All other materials were obtained from commercial sources. A Hewlett-Packard 8450A UV/vis or a Varian Cary 219 spectrophotometer was used for all optical measurements.

Apocytochrome b_5 was prepared by the method of Teale (1959) at 4 °C. Specifically, 500 nmol of cytochrome b_5 in 5.0 mL of 0.10 M KP_i, pH 7.0, was brought to pH 2.0 by slow addition of cold 2.0 N HCl. Equal volumes of butanone, prechilled to −20 °C, were used repeatedly to extract the red heme until the organic layer appeared colorless. The apoprotein was dialyzed against 20 mM Tris–acetate/0.2 mM EDTA, pH 8.0, for 24 h with three buffer changes (Morgan & Coon, 1984).

Iron(III) chlorin or oxochlorin was dissolved in a minimal volume of 1:1 ethanol/20 mM Tris, pH 7.4. Over a period of 1 h, the green derivative was titrated into the apoprotein solution. The progress of the reconstitution was monitored spectrally at 670 nm, at which the presence of excess chlorin could be detected.

The reconstituted protein was purified over a Sephadex G-25 column (3.5 × 13.5 cm) equilibrated in 20 mM Tris–acetate/0.2 mM EDTA, pH 8.0. The free chromophores adsorbed to the resin, and the protein was eluted in the void volume. An Amicon ultrafiltration cell, fitted with an Amicon YM-10 membrane, was used for concentration, followed by storage in liquid nitrogen.

Extinction coefficients for the reconstituted proteins were determined by a pyridine–hemochrome assay (Paul et al., 1953). The protein was diluted to obtain an approximate concentration range of 100–200 μM on the basis of the protein absorbance at 280 nm. To a 1.0-mL aliquot of the substituted cytochrome b_5 were added 250 μL of pyridine and 125 μL of 1.0 N NaOH followed by reduction with a few crystals of sodium dithionite. The absorbance was measured immediately.

EPR spectra of native cytochrome b_5 were determined in the laboratory of Dr. P. Debrunner at the University of Illinois with a Bruker ER 200 DESR instrument and a Bruker microwave bridge ERO42 MRH. Spectra for the reconstituted proteins were recorded in the Illinois ESR Research Center on a Bruker ER 220D-SRC equipped with a Bruker microwave bridge ERO42MRH, helium dewar, Varian gaussmeter,

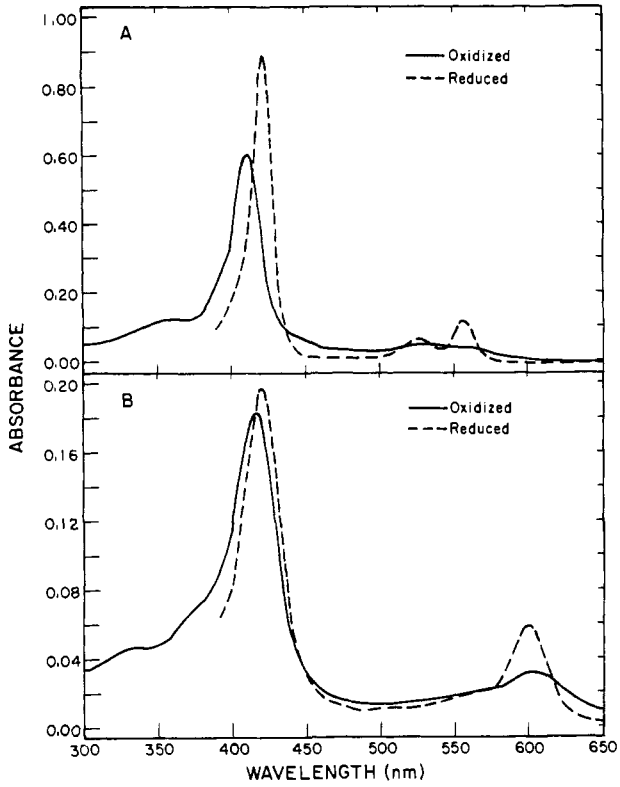

FIGURE 2: Optical spectra of cytochrome b_5 (A) and cytochrome b_5/ochl (B) in 50 mM KP_i, pH 7.0 at 25 °C.

