Synthesis, bacterial expression, and mutagenesis of the gene coding for mammalian cytochrome $b_5$

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ABSTRACT We have totally synthesized a gene that codes for rat hepatic cytochrome $b_5$. The 5’ flanking region was designed for efficient expression of this gene in Escherichia coli by incorporating an optimum ribosome binding site and spacer region. Both a soluble form, analogous to the protease-treated microsomal protein, as well as the complete cytochrome with hydrophobic membrane anchor, was constructed and expressed. Transformants with the gene for the soluble protein overproduce authentic cytochrome $b_5$ to a level of 8% of the total cell protein. The complete cytochrome is expressed to a lesser extent with most of the protein found in the cell membrane fraction. This represents complete synthesis and bacterial expression of a mammalian metalloprotein gene. Cytochrome $b_5$ is normally a six-coordinate low spin heme protein with histidine-39 and histidine-63 as axial ligands. We have replaced histidine-63 with a methionine residue by cassette mutagenesis, utilizing specific restriction enzyme sites engineered into the synthetic gene. The resultant protein has histidine-39 as sole axial ligand and is five-coordinate high spin heme protein in the ferric resting state, as indicated by optical and electron spin resonance spectroscopy. The ability to generate mutant cytochrome $b_5$ in high yield is a crucial step in understanding heme protein folding, protein–protein recognition and binding, and biological electron transfer processes.

Cytochrome $b_5$ is a small heme protein that plays a central role in a variety of electron transfer reactions related to fatty acid desaturation (1–3), hepatic cytochrome P-450 reduction (4–7), and regeneration of ferrous hemoglobin in erythrocytes (8, 9). The complete hepatic protein is composed of two domains, a soluble heme-containing core and a hydrophobic COOH-terminal tail anchored in the microsomal membrane (10), whereas erythrocyte cytochrome $b_5$ acts as a soluble agent in cytoplasmic hemoglobin reduction. The amino acid sequence of hepatic cytochrome $b_5$ from several species has been determined, and their primary structures are highly conserved (11, 12). The three-dimensional crystal structure of the tryptic fragment of bovine liver cytochrome $b_5$ has been determined by x-ray diffraction to a resolution of 2 Å (13). A multitude of spectroscopic, kinetic, and magnetic resonance techniques have been applied to this cytochrome and its interaction with physiological and nonphysiological electron transfer partners. The wealth of this molecular information makes cytochrome $b_5$ an ideal target for site-directed mutagenesis experiments designed to probe the molecular mechanisms of electron transfer, the control of heme protein redox potential, the specificity of protein–protein interactions, and the dynamics of heme protein folding. These endeavors would be greatly aided by efficient expression of the mammalian cytochrome $b_5$ coding sequences in a microbial system. Unfortunately, past attempts at the expression of mammalian proteins in microbial hosts have met with limited success (14, 15). For example, constructions in which a cDNA clone of myoglobin or hemoglobin are juxtaposed to an RNA polymerase promoter, ribosome binding site, and translation initiator codon of a suitable expression vector have not resulted in the synthesis of measurable heme protein. If these mammalian cDNA sequences are fused to short regions of the $C_9$ gene, small amounts of holoprotein can be synthesized in Escherichia coli and subsequently cleaved from the fusion protein by proteolytic digestion. It is not known whether the lack of high-level production of mammalian heme proteins in E. coli is due to lack of mRNA or protein stability, difficulty in translation as a result of a poor choice of codon usage, or by the inability of E. coli to synthesize heme at rates necessary for efficient incorporation into the nascent polypeptide chain.

To address these questions and provide a means for producing native and mutant cytochrome $b_5$ in the large quantities and high purity needed for biophysical measurements, we have utilized de novo gene synthesis, involving annealing and ligation of separately synthesized single-stranded oligodeoxynucleotides that together constitute the coding sequence for the catalytic soluble core, or the complete molecule of rat hepatic cytochrome $b_5$. Included are a 5’ flanking region containing a unique bacterial ribosome binding site, optimized spacer region, and initiator methionine. This approach also allows the choice of codons optimized for translation in E. coli and insertion of unique restriction endonuclease sites to aid various DNA manipulations. The ligated product of these synthetic genes was cloned into a plasmid (pUC13) and transformed into E. coli. Transformants containing the soluble core of cytochrome $b_5$ produce holoprotein containing the protoporphyrin IX prosthetic group in amounts up to 8% of the total cellular protein, with the transformed cells containing the cytochrome $b_5$ sequences appearing bright red due to high protein production and efficient heme incorporation. The complete cytochrome $b_5$ gene including the membrane anchor domain is also efficiently expressed in E. coli, with incorporation of the holoprotein into the membrane fraction of the cell. Cytochrome $b_5$ isolated and purified to homogeneity from E. coli by successive ion exchange and sizing chromatography was found to have the same physical properties and NH$_2$-terminal amino acid sequence as the corresponding mammalian liver microsomal protein. The results described in this communication represent complete synthesis and bacterial expression of a mammalian membrane metalloprotein gene.

