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Gene Synthesis, Bacterial Expression, and ^{1}H NMR Spectroscopic Studies of the Rat Outer Mitochondrial Membrane Cytochrome b_5†

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ABSTRACT: The gene coding for the water-soluble domain of the outer mitochondrial membrane cytochrome b_5 (OM cytochrome b_5) from rat liver has been synthesized and expressed in *Escherichia coli*. The DNA sequence was obtained by back-translating the known amino acid sequence [Lederer, F., Ghrir, R., Guiard, B., Cortial, S., & Ito, A. (1983) *Eur. J. Biochem. 132*, 95–102]. The recombinant OM cytochrome b_5 was characterized by UV–visible, EPR, and ^{1}H NMR spectroscopy. The UV–visible and EPR spectra of the OM cytochrome b_5 are almost identical to the ones obtained from the overexpressed rat microsomal cytochrome b_5 [Bodman, S. B. V., Schyler, M. A., Jollie, D. R., & Sligar, S. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9443–9447]. The one-dimensional ^{1}H NMR spectrum of the OM cytochrome b_5 indicates that the rhombic perturbation of the ferric center is essentially identical to that in the microsomal beef, rabbit, chicken, and rat cytochromes b_5. Two-dimensional ^{1}H NMR spectroscopy (NOESY) and one-dimensional NOE difference spectroscopy were used to assign the contact-shifted resonances that correspond to each of the two isomers that result from the rotation of the heme around its α–γ-meso axis. The assignment of the resonances allowed the determination of the heme orientation ratio in the OM cytochrome b_5, which was found to be 1.0 ± 0.1. It is noteworthy that the two cytochromes b_5 that have similar populations of the two heme isomers (large heme disorder) originate from the rat liver.

Microsomal cytochrome b_5 is a small (15 kDa) heme protein that is essential in a variety of electron-transfer reactions related to fatty acid desaturation (Strittmatter et al., 1974). The intact protein is composed of two parts: a heme-containing (water-soluble) domain and a hydrophobic domain that is used to anchor the protein to the microsomal membrane (Spatz & Strittmatter, 1971). The structure of the water-soluble lipase-cleaved fragment of bovine liver microsomal cytochrome b_5, determined by single-crystal X-ray crystallography, has been reported to a resolution of 2 Å (Mathews et al., 1971, 1979; Mathews, 1980). A gene coding for the lipase-solubilized bovine microsomal cytochrome b_5 has been synthesized and expressed in *Escherichia coli* (Funk et al., 1990). Furthermore, ^{1}H NMR experiments have shown that in solution the native bovine microsomal cytochrome b_5 is a mixture of two interconvertible isomers that differ by a 180° rotation about the α,γ-meso axis of the heme. The more stable isomer is the one with the heme oriented as in Figure 1, isomer A (Keller & Wüthrich, 1980; La Mar et al., 1981; McLachlan et al., 1986a). Bodman et al. (1986) reported the gene synthesis and bacterial expression of the complete and water-soluble tryptic fragment of the rat hepatic microsomal cytochrome b_5, whose ^{1}H NMR spectrum indicates the presence of interconvertible isomers with very similar relative stabilities (A:B = 1.6:1) (Lee et al., 1990). It was indicated that in the bovine microsomal protein, orientation A is enthalpically favored by 1.4 kcal/mol, whereas in the rat microsomal b_5, orientation B is enthalpically favored by 2.4 kcal/mol but orientation A is still slightly favored at 25 °C (A:B = 1.6:1) due to entropic factors (Lee et al., 1990). It has also been pointed out that one of the important factors that dictates heme orientation is the steric destabilization of a vinyl substituent at position c in the heme (Figure 1) by a hydrophobic cluster of amino acids at positions 23 and 25 (Lee et al., 1990).

Although extensive NMR investigations of the trypsin- or lipase-cleaved fragments of bovine, rat, and chicken microsomal cytochrome b_5 have been reported (McLachlan et al., 1986a,b, 1988; Lee et al., 1990), there have not been previous NMR studies of the rat outer mitochondrial membrane (OM) cytochrome b_5. This protein was first isolated and partially purified from rat liver, specifically from the outer membranes of mitochondria, by proteolytic cleavage (Fukushima & Sato, 1972), and later by detergent solubilization (Nisimoto et al., 1977). Ito (1980) purified the proteolytically cleaved OM cytochrome b_5 to homogeneity and carried out immunological reactions with rabbit antibodies prepared against microsomal cytochrome b_5 and OM cytochrome b_5 (Ito, 1980). These experiments demonstrated that the OM cytochrome b_5 and the microsomal b_5 isolated from rat liver are two distinct proteins. OM cytochrome b_5 has been shown to participate in the outer mitochondrial membrane rotenone-insensitive NADH–cytochrome *c* reductase system that is not coupled to oxidative phosphorylation (Sottocasa et al., 1967), suggesting to us that OM cytochrome b_5 might be a physiological redox partner of cytochrome *c*. Lederer and co-workers (Lederer et al., 1983) reported the complete amino acid sequence of the heme binding domain of OM cytochrome b_5 and showed that 53 out of 91 amino acids of the rat OM and microsomal cytochromes b_5 are identical. The protein sequence of rat OM cytochrome b_5 is shown in Figure 2, together with the amino acid sequences of rat, beef, rabbit, human, and chicken microsomal cytochromes b_5 [see Ozols (1989) and references cited therein]. It is noteworthy that although these proteins are remarkably similar, some of the amino acids that line the heme binding site differ between rat OM and the microsomal cytochromes b_5. The hydrophobic region around