and EIP microwave 548A frequency meter. Data manipulation was carried out with an IBM PC and in-house software (Morse, 1987). A 50.0-μL aliquot of 100 μM cytochrome b_5 or its analogue was transferred into a 9-in. quartz tube (o.d. 4 mm, i.d. 3 mm) and frozen in liquid nitrogen. The EPR spectra of the free oxochlorin and chlorin dimethyl ester hemes axially liganded by methylimidazole were determined in a solvent of CH_2Cl_2/toluene at 77 K.

Reduction potentials of cytochrome b_5 and its substituted analogues were measured spectrophotometrically (Dutton, 1978), with the mediator dye methylene blue. A 1.0-mL solution of 20 mM EDTA, 5.0 μM methylene blue, 5.0 μM cytochrome b_5 or its chlorin derivative, and 50 mM KP_i, pH 7.0, was transferred to a Thunberg cuvette and degassed by a vacuum pump to ensure anaerobic conditions. Electron transfer was induced photometrically by periodic exposures of 1–5 s to a xenon arc lamp. Reduction was monitored at 668 nm for methylene blue and at the reduced Soret for cytochrome b_5. Complete reduction was assured by addition of sodium dithionite crystals.

Electron transfer velocities to cytochrome *c* (Williams, 1977) were determined in a system which included 500 μM NADH, 2.0 μM cytochrome b_5 or its reconstituted analogues, 0.004% Triton X-100 (Mihara & Sato, 1975), 50 mM KP_i, pH 6.8, 250 μM horse heart cytochrome *c*, and catalytic quantities of cytochrome b_5 reductase from *Pisum sativum* (Jollie et al., 1987). Reduction of cytochrome *c* was monitored at 550 nm at 5-s intervals until reduction was complete, typically under 3 min.

Results

Reconstituted cytochrome b_5 was prepared as described under Experimental Procedures. A 96% yield of dialyzed apocytochrome b_5 was typically recovered from the butanone heme extraction. Less than 1% native heme contaminants were present as determined optically at 420 nm following reduction with dithionite. The apoprotein was reconstituted with an iron(III) chlorin or oxochlorin, whose structures are shown in Figure 1, and purified by gel filtration, producing a final yield of 58%. This high yield of product demonstrated the inherent stability of cytochrome b_5.

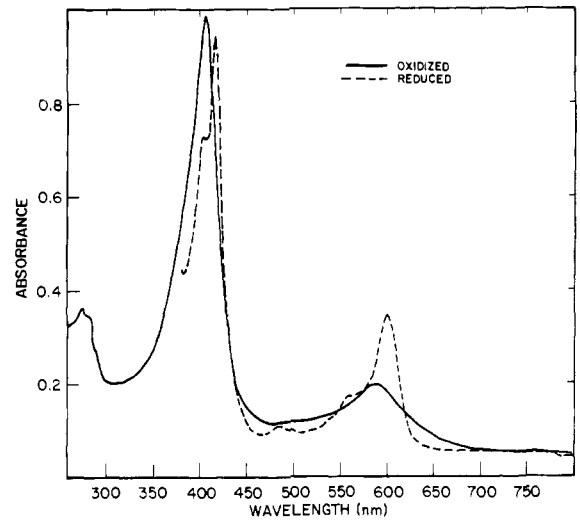

FIGURE 3: Optical spectra of cytochrome b_5/chl in 50 mM KP_i, pH 7.0 at 25 °C.