This successful expression of cytochrome $b_5$ also dictates that the same methods can be used to construct mutant cytochromes $b_5$ and to investigate their function in the membrane of the cell. This communication demonstrates the utility of this approach for the production of mutant cytochromes $b_5$ with our report of the construction and expression of genes coding for mutant cytochrome $b_5$ containing specific amino acid substitutions at the histidine-63 site known to function in the axial ligand binding of the heme of cytochrome $b_5$. 
METHODS

The enzymes used for the various experiments described below were purchased from International Biotechnologies (New Haven, CT). Protocols for standard procedures such as plasmid isolations, transformations, and colony hybridizations were those published by Maniatis et al. (16).

Design of the Gene for Soluble Heme-Containing Domain of Cytochrome \( b_5 \). Specific single-stranded oligodeoxyribonucleotides were synthesized and purified by HPLC in the University of Illinois Biotechnology Center using an Applied Biosystems (Foster City, CA) 380A synthesizer. Individual oligodeoxyribonucleotides shown in Fig. 1, comprising three separate regions between the \( Pst \) I and \( Sal \) I sites, the \( Sal \) I and \( Xho \) I sites, and the \( Xho \) I and EcoRI sites were 5'-phosphorylated in the presence of T4 polynucleotide kinase and ATP. These resultant 5'-phosphodeoxyribonucleotides were then ligated and incorporated into three separate pUC13 plasmids that had been linearized by earlier double digestion with the appropriate pairs of restriction endonucleases. The pUC13 plasmid used in these constructions had been previously modified with a small \( Xho \) I-specific DNA sequence inserted into the BamHI site of the linker to create a site for cleavage by \( Xho \) I. Separate transformations into \( E. coli \) strain TB-1 (\( \pi^+ \) derivative of JM3; T. O. Baldwin, T. Thorne, and M. J. also marketed by Bethesda Research Laboratories) of these three pUC13 plasmids containing the three different gene segments followed by growth of the transformed cells allowed preparation of large amounts of plasmid DNA by standard techniques. The separate cytochrome \( b_5 \) gene segments were then excised by digestion with the appropriate pair of restriction endonucleases (e.g., \( Pst \) I and \( Sal \) I for the gene segment governing the synthesis of the NH2-terminal segment) and were subsequently purified by N,N,N',bisacrylamide/polyacrylamide gel electrophoresis (Bio-Rad). The three isolated gene segments were then ligated and incorporated into pUC13 to constitute a complete gene for the soluble segment of cytochrome \( b_5 \). Transformation of this recombinant plasmid into \( E. coli \) strain TB-1 results in the efficient production of cytochrome \( b_5 \), as evidenced by bright red colonies.

Purification of the Synthetic Soluble Core Domain of Cytochrome \( b_5 \). \( E. coli \) TB-1 cells containing plasmids harboring the gene shown in Fig. 1 were grown in overnight shake flasks, harvested by centrifugation, and lysed with 4 vol of 50 mM Tris-HCl, pH 8.0/0.1 mM dithiothreitol/1.0 mM EDTA/RNase A (1 unit/ml)/DNase (16 units/ml)/lysozyme (3.0 mg/ml). Cell debris was removed by centrifugation, and the supernatant was applied to a DEAE-Sephadex (LKB) column (2.6 \times 9.4 cm) equilibrated in 50 mM Tris-HCl, pH 8.0/0.1 mM dithiothreitol/1.0 mM EDTA and eluted with a linear KCl gradient. Fractions containing cytochrome \( b_5 \) were concentrated by ultrafiltration and applied to a Biogel P-30 (Bio-Rad) gel filtration column (1.9 \times 73 cm). A final DEAE-Sephadex column afforded homogeneous protein, which was stored in liquid nitrogen. Extinction coefficients were calculated from overall heme content determined by pyridine hemochromogen assay (17).