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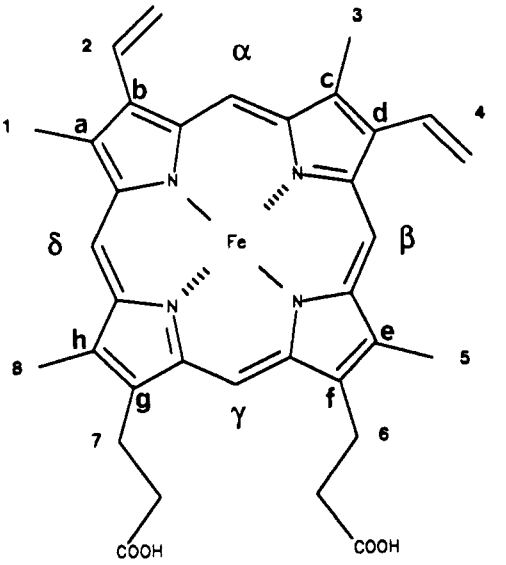
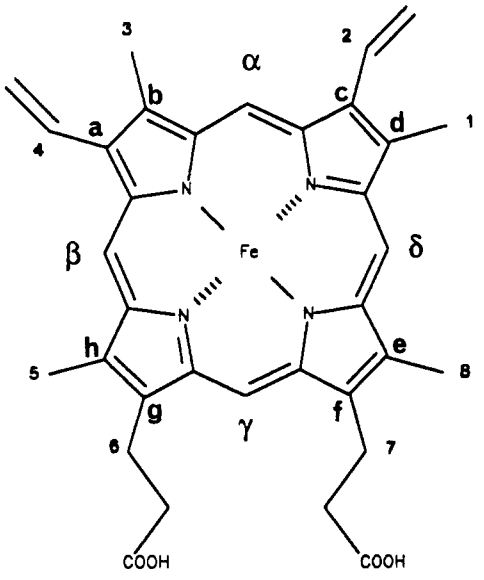


Isomer A



Isomer B

FIGURE 1: Diagram of the two heme orientations which differ by a 180° rotation about the α,γ-meso axis which results in heme disorder in the outer mitochondrial membrane cytochrome b_5 and other microsomal cytochromes b_5. Letters a through h refer to the labeling system that is defined by the heme position and is independent of heme orientation.

Leu-23 in the bovine protein, which is thought to dictate heme orientation (Lee et al., 1990), has several substitutions which potentially can affect the heme orientation ratio in the OM cytochrome b_5. Walker and co-workers (Walker et al., 1988) reported the calculated reduction potentials for the pure A and B heme orientations of the trypsin-cleaved bovine microsomal cytochrome b_5 to be +0.8 and −26.2 mV, respectively, which indicates that there may be a correlation between structure (e.g., heme orientation) and reduction potential.

Ready access to the rat OM cytochrome b_5, therefore, would make it possible to carry out experiments designed to further unravel the factors that determine heme orientation as well as redox and kinetic properties of this protein. Moreover, studying a protein from a different cell compartment, which is similar but not identical to the microsomal proteins, can provide the opportunity to understand the subtleties that control heme orientation and its correlation with reduction potentials and mechanisms of electron transfer. Careful comparisons of the physicochemical properties of the rat OM cytochrome b_5 with the much better characterized beef and rat microsomal cytochromes b_5 might provide some insight into why there are two different rat hepatic cytochromes b_5. Finally, ready access to site-directed mutants can prove extremely useful in testing hypotheses aimed at explaining any of the properties mentioned above.

To these ends, we decided to synthesize the gene that would encode the water-soluble domain of the rat OM cytochrome b_5 and express it in *E. coli*. Gene synthesis provides several advantages over traditional cloning techniques, in that the former allows the incorporation of frequently used codons, appropriate start and stop sequences, and an efficient ribosome binding site and the incorporation of restriction sites appropriate for future site-directed mutagenesis. In this paper, we describe the synthesis and expression of the gene. In addition, the purified protein has been characterized by UV–vis, EPR, and NMR techniques. The assignment of the contact-shifted resonances in the NMR spectrum of the rat OM cytochrome b_5 in the two heme orientations has been carried out by a combination of one-dimensional (steady-state NOE) and two-dimensional (NOESY) ^{1}H NMR experiments.

EXPERIMENTAL PROCEDURES

Protocols for standard procedures such as plasmid isolations, transformations, restriction endonuclease reactions, and ligations were those published by Sambrook et al. (1989). Protocols which had to be modified or optimized are described in detail in the following section. The dNTP, isopropyl β-thiogalactoside, and enzymes used for the experiments described were purchased from Gibco BRL, Grand Island, NY. The sequenase kit was purchased from U.S. Biochemical, Cleveland, OH. The pBS+ plasmid and XL1-blue *E. coli* cells were purchased from Stratagene, La Jolla, CA. The pET-11a plasmid and BL21(DE3) *E. coli* cells were purchased from Novagen, Madison, WI.

Design and Synthesis of the Gene That Encodes the Water-Soluble Domain of the Rat OM Cytochrome b_5. The DNA and amino acid sequences of the target gene are shown in Figure 3. The first and last 15 base pairs were added to the original sequence of the gene in order to add stop signals and restriction endonuclease sites (*Eco*RI, *Nde*I, *Bam*HI) convenient for cloning and subcloning. Restriction sites (*Bst*EII, *Sac*II, *Nco*I, *Sma*I, *Bst*BI, *Sca*I, and *Bsp*MII) were included along the target gene to facilitate future site-directed mutagenesis. This was accomplished by back-translating the amino acid sequence to an ambiguous nucleotide sequence using the program NPRTDNA (Little & Mount, 1984) and searching this ambiguous sequence using a pattern-matching program and a list of restriction endonuclease sites (Mount & Conrad, 1984). Codons frequently used by *E. coli* (Ikemura, 1985) were introduced without altering the amino acid sequence.