Table I: EPR Parameters of Cytochrome b_5 and Cytochrome b_5/ochl

	g_z	g_y	g_x	Δ/λ	V/λ	V/Δ
cytochrome b_5	3.059	2.231	1.444	3.333	1.654	0.496
cytochrome b_5/ochl	2.566	2.375	1.756	2.857	3.28	1.151
oxochlorin dimethyl ester	2.57	2.38	1.75	2.83	3.24	1.14
chlorin dimethyl ester	2.510	2.380	1.749	2.590	3.485	1.346

As shown in Figure 2B, oxidized cytochrome b_5/ochl is characterized optically by a Soret at 416 nm, a shoulder at 338 nm, and a band at 602 nm. The optical spectrum is thus red-shifted as compared to that of native cytochrome b_5 with an iron(III) protoporphyrin IX prosthetic group which has maxima at 410, 532, and 558 nm (Figure 2A). On the basis of the assay, the extinction coefficient for the cytochrome b_5/ochl Soret was found to be 152 mM^{-1} cm^{-1}. Dithionite reduction produced a Soret with increased intensity at 420 nm in both cytochrome b_5 and its oxochlorin derivative. The visible region of the reduced spectra differs, with maxima at 508, 554, and 600 nm for cytochrome b_5/ochl and at 555 and 527 nm for cytochrome b_5. The optical spectra for cytochrome b_5/chl may be found in Figure 3. Maxima for the oxidized protein are at 404 and 586 nm. The extinction coefficient for the Soret was determined by a heme chromogen assay to be 133 mM^{-1} cm^{-1}. The reduced protein displayed maxima at 406, 418, 455, 563, and 600 nm.

Spectral data of cytochrome b_5/ochl derived from EPR at 41.5 K indicate a low-spin protein with *g* values of 2.566, 2.375, and 1.756 (Table I) with respective widths of 1.130×10^{-2} T (113.0 G), 4.23×10^{-3} T (42.3 G), and 1.270×10^{-2} T (127.0 G) (Figure 5). It should be noted that the oxochlorin dimethyl ester axially liganded by methylimidazole has essentially identical values of 2.57, 2.38, and 1.75. The spectrum of the reconstituted protein is found to be compressed relative to that of cytochrome b_5 which has values of 3.059, 2.231, and 1.444 and respective widths of 9.18×10^{-3} T (91.86 G), 1.342×10^{-2} T (134.26 G), and 3.10×10^{-2} T (310.06 G) (Figure 4). The rhombic component of cytochrome b_5/ochl is calculated to be 3.287, reflecting a distortion of the electron density in the planar chlorin ring due to the break in conjugation as

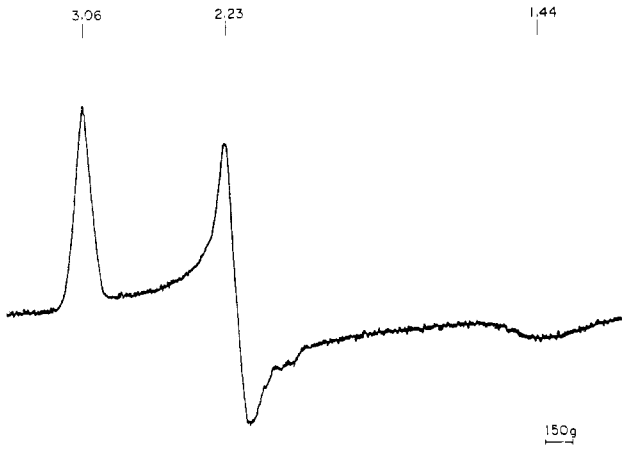

FIGURE 4: EPR spectra of cytochrome b_5 in 50 mM KP_i, pH 7.0. Experimental conditions: 16-dB power at 42 K, 9.445-GHz microwave frequency, 10-G modulation amplitude, and 100-kHz modulation frequency.