Gene Design and Construction of the Complete Gene for Cytochrome \( b_5 \). The synthesis of the complete gene for cytochrome \( b_5 \) is aided by partial reconstruction of the DNA sequence constructed for the soluble domain of cytochrome \( b_5 \) described above. Oligodeoxyribonucleotides constituting the coding sequence from the \( Xho \) I site to the junction of the two cytochrome \( b_5 \) domains (\( Bcl \) I site in Fig. 4; EcoRI site in Fig. 1) were combined with additional oligodeoxyribonucleotides that together comprise the sequence for the hydrophobic tail domain. The oligodeoxyribonucleotides that span the junction of the two domains differ from those used in the gene for the soluble fragment in the lack of translation termination codons and the inclusion of a unique \( Bcl \) I restriction site at the junction of the two domains. These oligodeoxyribonucleotides were 5'-phosphorylated and annealed as described, and the annealed product was ligated to the \( Xho \) I site of the soluble domain and the EcoRI cloning site of pUC13.

Analysis of Cellular Distribution of Cytochrome \( b_5 \). Cells from 250-mL cultures were harvested, the pellets were suspended in 1 ml of 10 mM Tris-HCl, pH 8.0/1 mM dithiothreitol/20% (wt/vol) sucrose containing 100 units each of DNase and RNase, ruptured by sonication, and whole cells were removed by centrifugation at 10,000 \times g for 10 min at 4°C. After dilution of the supernate with 10 mM Tris-HCl, pH 8.0/1 mM dithiothreitol, the total membrane fraction was separated from the soluble fraction by ultracentrifugation at 110,000 \times g for 3 hr at 4°C. The membrane pellets were dissolved in a minimal volume of 10 mM Tris-HCl, pH 8.0/1 mM dithiothreitol. The distribution of the complete and soluble catalytic core forms of cytochrome \( b_5 \) in the membrane or cytoplasmic fraction of \( E. coli \) was analyzed by NaDodSO4/PAGE and subsequent staining with Coomassie brilliant blue.

Site-Directed Mutagenesis of the Codon for the Histidine-63 Axial Ligand. Plasmids carrying the synthetic gene for the soluble catalytic core of cytochrome \( b_5 \) were exhaustively digested with Bal I and \( Xho \) I. Oligodeoxyribonucleotides constituting both strands of the DNA region from Bal I to \( Xho \) I (Fig. 1) with an altered codon at position 63 were annealed and ligated into the double-digested plasmid. Transformant \( E. coli \) cells were characterized by the presence of plasmid DNA that lacked the Bal I restriction site from the construction and the mutant DNA sequence confirmed by Sanger sequencing (18).