Four single-stranded oligonucleotides were chemically synthesized and purified in the University of Arizona Biotechnology Center. The four single-stranded oligonucleotides were designed so that oligonucleotides 1 and 2 and oligonucleotides 3 and 4 are self-primed (Figure 3 and Scheme A). Typically, 1 μL (1 μg/μL) of each single-stranded nucleotides

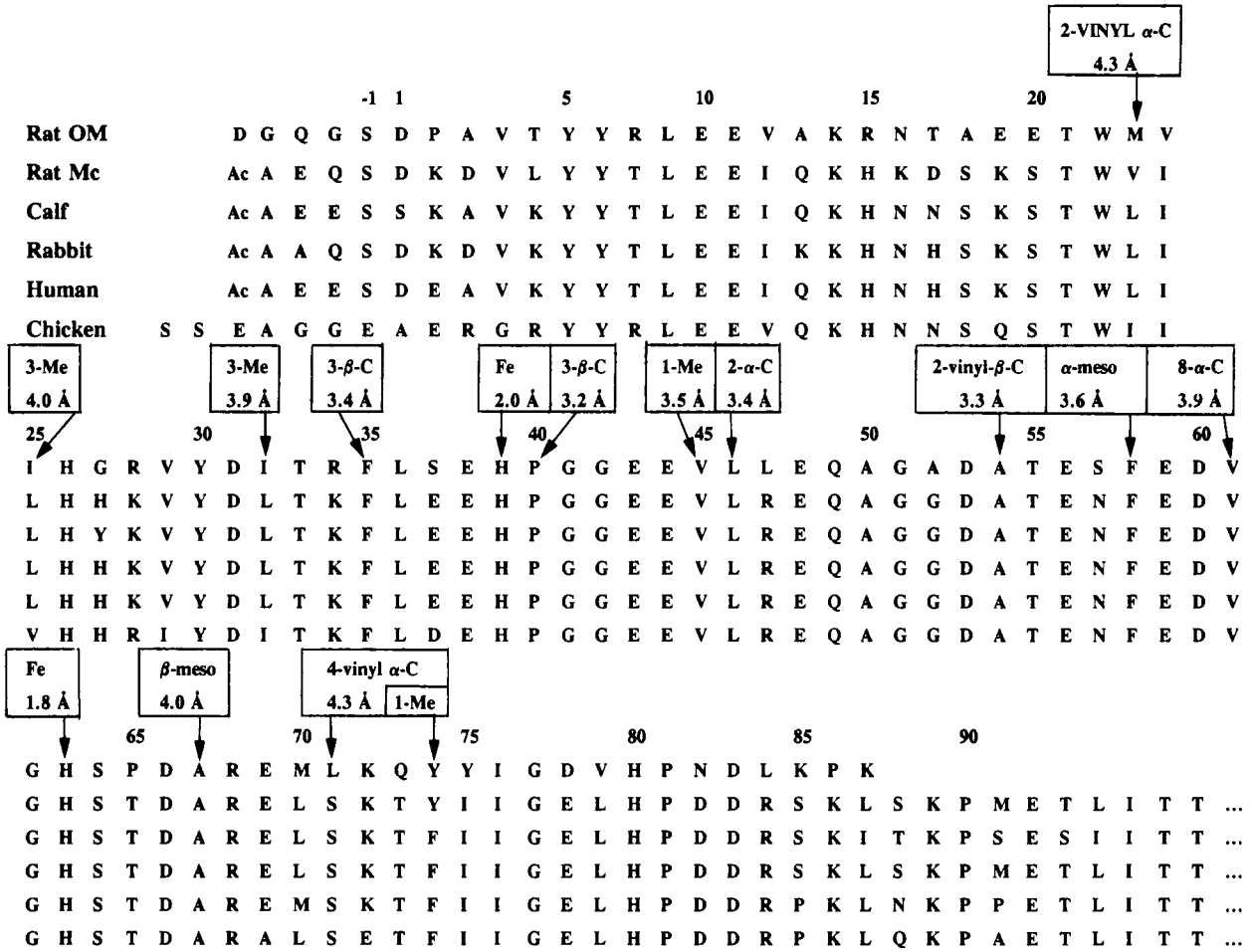


FIGURE 2: Amino acid sequences of selected cytochromes b_5. The residue numbering system is that of Mathews et al. (1979). Heme contacts with protein side chains are given for isomer B of microsomal cytochrome b_5 (Mathews et al., 1979; Lee et al., 1990). The heme numbering system is that shown in Figure 1B.

1 and 2 or nucleotides 3 and 4 was taken to a final volume of 20 μL with sequenase buffer. The solution was heated to 70 °C for 5 min and then cooled to 50 °C for 10 min. After addition of 2 μL of a dNTP mixture (2.5 mM in each dNTP) and 13 units of sequenase version 2.0 and incubation at 37 °C for 30 min, solutions were heated to 70 °C to inactivate the sequenase and then taken to a final volume of 100 μL. A 50-μL portion of each resulting solution was digested with 50 units of *Sma*I in a final volume of 100 μL for 3 h (Scheme A), to produce phosphorylated ends, and then purified by phenol–chloroform extraction and ethanol precipitation. The two double-stranded oligonucleotides were ligated together with 4 units of T4 DNA ligase in 40 μL of ligase buffer in order to obtain the total synthetic gene. The solution containing the OM cytochrome b_5 gene was purified by phenol–chloroform extraction and ethanol precipitation, digested with *Eco*RI and *Bam*HI (50 units each) in 200-μL total volume, and purified again by phenol–chloroform extraction and ethanol precipitation. DNA was resuspended in water and cloned into the pBS+ plasmid, which had been linearized by digestion with *Eco*RI and *Bam*HI. The recombinant plasmid (MRL1) was used to transform *E. coli* strain XLI-Blue for amplification and sequencing of the target gene. Once the sequence of the gene was established by Sanger sequencing, it was excised from the pBS+ plasmid by double-digestion with *Nde*I and *Bam*HI and purified by agarose gel electrophoresis. The purified DNA was ligated into the plasmid pET 11-a (Studier & Moffat, 1986) that had been digested with *Nde*I and *Bam*HI. Transformation of *E. coli* strain BL21-(DE3) with this recombinant plasmid (MRL2) resulted in single colonies that upon induction with IPTG (isopropyl β-thiogalactoside) synthesize protein (OM cytochrome b_5 in this case) very efficiently (Studier & Moffat, 1986). Approximately 30% of the total protein in the induced cells is OM cytochrome b_5.