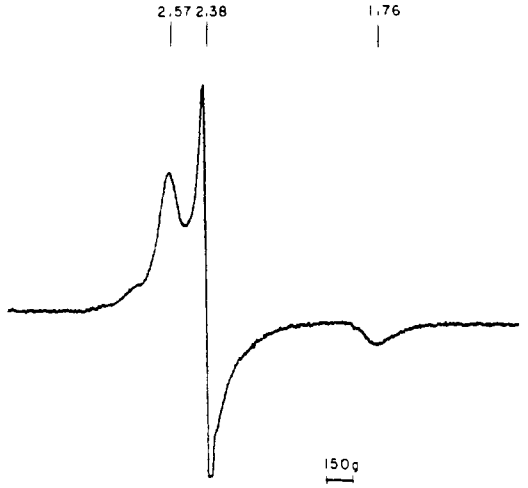

FIGURE 5: EPR spectra of cytochrome b_5/ochl in 50 mM KP_i, pH 7.0. Experimental conditions: 16-dB power at 20 K, 9.4913-GHz, 100-kHz modulation frequency, and 12.5-G modulation amplitude.

well as to the electron withdrawing effects of the carbonyl substituent at position 4 of the chlorin. As is indicated by the axial component at 2.857, the ligands compensate for the electron-deficient system by donating an increased amount of electron density in comparison to cytochrome b_5 which has axial and rhombic components of 3.333 and 1.654, respectively.

The axial and rhombic components ($\Delta/\lambda = 2.59$; $V/\lambda = 3.49$) of the methylimidazole axially liganded chlorin compound also reveal in-plane electronic distortion. When reconstituted into pure cytochrome b_5 ($R_Z > 6.0$), multiple species are revealed in the EPR spectrum. Extensive, repeated purification by sizing and anion exchange chromatography under a variety of buffer, pH, and salt conditions yields a single band on an SDS gel, but the multiple EPR states persist. Since the protein is homogeneous, these states have been attributed to a mixture of heme orientations due to heme disorder in the prosthetic group pocket and strained axial ligation which have been previously documented (Walker et al., 1986; LaMar et al., 1981).

Reduction potentials can also be used to probe the electronic properties of the porphyrin conjugation system. Cytochrome b_5 reconstituted with the chlorin displayed a reduction potential of -52 ± 5 mV in 50 mM KP_i, pH 7.0 at 26 °C. The observed potential for native cytochrome b_5 is 5 ± 1 mV. This comparison is consistent with the trend found in model heme chemistry, where iron chlorin derivatives are characteristic of lower reduction potentials of up to 50 mV as compared to the corresponding porphyrins (Chang & Fajer, 1980; Stolzenberg et al., 1981). As predicted for an oxochlorin derivative, cytochrome b_5/ochl had a reduction potential within -1 ± 6 mV to that of the native protein.

The electron transfer velocities of cytochrome b_5 and its reconstituted derivatives to cytochrome c were found to be dependent on cytochrome b_5 reductase and the electron donor, NADH. The relative velocities were determined at 26 °C in 50 mM KP_i, pH 6.8, and found to be 1.14 ± 0.19 (native), 0.86 ± 0.04 (b_5/chl), and 0.39 ± 0.02 μmol min^{-1} mg^{-1} (b_5/ochl).

Discussion

Heme proteins are involved in a multitude of diverse biological activities, including electron transfer, oxygen transport, and a wide variety of oxidative reactivities. This diversity has prompted extensive investigation of the physicochemical properties of numerous heme proteins in order to probe the nature of their distinct mechanisms. Several approaches have been applied, with particular focus on the common prosthetic group, iron protoporphyrin IX. One such approach has been to substitute the iron center with other transition metal analogues (Wagner et al., 1981; Gelb et al., 1982; Morgan & Coon, 1984). Steric and orientation phenomena of the heme proteins' active sites and the prosthetic group ligands have been examined by use of chemically modified free porphyrin derivatives in solution (Suslick & Reinert, 1985). Also, a variety of biophysical methods have been used to evaluate the effects of the axial ligand orientation toward the heme by utilizing synthetically altered ligands (Walker et al., 1986). More recently, the axial ligand fields of the heme proteins are being actively investigated by site-specific mutagenesis (von Bodman et al., 1986; Sligar et al., 1987).

