Expression of Cytochrome \( b_5 \) in Yeast and Characterization of Mutants of the Membrane-anchoring Domain*

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The soluble and membrane-bound forms of the synthetic rat cytochrome \( b_5 \) gene have been expressed in Saccharomyces cerevisiae. In order to examine the topology and function of the COOH-terminal membrane binding domain of cytochrome \( b_5 \), mutants have been constructed, expressed, purified, and partially characterized. Pro-115 is located in the middle of the putative \( \alpha \)-helical membrane-anchoring domain of cytochrome \( b_5 \) and has been hypothesized to give rise to either a hairpin-like loop or \( \pm 26^\circ \) kink in the helix, depending on whether it exists, respectively, in the cis or trans configuration. The Pro-115 \( \rightarrow \) Ala mutant, which is expected to have a straight transmembrane helix, inserted normally into the endoplasmic reticulum and exhibited wild type levels of activity in yeast microsomes and in vitro in the cytochrome P-450 mixed function oxidation system. Since a hairpin structure does not appear to be essential, it is likely that the membrane binding domain of cytochrome \( b_5 \) spans the membrane.

Characterization of the truncated cytochrome \( b_5 \) molecule, Pro-115 \( \rightarrow \) Stop, lacking 19 amino acids at the COOH terminus indicates that the distal part of the membrane binding domain of cytochrome \( b_5 \) is necessary for in vitro binding to the endoplasmic reticulum and for functioning with its membrane-associated electron transfer partners.

Replacement of Ser-104 to Met-125, the putative membrane-anchoring domain of cytochrome \( b_5 \), with 22 leucine residues results in a protein which targets to the endoplasmic reticulum but the extent of its reduction is only 50% of that of the wild type in yeast microsomes. In vitro, the polyleucine mutant is unable to support substrate oxidation by cytochrome P-450.

The mutation of Ala-131 and Glu-132, amino acids flanking the transmembrane domain, to lysines resulted in a protein with normal membrane topology and function.

Cytochrome \( b_5 \) is an amphipathic protein which contains two domains; one is a water-soluble heme binding domain and the second is a hydrophobic membrane-anchoring domain. The membrane-bound cytochrome \( b_5 \) is a component of the NADH-dependent \( \Delta 9 \) stearyl-CoA desaturase system (Strittmatter et al., 1974; Oshino, 1982). With selected substrates cytochrome \( b_5 \) may also provide the second electron to cytochrome P-450 for its NADPH-dependent mixed function oxidations (Hildebrandt and Estabrook, 1971). In this regard it has been demonstrated both in a reconstituted system and intracellularly that the oxidation of certain substrates, for example the anesthetic methoxyflurane, proceeds at an appreciable rate only in the presence of cytochrome \( b_5 \) (Canovadavis and Waskell, 1984; Aoyama et al., 1990; Vatsis et al., 1982). The soluble form of cytochrome \( b_5 \) is found in red blood cells, where it maintains hemoglobin in a reduced state (Hegesh et al., 1986).

The structure and topology of the membrane-anchoring domain of cytochrome \( b_5 \) is unknown. Thermodynamic considerations predict that the generally large and unfavorable free energy of burying charged and polar amino acids will result in their infrequent occurrence in the hydrophobic membrane bilayer. Another consideration is hydrogen bond formation. In order to remove a polypeptide chain from water and insert it into the lipid bilayer, the carbonyl and amino groups of the protein must be maximally hydrogen-bonded to one another if the change in free energy accompanying insertion is to be favorable (Singer, 1990). The simplest structure that forms the maximum number of hydrogen bonds is the \( \alpha \)-helix; in contrast, in a two-stranded anti-parallel \( \beta \) structure only one-half of the carbonyl and amino groups are hydrogen-bonded to one another. These considerations led Singer (1971) to suggest that the membrane binding regions of proteins "may all be largely in the \( \alpha \)-helical conformation." In the two membrane proteins, photosynthetic reaction center and bacteriorhodopsin, where relatively high resolution structural information exists, this prediction has proven to be correct (Deisenhofer et al., 1985; Henderson et al., 1990). However, there are reliable, albeit low resolution, crystallographic data which suggest the presence of \( \beta \) structure in the porins. Each porin molecule consists of a 16-stranded \( \beta \)-barrel which traverses the membrane as a tube (Jap, 1989; Weiss et al., 1990).

A hydrophobic plot of the cytochrome \( b_5 \) sequences demonstrates a single hydrophobic stretch of 23 amino acids at the COOH terminus which has been proposed as the membrane anchor of the protein (Ozols, 1989). The membrane binding domain of microsomal cytochrome \( b_5 \) has been proposed to be a conventional transmembrane \( \alpha \)-helix (Holloway and Buchheit, 1990; Takagaki et al., 1983a, 1983b). However, other investigators (Ozols, 1989; Dailey and Strittmatter, 1978; Enoch et al., 1979) have proposed that the carboxyl and amino termini are on the cytoplasmic side due to a hairpin loop in the \( \alpha \)-helical hydrophobic segment (Fig. 1). The corollary is that the hydrophobic domain does not extend into the lumen of the endoplasmic reticulum.