Expression and Purification of the Water-Soluble Domain of the Rat OM Cytochrome b_5. A single colony of *E. coli* cells, BL21(DE3), containing the recombinant pET 11-a plasmid (MRL2) was grown overnight (37 °C) in 4 mL of LB medium containing ampicillin (10 mg/L). The overnight culture was used to inoculate four 1-L culture flasks of LB medium with the same concentration of ampicillin, and the cells were grown at 37 °C until the OD_{600} reached a value between 0.8 and 1.0 (approximately 4.5 h). IPTG was added to a final concentration of 0.5 mM, and the cells (orange in color due to high levels of OM cytochrome b_5) were harvested 3.5 h later by centrifugation. Approximately 16 g of cells was obtained and was lysed using a modification of the method described by Sambrook et al. (1989). The cells were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, and 100 mM NaCl, pH 8.0) and lysed with lysozyme (1.3 mL, 10 mg/mL) in the presence of PMSF (phenylmethanesulfonyl fluoride, 130 μL, 50 mM) for 40 min at 4 °C. Deoxycholic acid was added (64 mg), and the lysate was incubated at 37 °C until it became viscous. DNase I (320 μL, 1 mg/mL) was then added, and the lysate was incubated at room temperature until the lysate was no longer viscous. Cell debris was removed by centrifugation, and the supernatant containing the OM cytochrome b_5 was desalted in a Sephadex G-25 column. The chromatographic fractions containing the OM cytochrome b_5

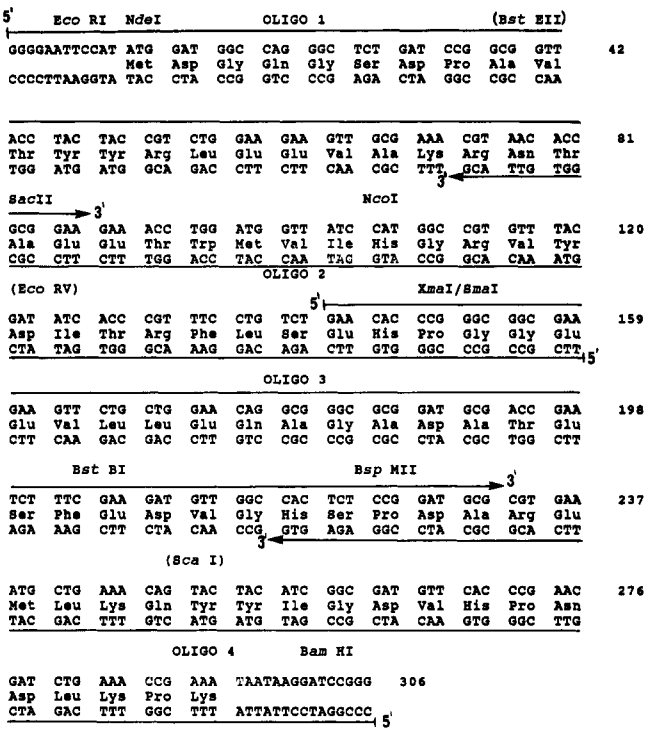
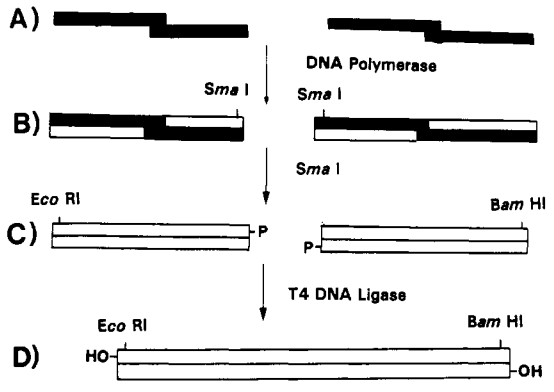


FIGURE 3: Amino acid sequence of the water-soluble tryptic fragment of OM cytochrome b_5 (Lederer et al., 1983) and the corresponding DNA sequence obtained by back-translation of the amino acid sequence. Initiator methionine, codons frequently used by *Escherichia coli*, restriction endonuclease sites, and stop codons were engineered as discussed under Experimental Procedures. The arrows indicate the length of the synthetic single-stranded oligonucleotides. Restriction enzyme sites shown are unique except for the ones shown in parentheses. *Sca*I is present in pBS+ and in pET11a. *Bst*EII is present in the pET11a plasmid.

Scheme A



were followed by monitoring the absorbance ratio A_{280}/A_{423}. The desalted fraction was applied to a DEAE-Sephacel column equilibrated with 50 mM Tris and 10 mM EDTA (pH 7.8) and eluted with a linear NaCl (0–250 mM) gradient. Fractions containing the OM cytochrome b_5 were concentrated by ultrafiltration and further purified in a Sephacel G-50 column equilibrated with 100 mM NaCl and 20 mM Tris, pH 7.8, from which fractions with an absorbance ratio (A_{280}/A_{423}) of 0.2 ± 0.01 were collected in order to obtain homogeneous protein. Typically, 4 L of culture media provided 40 mg of homogeneous OM cytochrome b_5.

Sequential Edman degradation of 360 pmol of homogeneous protein in an Applied Biosystems 477A protein sequencer yielded a methionine amino-terminal residue. The 19 subsequent residues were found to have a sequence that is identical with the one predicted by the coding sequence: Asp-Gly-Gln-Gly-Ser-Asp-Pro-Ala-Val-Thr-Tyr-Tyr-Arg-Leu-Glu-Glu-Val-Ala-Lys.

^1H NMR Studies. ^1H NMR spectra were recorded at 25 °C on a Bruker AM-500 spectrometer operating in the quadrature mode with a proton frequency of 500.13 MHz. OM cytochrome b_5 solutions (1 mM) were obtained by dissolving freeze-dried protein in D_2O; the pH* was adjusted by the addition of DCl or NaOD and measured using an Ingold microcombination electrode. The pH* values were not corrected for the isotope effect.

Steady-state NOE difference experiments were carried out as described by Nehaus and Williams (1989) and McLachlan et al. (1986b). Two-dimensional phase-sensitive NOE experiments (NOESY) were carried out using the method of States et al. (1982). Spectra were collected with presaturation of the residual HDO peak during the relaxation delay (400 ms). A mixing time of 50 ms was found to be optimal for the detection of the majority of cross-peaks arising from contact-shifted resonances. A random variation of 10% of the mixing time was allowed in order to cancel the scalar correlation effects. Data sets consisting of 256 experiments, each collected with 2K data points, were typically obtained. The data sets were processed in an IRIS work station using the program FELIX (Hare Research Inc.). Before Fourier transformation, the t_2 dimension was multiplied by a 90°-shifted squared sine bell function; after Fourier transformation, the base line was corrected using the SPLINE function. Before transformation, the t_1 dimension was multiplied by a 90°-shifted squared sine bell function. The data sets were not symmetrized.