The goal of this investigation is to focus on the macrocycle and probe the ring saturation effect by substituting chlorins into a heme protein. Cytochrome b_5, a low molecular weight electron transfer protein, was an obvious choice from the pool of heme proteins for several reasons. Native cytochrome b_5 is well characterized by several techniques including X-ray crystallography, electron paramagnetic resonance, and nuclear magnetic resonance (Mathews & Argos, 1975; von Bodman et al., 1986; McLachlan et al., 1986). The apo- and holoproteins are extremely stable, facilitating reconstitutions of heme analogues under harsh acidic conditions with a high recovery rate of over 95%. Also, the heme is bound in a hydrophobic cleft with access to solvent enabling easy extraction of the heme. Furthermore, the soluble domain of cytochrome b_5 which contains the iron porphyrin prosthetic group has been synthesized and successfully expressed in *Escherichia coli* in high yield, providing an abundant source of the protein (von Bodman et al., 1986).

Cytochrome b_5 has been the target of other reconstitutions with heme derivatives. Manganese protoporphyrin IX was substituted into rabbit liver cytochrome b_5, and its effects were evaluated in a reconstituted system with microsomal cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine (Morgan & Coon, 1984). This system provided an elegant means to separate protein–protein association from redox transfer events. The manganese protein derivative was found to be inhibitory in two native reactions, 7-ethoxycoumarin deethylation and acetanilide p-hydroxylation. Also, in contrast to the native protein, manganese-substituted cytochrome b_5 remained fully oxidized in the reconstituted system. Cytochrome b_5 has also been re-

constituted with deuteroheme and manganese mesoheme in a comparative study of reduction rates (Rogers & Strittmatter, 1974). In this report a synthetic chlorin was reconstituted into cytochrome b_5, and its properties were characterized on the basis of reduction potentials, enzymatic activity, and optical and electron paramagnetic resonance spectroscopies.

Chlorin, a two-electron-reduced analogue of porphyrin containing one less double bond in the conjugated ring, is found in several biological systems. Proteins which possess chlorin-type prosthetic groups include bacterial oxidase cytochrome *d* (Timkovich et al., 1985), myeloperoxidase, which is involved in the immune system (Sibbet & Hurst, 1984), and the oxygen carrying protein sulfmyoglobin (Andersson et al., 1984). Unfortunately, the prosthetic groups of these latter two are unstable when isolated, impeding explicit identification and characterization.

The stable, synthetic chlorins used in this investigation and described in the introduction, were reconstituted with apocytochrome b_5. Optically, the oxidized oxochlorin protein species produced a red-shifted spectrum in the visible region with a Soret at 416 nm and a band at 602 nm. Maxima for cytochrome b_5/chl were found at 404 and 587 nm. The reduced oxochlorin protein derivative was characterized by a Soret at 420 nm and a maximum at 600 nm, while the reduced cytochrome b_5/chl displayed bands at 406, 418, 455, 563, and 600 nm. Electron paramagnetic resonance produced a rhombic spectrum compared to that of native cytochrome b_5 for cytochrome b_5/ochl, but an identical spectrum to the free oxochlorin with bis(imidazole) axial ligation indicated the desired ligation of the oxochlorin to the native histidine residue ligands in the heme pocket.

Both of the reconstituted proteins were found to be capable of accepting and donating electrons in an in vitro native cytochrome b_5 electron transfer system. The electron transfer velocities to cytochrome *c* from the chlorin- and oxochlorin-reconstituted cytochrome b_5 in a system with NADH and cytochrome b_5 reductase were found to be slightly decreased relative to that of the native protein. The rate decrease, at least in cytochrome b_5/chl, may be partially related to a decrease in the reduction potential of 52 mV upon chlorin reconstitution, thus changing the driving force. Another parameter which may influence the reduction of cytochrome *c* is molecular recognition between the redox partners. Molecular recognition between cytochrome *c* and cytochrome b_5 has been extensively investigated (Salemme, 1986; Rodgers et al., 1988), implicating specific charged amino acid residues and one heme propionic acid group in salt bridges which orient the proteins for electron transfer. Since these chlorin compounds differ slightly in their peripheral side chains, small adjustments in the tertiary structure of cytochrome b_5 may result, causing a disruption in these specific molecular recognition sites. In the case of cytochrome b_5/ochl, the presence of the keto group on the ring may alter the orientation of the heme group in the protein pocket. It is interesting to note that in cytochrome cd_1 of *Pseudomonas aeruginosa*, the electron transfer rate between the *c* heme and the d_1 heme (dione heme) in which two keto groups are present on the ring (Wu & Chang, 1987) is also relatively slow (Schichman & Gray, 1981). Further work, particularly on the intrinsic electron transfer properties of these keto heme derivatives, is clearly needed to fully understand these results.