RESULTS AND DISCUSSION

The gene coding for rat hepatic form of microsomal cytochrome \( b_5 \) was chosen for synthesis to take advantage of the detailed full-length sequence data available from the Ozols laboratory (12). For the soluble core of cytochrome \( b_5 \), synthetic oligodeoxyribonucleotides ranging in length from 28 to 48 base pairs were designed to comprise both sense and anti-sense strands of the engineered DNA segments as shown in Fig. 1 and as described in Methods. In addition to circumventing lengthy mammalian cDNA cloning and subsequent gene isolation procedures, this total synthetic approach allows the design of a gene suitable for expression in \( E. coli \) by including optimal sequences for a ribosome binding site complementary to the 16S \( E. coli \) tRNA and the spacer and initiator methionine sequences previously shown by us to favor high levels of expression of the \( Pseudomonas \) cytochrome P-450cam gene in \( E. coli \) (19). In addition, the direct synthesis of the entire cytochrome \( b_5 \) gene provides the opportunity to choose codon sequences that are optimal for the \( E. coli \) translation apparatus and enables the introduction of a variety of unique restriction sites bordering particular areas of interest within the gene, thus allowing subsequent facile mutagenesis of these target regions in the protein structure. Oligodeoxyribonucleotides corresponding to three major divisions of the cytochrome \( b_5 \) gene were separately annealed, ligated, and cloned into \( E. coli \) using a pUC vector system as described in Methods. These three fragments from the separate clones were then combined, and further transformed into the same cell line. Colonies were screened for retention of \( Sal \) I, \( Bal \) I, \( Xho \) I, \( Pst \) I, and EcoRI sites and by pairwise restriction mapping. The selected plasmid insert was completely sequenced by the Sanger technique after subcloning into M13. Transformation of a recombinant plasmid containing the complete correct DNA sequence for the soluble fraction of cytochrome \( b_5 \), corresponding to the
protease solubilized fraction from hepatic microsomal membranes as shown in Fig. 1, results in the growth of visibly red E. coli cells due to the production of cytochrome b₅ protein that constitutes >8% of the total cellular protein. This protein is found entirely in the cytoplasmic cellular fraction (Fig. 2) and is purified by homogeneity according to the procedures described in Methods. The resulting homogenous cytochrome b₅ has all the physical properties of the protein isolated from hepatic microsomes, including an oxidized protein Soret band at 410 nm with an extinction coefficient of 130 M⁻¹cm⁻¹, a reduced protein Soret band of increased intensity at 423 nm with an extinction coefficient of 195 M⁻¹cm⁻¹, and visible bands at 555 and 527 nm, respectively (Fig. 3). The absorption difference spectrum between the oxidized and reduced forms of the engineered gene product also agrees with that of the tryptic cytochrome b₅ purified from mammalian tissue, having a characteristic minimum at 409 nm and a maximum at 424 nm with a differential extinction coefficient of 190 M⁻¹cm⁻¹. The synthetically constructed soluble segment of cytochrome b₅ is predicted to have a molecular weight of 13,603 (98 amino acids).
acids plus the heme prosthetic group), which is confirmed by gel electrophoretic analyses of the purified protein isolated from transformed E. coli cells (data not shown). Sequential Edman degradation of 4 nmol of the purified protein by an Applied Biosystems sequencer yields an alanine amino-terminal (3 nmol residue and the 13 subsequent residues Glu (1.4 nmol), Gln (2 nmol), Ser (0.2 nmol), Asp (0.7 nmol), Lys (2.5 nmol), Asp (0.9 nmol), Val (2.7 nmol), Lys (2.0 nmol), Tyr (1.3 nmol), Thr (1.0 nmol), Leu (2.8 nmol), and Glu (1.1 nmol) that are identical with the native rat liver cytochrome b5 sequence, demonstrating that a lac fusion protein is not produced and that there is a complete removal of the formyl methionine initiator codon. Total amino acid analysis confirms the expected amino acid composition predicted by the coding sequence. The purified protein is low spin in the ferric resting state, being characterized by ESR g values of 1.41, 2.22, and 3.05—completely identical to values in the literature.

Full understanding of the molecular basis for electron transfer and protein-protein interaction between cytochrome b5 and its protein electron transfer partners requires studies with the complete cytochrome b5 protein, including the hydrophobic membrane domain. We thus extended our gene synthesis effort with eight additional oligodeoxyribonucleotides (Fig. 4) to generate the coding sequence for the entire microsomal membrane protein. Our approach utilized the Xho I restriction endonuclease site of the synthetic soluble domain to accommodate oligodeoxyribonucleotides comprising the coding sequence of the membrane anchor and providing a unique Bcl I restriction site at the junction of the two domains prior to translation termination (Fig. 4). The complete cytochrome b5 construction was verified by Sanger sequencing.

Cells transformed with recombinant plasmids containing the complete cytochrome b5 gene express ~1/10th as much cytochrome b5 as cells containing the synthetic gene for the soluble domain of cytochrome b5, as judged by whole-cell optical spectroscopy and NaDODSO4/PAGE. NaDODSO4/PAGE analyses of subcellular fractions of cells expressing this complete gene for mammalian cytochrome b5 establish that much of the complete protein product copurifies with the bacterial membrane, although some of the expressed protein is also found in the cytoplasmic fraction (Fig. 2). Detergent solubilization and purification readily purifies this membrane-associated form of cytochrome b5.

The successful engineering, cloning, and expression of a gene for both the soluble catalytic core segment and the complete membrane-associated form of cytochrome b5 provides an excellent means for obtaining large amounts of purified protein for biophysical and chemical studies. More importantly, this method of genetic engineering allows us to clone and express a wide variety of mutant cytochrome b5 structures with specifically altered amino acids. Primary targets for our initial mutagenesis studies include substitution of the histidine axial ligands (histidine-39 and histidine-63), alteration of surface charge distributions, modification of the hydrophobic membrane anchor domain, and modification of residues that potentially control the redox potential and electron transfer rate in this important cytochrome of mammalian physiology.