EPR and Optical Spectral Studies. The EPR spectrum was acquired using an ESP 300 E Bruker spectrometer operating at 9.38 GHz with 200 μW of microwave power. The modulation frequency was 100 kHz, and the modulation amplitude was 10 G. The spectrum was acquired at 4.2 K using the Model ESR 900 Oxford instrument cryostat. The UV–visible spectra were acquired using a diode array 8451A Hewlett Packard spectrophotometer.

RESULTS

Gene Synthesis and Bacterial Expression. The synthetic gene was assembled from two pairs of self-priming single-stranded oligonucleotides, as shown in Figure 3 and in Scheme A. Oligonucleotides 1 and 2 were each 87 bases long, and oligonucleotides 3 and 4 were each 90 bases long. The 5′ ends of the double-stranded oligonucleotides were not phosphorylated with T4 polynucleotide kinase and ATP. Instead, phosphorylation of the desired ends was achieved by digesting the double-stranded oligonucleotides with *Sma*I (Scheme A). Since only ends resulting from *Sma*I digestion were phosphorylated and reactive with DNA ligase, the number of side products for the ligation of the two double-stranded oligonucleotides was reduced. One of the products of the ligation reaction was the total gene that encodes for the OM cytochrome b_5.

The OM cytochrome b_5 gene was inserted in the pBS + plasmid, sequenced by the Sanger technique, and shown to have the correct sequence (data not shown). The synthetic gene (*Nde*I to *Bam*HI) was subcloned into the T7 expression vector pET11-a (Studier & Moffat, 1986). Upon addition of IPTG to a final concentration of 0.5 mM, OM cytochrome b_5 was expressed at high levels, and the cells became orange in color. Cells were harvested and the protein was purified to homogeneity as described under Experimental Procedures.

The homogeneous OM cytochrome b_5 has an absorption spectrum (Figure 4) very similar to the one obtained for the

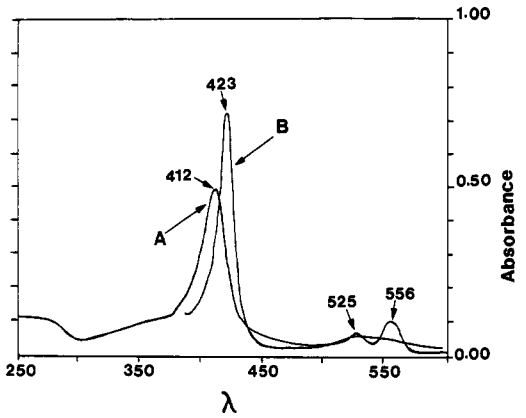


FIGURE 4: UV–visible spectra of the (A) oxidized and (B) reduced forms of OM cytochrome b_5.

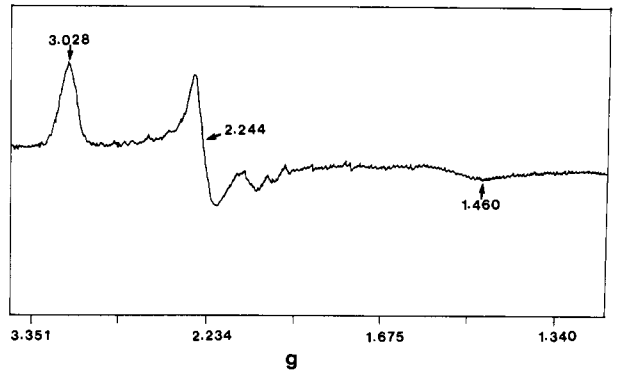


FIGURE 5: X-band EPR spectrum of the OM cytochrome b_5 recorded at 4.2 K.

hepatic microsomal cytochrome b_5. The Soret bands of the oxidized and reduced proteins occur at 412 and 423 nm, respectively, and visible bands for the reduced protein occur at 525 and 556 nm.

The EPR spectrum of the homogeneous OM cytochrome b_5 (Figure 5) is characteristic of a low-spin ferric hemoprotein, with g values of 3.03, 2.24, and 1.46. These values are almost identical to the ones obtained for the overexpressed rat microsomal cytochrome b_5 (3.05, 2.24, and 1.46) (Bodman et al., 1986), as well as those of the bovine microsomal (3.03, 2.23, and 1.43) (Bois-Poltoratsky, & Ehrenberg, 1967) and erythrocyte cytochromes b_5 (3.03, 2.23, and 1.39) (Passon et al., 1972).

^1H NMR Spectroscopy. The ^{1}H NMR spectrum of the rat OM cytochrome b_5 (Figure 6) indicates that the protein as isolated is in the low-spin ferric state, which is in agreement with the EPR results (see above). In addition, the large number of peaks in the low-field portion of the ^{1}H NMR spectrum of OM cytochrome b_5 indicates that this protein in solution consists of two isomers which differ by 180° rotation of the heme about the α,γ-meso axis. Furthermore, heme disorder in the OM cytochrome b_5 seems to be the largest observed so far, with an isomeric ratio of 1.0 ± 0.1 at equilibrium. This number was found by assigning three hydrogens to the area corresponding of the 5Me resonance of isomer A (Figure 6). The area of the peak corresponding to the 8Me resonance of isomer B was then evaluated and the isomeric ratio calculated. These two resonances were chosen because A 5Me and B 8Me are in similar chemical environments in the two isomers, and thus have similar chemical shifts, therefore eliminating any possible differences in apparent signal intensity owing to possible differences in excitation power. The equilibrium ratios for the rabbit (La Mar et al., 1981), bovine (McLachlan et al., 1986a), chicken, and recombinant rat (Lee et al., 1990) microsomal cytochromes b_5 are 10, 8.9, 20, and 1.6, respectively.