As would be expected, the macrocycle's π system contributes to the activity and electronic properties of the protein. Alteration of orbital energies and symmetries of the heme prosthetic group in the chlorin-reconstituted cytochrome b_5 is reflected by electron paramagnetic resonance and optical spectroscopies which display unique bands. However, the reduction potential and electron transfer velocities of the chlorin-reconstituted protein derivatives differ only slightly from those of the native cytochrome b_5 and in general agree well with properties of six-coordinate model compounds.

In summary, this work demonstrates that cytochrome b_5 can be reconstituted to yield structurally and functionally well-defined homologues containing a chlorin or oxochlorin heme prosthetic group.

Acknowledgments

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Registry No. Cytochrome b_5, 9035-39-6; iron(III) chlorin, 117828-53-2; iron(III) oxochlorin, 117828-52-1; oxochlorin dimethyl ester, 98821-80-8; chlorin dimethyl ester, 117828-54-3.

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Partitioning of Exchangeable Fluorescent Phospholipids and Sphingolipids between Different Lipid Bilayer Environments†

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ABSTRACT: Exchangeable phospho- and sphingolipid probes (phosphatidylcholine, -ethanolamine, -serine, and -glycerol, phosphatidic acid, sphingomyelin, cerebroside, and sulfatide) have been synthesized in which one acyl chain is substituted with a fluorescent bimanyl, 7-(dimethylamino)coumarin-3-yl, or diphenylhexatrienyl group. The distribution of these probes between two different populations of lipid vesicles can be readily monitored by fluorescence intensity measurements, as described by Nichols and Pagano [Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720-1726], when one of the vesicle populations contains a low mole fraction of a nonexchangeable quencher, (12-DABS)-18-PC. The probes examined in this study exchange between phospholipid vesicles on a time scale of minutes, with kinetics indicating that the transfer process takes place by diffusion of probe monomers through the aqueous phase. As expected, lipid probes with different charges differ markedly in their equilibrium distributions between neutral and charged lipid vesicles. However, probes with different polar headgroups differ only modestly in their relative affinities for vesicles composed of "hydrogen-bonding" lipids (PE and PS) vs "non-hydrogen-bonding" lipids (PC and PG or *O*-methyl-PA). Probes with different headgroups also show modest, albeit reproducible, differences in their relative affinities for cholesterol-containing vs cholesterol-free PC/PG vesicles. Our results suggest that lipids with different headgroup structures may mix more nearly ideally in liquid-crystalline lipid bilayers than would be predicted from previous analyses of the phase diagrams for binary lipid mixtures.

The nature of the lateral interactions between different lipid species can strongly influence the physical properties and the organization of bilayer membranes that contain multiple lipid components. Because of this fact, numerous studies have examined the interactions between different types of polar lipids in bilayers, using a variety of physical techniques [for reviews, see Lee (1977), Gaffney and Chen (1977), Mabrey and Sturtevant (1978), Melchior and Steim (1979), McElhaney (1984), Keough and Davis (1984), Thompson and Tillack (1985), Brauner and Mendelsohn (1986), and Curatolo (1987)]. To date, most thermodynamic information regarding the interactions between different lipids in bilayers has been derived from analyses of the phase diagrams for various binary mixtures of phospho- and sphingolipids. The principles of regular solution theory are often applied in such analyses to estimate quantitatively how differences in the headgroups and/or the acyl chains of different lipid species affect their free energy of mixing (Lee, 1977; van Dijck et al., 1977; Von Dreele, 1978; Cheng, 1980; Keough & Davis, 1982).

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