To demonstrate the utility of this approach, we chose to alter the histidine axial ligand at amino acid position 63. A prime consideration in selecting the amino acid to be replaced at this site is the realization that altered amino acid side chains in an interior structure of a protein can potentially generate large interstitial spaces that can be occupied by solvent. This is particularly important near the regions of cytochrome b5 structure, which provide close van der Waals contact with the hydrophobic surface of the protoporphyrin IX prosthetic group. Replacement of histidine-63 with an alanine-63 by cassette mutagenesis, schematically illustrated in Fig. 1, yields a protein that fails to incorporate heme during cell fermentation and that is not reconstituted with exogenous heme after purification of the apoprotein. Mutant cytochrome b5 protein with a methionine substitution at position 63 served two experimental purposes depending on whether the methionine residue would form a functional coordinate bond to the heme iron. Examination of the native and proposed mutant structures of the catalytic core of cytochrome b5 on an IRIS 2400 molecular graphics system suggests that the methionine sulfur would be at too great a distance from the heme iron to form an axial ligand unless substantial movement of the prosthetic group were to take place. If sufficient structural relaxation did occur, a histidine-39–methionine-63 ligation scheme would mimic the ligand structure of the c-type cytochromes with their well-defined optical and magnetic resonance properties. The extra volume of the methionine side chain, however, should in any case tend to exclude water from the porphyrin surface and hence favor prosthetic group incorporation. Generation of a methionine-63 mutation of the soluble form of cytochrome b5 as described

![Fig. 4. Additional nucleotide sequence to attach the membrane anchor domain to the soluble fragment of cytochrome b5.](image-url)
in Methods resulted in the production of the apo form of the cytochrome in high yield. Purification of this apoprotein following the identical procedure for the wild-type holoprotein allowed facile reconstitution with heme to form the intact mutant protein. This methionine-63 cytochrome $b_5$ displayed an axial high spin ESR signal ($g = 6$) and optical spectra in the ferric form, which suggests that the methionine sulfur is not bonded to the heme iron. Consistent with this interpretation, the reduced protein was found to readily bind carbon monoxide with a 420 nm Soret maxima similar to that observed for myoglobin and hemoglobin. It thus appears that the methionine-63 protein is a stable five-coordinate heme protein. We are now in the process of replacing both histidine-63 and histidine-39 with a variety of amino acids with the goal of defining the structural features responsible for the control of carbon monoxide and oxygen binding affinity and reactivity.

**SUMMARY**

We have successfully demonstrated the feasibility of expressing a mammalian heme protein in *E. coli* via total gene synthesis. Specifically, the synthetic gene coding for the soluble heme-containing domain of cytochrome $b_5$ is expressed in high yields to produce intact enzyme that demonstrates physical properties identical to the corresponding mammalian liver microsomal protein. Moreover, the efficient synthesis of the holoenzyme dictates that the *E. coli* heme synthesis machinery is able to provide the additional quantities of the heme prosthetic group necessary. Analyses of subcellular fractions of *E. coli* cells show that the synthetic heme-containing soluble segment of cytochrome $b_5$ resolves in the cytoplasmic fraction with no evidence for inclusion body formation. It thus appears that proper choice of codons to match the tRNA pools of the host organisms is an important aspect of heterologous protein expression. Neither protein nor message stability appears to limit holoprotein production.

Attachment of the membrane anchor fragment to the soluble cytochrome $b_5$ generates an intact cytochrome that is primarily inserted into the membrane fraction of *E. coli*. This represents the first example of the incorporation of a foreign mammalian membrane protein into a bacterial bilayer compartment. At present, the reason for the somewhat lower expression of the complete cytochrome $b_5$ gene is not understood.

The incorporation of restriction sites surrounding the heme axial ligands of cytochrome $b_5$ has allowed conversion of this normal six-coordinate low-spin heme protein to a five-coordinate high-spin cytochrome by replacing histidine-63 with a methionine ligand. This modified protein is able to reversibly bind exogenous ligands such as carbon monoxide. The ability to alter metal ligation geometry in metalloproteins opens up the exciting possibility of understanding the role of ligand geometry and type in determining metal site reactivity and physical and spectroscopic properties.

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