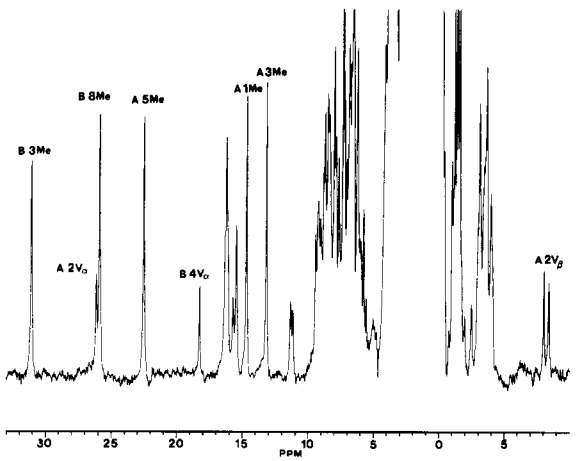


FIGURE 6: ^{1}H NMR spectrum of the OM cytochrome b_5 at 25 °C and pH* 7.2. The spectrum shows the assignments of the contact-shifted resonances to their corresponding heme substituents in isomers A and B.

Assignments of Contact-Shifted Resonances in the Rat OM Cytochrome b_5. ^{1}H NMR assignments of heme resonances in ferricytochromes were originally approached by nuclear Overhauser effect based assignments of the reduced protein, followed by connectivities to the oxidized protein by saturation transfer via electron exchange (Keller & Wüthrich, 1980) and by direct reconstitution of apoprotein with labeled hemes (La Mar et al., 1981; McLachlan et al., 1986a). A more recent approach is the direct assignment of contact-shifted resonances in paramagnetic proteins via the nuclear Overhauser effect. These types of assignments have been carried out in one dimension via steady-state NOEs (Unger et al., 1985; McLachlan et al., 1986b; Lee et al., 1990, 1991) and in two dimensions via NOESY experiments (McLachlan et al., 1988; Emerson & La Mar, 1990; Yu et al., 1990). An empirical assignment strategy from which the presence of heme disorder could be rapidly inferred was reported by Lee et al. (1990). The high-frequency region of the ^{1}H NMR spectrum of the rat OM cytochrome b_5 (Figure 6) shows two sets of peaks of essentially identical intensity. The relative intensity of the two sets of peaks remained constant upon incubation of the protein at 37 °C for 16 h. These peaks were assigned from the two-dimensional NOESY map (Figure 7 and Table I) using as a starting point the assignments obtained from the empirical strategy reported by Lee et al. (1990).

For orientation A: Starting with the 2Vα resonance at 26.13 ppm (1), cross-peaks are observed to 1Me at 14.70 ppm (2), to meso-Hα at −3.07 ppm (3), and to 2Vβ at −7.89 ppm (seen on NOESY slices and on steady-state NOE experiments). Meso-Hα (3) gives a cross-peak to 3Me at 13.18 ppm (4). 1Me (2) gives a cross-peak to meso-Hδ at 9.25 ppm (5). 5Me at 22.45 ppm (6) gives a cross-peak to meso-Hβ at 9.30 ppm (7) and to 6Hα at 16.13 ppm (8). Resonances due to 8Me, meso-Hγ and to 7Hα and 7Hβ could not be unambiguously assigned. From steady-state NOE experiments, it was determined that the 2V group is rapidly reorienting in the NMR time scale, since irradiation of the 2Vα resonance gives an NOE to both 1Me and meso-Hα resonances, as obseved by McLachlan et al. (1988).

For orientation B: Starting with the 3Me resonance at 31.04 (9), an NOE is observed to the 4Vα resonance at 18.18 ppm (10) and to the meso-Hα resonance at −2.36 ppm (11). The 4Vα resonance (10) gives a cross peak to the meso-Hβ

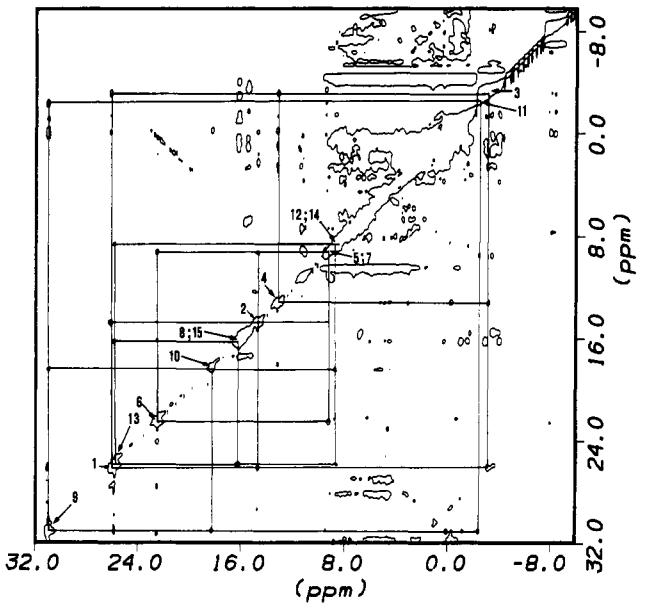


FIGURE 7: ^{1}H NMR NOESY map of the OM cytochrome b_5 at 25 °C and pH* 7.2.

resonance at 8.81 ppm (12). The 8Me resonance at 25.86 ppm (13) gives an NOE to the meso-Hδ resonance at 8.63 ppm (14) resonance and to the 7Hα resonance at 16.3 ppm (15). Resonances due to 5Me, meso-Hγ, 6Hα and -β, and 7Hα and -β could not be unambiguously assigned.

DISCUSSION

Rat OM Cytochrome b_5 Gene Synthesis. Current technology has made it possible to obtain relatively long single-stranded oligonucleotides in an expedient manner, thus making gene synthesis accessible (Springer & Sligar, 1987; Bodman et al., 1986; Chang et al., 1991). When the protein sequence is available, either from the amino acid or from the DNA sequence, gene synthesis presents several advantages. It circumvents lengthy cDNA cloning and gene isolation procedures, and it is possible to introduce silent mutations that result in frequently used codons and/or useful restriction sites without altering the amino acid sequence of the protein (Little & Mount, 1984; Mount & Conrad, 1984). Rare codons can result in poor expression or lack of expression of the target gene. In addition, optimum sequences for complementarity with the expression system can be easily introduced in the design of the gene; these include optimum ribosome binding site, spacer, initiator methionine sequence, and the introduction of unique restriction enzyme sites along the gene for future site-directed mutagenesis.

Expression of the OM cytochrome b_5 using the pET-11a system resulted in rapid and efficient protein synthesis. A typical 4-L culture yielded approximately 40 mg of OM cytochrome b_5 after purification.

Heme Disorder in the Rat OM Cytochrome b_5. Heme disorder and/or the orientation of protoporphyrin IX in a hemoprotein can be determined readily, provided that the ^{1}H NMR spectrum of the contact-shifted resonances can be assigned to their heme positions. The ^{1}H NMR spectrum of rat liver OM cytochrome b_5 shows that the axial binding and resulting rhombic perturbation of the ferric center are similar to those observed for microsomal cytochromes b_5 (beef, rabbit, chicken, and rat). The axial binding and rhombic perturbation of the heme center are responsible for the characteristic contact-shift pattern observed for cytochromes b_5 (La Mar et al., 1981) including the newly accessible OM cytochrome b_5. The range of shifts within the pattern arise from relatively slight changes in chemical environment, presumably brought about by changes in amino acids around the heme, as shown in Figure 2.

The assignments of the contact-shifted resonances for OM cytochrome b_5 (Table I) were carried out by a combination of the empirical method described by Lee et al. (1990) and nuclear Overhauser effect spectroscopy in one and two dimensions. In order to rapidly assess the presence of heme disorder and the approximate ratio of the two heme orientations, it is necessary to find the resonances that arise from heme position b and e in Figure 1A,B. These positions give rise to the larger contact shifts for a given substituent in the heme and correspond to 2Vα and 5Me, respectively, in orientation A and to 3Me and 8Me in orientation B (Lee et al., 1990). The presence of a peak between 30 and 32 ppm (B 3Me) has previously been shown to be indicative of the presence of the B isomer, and its intensity relative to the other resonances gives an indication of its concentration relative to the A isomer (Lee et al., 1990). The presence of a peak at the same chemical shift for OM cytochrome b_5, which was assigned to the 3Me substituent in isomer B via its NOE pattern, ensures that the latter isomer of OM cytochrome b_5 is in a very similar environment to that of the microsomal proteins investigated previously (Keller & Wüthrich, 1980; La Mar et al., 1981; McLachlan et al., 1986a; Lee et al., 1990). Once the rest of the contact-shifted resonances have been tentatively assigned by the empirical method, the interpretation of the NOESY map is easier and confirms or denies the identities of the resonances. In our experience, the region of the heme that provides intermediate contact shifts (positions a and c in Figure 1A,B) should be carefully analyzed, since relying only on the pattern from similar proteins can lead to misinterpretation of the NMR spectrum. For example, the methyl resonance corresponding to 1Me in isomer A in the OM cytochrome b_5 is shifted to higher frequency than that of the 3Me resonance in the same isomer. This is in contrast with the microsomal beef, rat, and chicken cytochromes b_5, in which the 3Me resonance in isomer A is shifted to higher frequency than that of 1Me in the same isomer (Lee et al., 1990). Steady-state NOEs can be used to corroborate assignments that are not totally clear from the NOESY map.

Heme disorder in the protein of this study is the largest reported so far for a cytochrome b_5, with an isomer ratio (A:B) of 1:1. It is worth noting that the two proteins with the largest heme disorder come from the rat liver, since the microsomal cytochrome b_5 (Lee et al., 1990) has an isomer ratio of 1.6. Furthermore, both proteins arise from synthetic genes overexpressed in *E. coli*, and it is possible that the folding process in the bacterial host does not yield proteins with identical conformations to those of the native enzymes. This could ultimately result in the high degree of heme disorder observed in both rat microsomal and rat OM cytochromes b_5. Successful site-directed mutagenesis experiments aimed at reconstructing the heme pocket based on information known about similar proteins (e.g., microsomal bovine or chicken cytochromes b_5) would indicate whether the bacterial host is capable of producing cytochrome b_5 with almost identical conformation to those of the native enzymes. Such experiments are currently underway in this laboratory.

It has been postulated (Lee et al., 1990) that Leu-23, which makes van der Waals contact with A 3Me in the crystal structure of bovine cytochrome b_5, could interact more strongly with the 2-vinyl group in isomer B (Figure 2) whereas in the rat microsomal cytochrome b_5, where Leu-23 has been replaced

Table I

assignment	native bovine cytochrome b_5, pH 6.75		recombinant OM rat cytochrome b_5, pH[f] 7.2		recombinant MC rat cytochrome b_5, pH[f] 7.41	
	shift[a,b]	shift[a,b]	shift[a,c]	shift[a,c]	shift[a,d]	shift[a,d]
1Me	11.45		14.70		10.7	−0.6
2Vα	27.43		26.13		27.4	10.8
2Vβ	−6.75, −7.04		−7.89			
meso-Hα	−2.89		−3.07	−2.36		
3Me	14.40	30.6	13.18	31.04	14.4	31.8
4Vα	5.38	17.9		18.18	5.1	16.8
4Vβ						
meso-Hβ	9.20		9.30	8.81		
5Me	21.84		22.45		20.4	
6Hα	15.66, 15.76		16.13			
6Hβ	−0.82, −1.56					
meso-Hγ	−0.27					
7Hα	18.97, −1.81	16.2		16.13		
7Hβ	1.57, −3.52					
8Me	2.70	27.2		25.86		24.3
meso-Hδ	9.79		9.25	8.63		

[a] Chemical shift in ppm referenced to DSS at 25 °C. [b] McLachlan et al. (1988). [c] This work. [d] Lee et al. (1990). [e] Protein overexpressed in *E. coli*. [f] pH* = pH readings not corrected for the deuterium isotope effect.

by a smaller valine, the 2-vinyl group in isomer B is less hindered, thus making isomer B more stable in the rat microsomal protein. In the case of leucine-25, which makes van der Waals contact with A 2V in the crystal structure of the bovine protein, it has been replaced by a smaller valine in the chicken microsomal cytochrome b_5, which diminishes the steric destabilization of the A 2V group, thus making isomer A very stable. In fact, it has been found that the isomeric ratio in the chicken protein is A:B = 20:1 (Lee et al., 1990). In the OM cytochrome b_5, Leu-23 has been replaced by a larger methionine, and, therefore, according to the previous analysis, it was expected that the 2-vinyl group in isomer B should experience large steric hindrance, thus making isomer B unstable. On the other hand, Leu-25 of both bovine and rat microsomal proteins is replaced by an isoleucine in the OM rat cytochrome b_5. Thus, it was expected that this residue would not affect heme orientation much differently than in the bovine microsomal cytochrome b_5. On the basis of above, it was expected that isomer A would be relatively more stable than isomer B and that the heme orientation ratio in the OM cytochrome b_5 should be A:B > 1:1.

The experimental observations reported in this paper (i.e., isomer ratio A:B = 1:1) are not in agreement with the analysis discussed above. It is possible, therefore, that the orientation of the heme may be controlled not only by steric effects but also by differences in protein folding of the recombinant proteins discussed above, or from water access to vacancies near the heme. These vacancies can in principle be created or deleted by mutations that interchange a hydrophobic amino acid near the heme by a hydrophilic one or vice versa. In addition, it may be possible to take advantage of the structural features that are common in the two rat cytochromes b_5, but are not shared by the other cytochromes b_5; for example, tyrosine-74 is common in both rat proteins, but it is replaced by a phenylalanine in the rest of the proteins whose sequences are shown in Figure 2. Phenylalanine-74 in the beef cytochrome b_5 is located in a hydrophobic patch which excludes access of water to one side of the heme, whereas the opposite side of the heme is relatively largely exposed to water, as observed from the crystal structure of beef cytochrome b_5 (Mathews et al., 1971, 1979) taken from the protein data bank and seen with the molecular graphics program SYBYL. It is possible that tyrosine in place of phenylalanine may provide water access to the heme by hydrogen-bonding water interactions with water molecules. Future site-directed mutagenesis will be aimed at probing these possibilities.

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REFERENCES

Bodman, S. B. V., Schuler, M. A., Jollie, D. R., & Sligar, S. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9443–9447.
Bois-Poltoratsky, R., & Ehenberg, A. (9167) *Eur. J. Biochem. 2*, 361–365.
Chang, T. K., Iverson, S. A., Rodrigues, C. G., Kiser, C. N., Lew, A. Y., Germanas, J. P., & Richards, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1325–1329.
Emerson, S. D., & La Mar, G. N. (1990) *Biochemistry 29*, 1545–1556.
Fukushima, K., & Sato, R. (1972) *J. Biochem. (Tokyo) 74*, 161–173.
Funk, W. D., Lo, T. P., Mauk, M. R., Brayer, G. D., MacGillivray, T. A., & Mauk, A. G. (1990) *Biochemistry 29*, 5500–5508.
Ikemura, T. (1985) *Mol. Biol. Evol. 2*, 13–34.
Ito, A. (1980) *J. Biochem. 87*, 63–71.
Keller, R. M., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 621*, 204–217.
La Mar, G. N., Burns, P. D., Jackson, J. T., Smith, K. M., Langry, K. C., & Strittmatter, P. (1981) *J. Biol. Chem. 256*, 6075–6079.
Lederer, F., Ghrir, R., Guiard, B., Cortial, S., & Ito, A. (1983) *Eur. J. Biochem. 132*, 95–102.
Lee, K. B., La Mar, G. N., Kehres, L. A., Fujinari, E. M., Smith, K. M., Pochapsky, T. C., & Sligar, S. G. (1990) *Biochemistry 29*, 9623–9631.
Lee, K. B., Jun, E., La Mar, G. N., Rezzano, I. N., Pardey, R. K., Smith, K. M., Walker, F. A., & Buttlaire, D. H. (1991) *J. Am. Chem. Soc. 113*, 3576–3583.
Little, J., & Mount, D. W. (1984) *Gene 32*, 67–73.
Mathews, F. S. (1980) *Biochim. Biophys. Acta 622*, 375–379.
Mathews, F. S., Argos, P., & Levine, M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 387–395.
Mathews, F. S., Gerwinsky, E. W., & Argos, P. (1979) *The Porphyrins* (Dolphin, D., Ed.) Vol. 7, pp 107–147, Academic Press, New York.
McLachlan, J., La Mar, G. N., Burns, P. D., Smith, K. M., & Langry, K. C. (1986a) *Biochim. Biophys. Acta 874*, 274–284.

McLachlan, S. J., La Mar, G. N., & Strittmatter, E. (1986b) *J. Am. Chem. Soc. 108*, 1285–1291.

McLachlan, S. J., La Mar, G. N., & Lee, K. B. (1988) *Biochim. Biophys. Acta 957*, 430–445.

Mount, D. W., & Conrad, B. (1984) *Nucleic Acids Res. 12*, 812–823.

Neuhaus, D., & Williamson, M. (1989) *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, pp 217–231, VCH Publishers, New York.

Nisimoto, Y., Takeuchi, F., & Shibata, Y. (1977) *J. Biochem. (Tokyo) 82*, 1257–1266.

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

Passon, P. G., Recd, D. W., & Hultquist, D. E. (1972) *Biochim. Biophys. Acta 275*, 51–61.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *J. Cell Biol. 32*, 415–438.

Spatz, L., & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1042–1046.

Springer, B. A., & Sligar, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8961–8965.

States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.

Strittmatter, P., Spatz, L., Cocovan, D., Rogers, M. J., & Redline, R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4565–4659.

Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113–130.

Unger, S. W., LeComte, J. T. G., & La Mar, G. N. (1985) *J. Magn. Reson. 64*, 521–526.

Walker, F. A., Emrick, D., Rivera, J. E., Hanquet, B. J., & Buttlaire, D. H. (1988) *J. Am. Chem. Soc. 110*, 6234–6240.

Yu, L. P., La Mar, G. N., & Rajarathnam, K. (1990) *J. Am. Chem. Soc. 112*, 9527–9534.