In order to investigate the topology of the membrane anchor...
of cytochrome $b_5$, Pro-115 was mutated using site-directed mutagenesis to the helix forming residue alanine. The cis-trans conformation at the X-Pro-115 bond should determine whether the COOH terminus is on the cytoplasmic (cis-X-Pro) or luminal (trans-X-Pro) side of the endoplasmic reticulum. Herein it is demonstrated that the Pro-115 $\rightarrow$ Ala mutant protein is indistinguishable from the native protein and therefore most likely exists in the transmembrane configuration.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Expression of Rat Apocytochrome $b_5$ in *S. cerevisiae*—Although numerous water-soluble eukaryotic proteins including a soluble form of cytochrome $b_5$ have been overexpressed in *Escherichia coli*, it is not usually possible to overexpress eukaryotic membrane proteins to the same extent as soluble proteins. For example, in *E. coli* the amphipathic form of cytochrome $b_5$ is only expressed at one-tenth the level of the soluble protein and is difficult to purify as an intact form (Beck von Bodman et al., 1986). The low yield of cytochrome $b_5$ in *E. coli* may be partially attributed to the fact that bacteria do not have an endoplasmic reticulum, with the result that intracellular processing is unlikely to be optimal. *S. cerevisiae* was therefore chosen as the expression vector. The synthetic genes of the soluble and membrane-bound forms of the rat cytochrome $b_5$ (Beck von Bodman et al., 1986) were cloned into a high copy plasmid of the yeast shuttle vector (pCGY1444) between the inducible yeast metallothionein (also known as chelatin) promoter and the phosphoglycerokinase transcription terminator (Etcheverry, 1990; Butt and Ecker, 1987; Etcheverry et al., 1986).

Expression of apocytochrome $b_5$ could be directly evaluated by SDS-polyacrylamide gels, since there was only a small amount of yeast protein that co-migrated with the expressed forms of the protein (Fig. 2). Following copper addition, the time course of expression of cytochrome $b_5$ was determined by visual inspection of the SDS gels and confirmed by scanning densitometry. The membrane bound and soluble forms of cytochrome $b_5$ are expressed to a similar extent; both bands were clearly among the most intense in the yeast cell extracts. At the time of copper addition cytochrome $b_5$ was not visible by Coomassie Blue staining (lane 2) and the protein pattern by SDS-polyacrylamide gels, since there was only a small amount of yeast protein that co-migrated with the expressed forms of the protein (Fig. 2). Following copper addition, the time course of expression of cytochrome $b_5$ was determined by visual inspection of the SDS gels and confirmed by scanning densitometry. The membrane bound and soluble forms of cytochrome $b_5$ are expressed to a similar extent; both bands were clearly among the most intense in the yeast cell extracts. At the time of copper addition cytochrome $b_5$ was not visible by Coomassie Blue staining (lane 2) and the protein pattern.

**Fig. 1. Model of the anchor of cytochrome $b_5$.** A, transmembrane form; B, hairpin loop form.

**Fig. 2. Expression of the membrane-bound and soluble forms of cytochrome $b_5$ in yeast.** Cells were grown and lysed as described under “Experimental Procedures.” Each well contains the lysate of $2 \times 10^6$ cells loaded on to a 13.5% SDS-polyacrylamide gel which was stained with Coomassie Blue. The arrowheads indicate the position of the expressed proteins. A, analysis of the expression of the membrane-bound form of cytochrome $b_5$. Lane 1, inv memb-bs 20B-12 which does not produce cytochrome $b_5$. Lane 2, memb-bs 20B-12 minus copper. Lanes 3-6, strain memb-bs 20B-12 with addition of 0.3 mM copper. Cells were harvested at 2.5 h (lane 3), 12 h (lane 4), 24 h (lane 5), and 32 h (lane 6) after addition of the copper to the growth medium. B, analysis of the expression of the soluble form of cytochrome $b_5$. Lane 1, inv memb-bs 20B-12, which does not produce $b_5$. Lane 2, sol-bs 20B-12 minus copper. Lanes 3-6, sol-bs 20B-12 with addition of 0.3 mM copper. Cells were harvested at 2.5 h (lane 3), 12 h (lane 4), 24 h (lane 5), and 32 h (lane 6) after addition of the copper.

1 Portions of this paper (including “Experimental Procedures” and Table 1S) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.
The amount of cytochrome bs remained stable until approximately 48 h after induction with copper. By 72 h the cytochrome bs concentration had begun to decrease presumably as a result of proteolysis in the stationary phase yeast (data not shown).

The number of molecules of apocytochrome bs expressed in a single yeast cell was quantitated on a Western blot (Fig. 3). The primary antibody was a mouse monoclonal IgG2 antibody which was raised against the membrane-bound form of rabbit cytochrome bs. Lane 2 of Fig. 3 shows only a single band migrating at the molecular weight of cytochrome bs. Approximately 1.8 x 10⁶ molecules of the membrane-bound cytochrome bs are expressed per cell, indicating that 45 mg of cytochrome bs are produced per liter of culture (1 x 10⁶ cells/ml). This is a greater than 20-fold increase over uninduced cells in which cytochrome bs could not be detected on the Western blot. Interestingly, the monoclonal anticytochrome bs antibody did not react on the Western blot with the soluble form of cytochrome bs (Fig. 3, lane 1) even though the SDS-polyacrylamide gel in Fig. 2B indicates it is expressed to approximately the same extent as the amphipathic form. This lack of reactivity of the monoclonal anticytochrome bs antibody with the soluble form of cytochrome bs made it impossible to use a Western blot to calculate the number of soluble cytochrome bs molecules found in a yeast cell. Also note that under the conditions of this experiment endogenous yeast cytochrome bs does not react with the antibody (Fig. 3, lane 1).

Expression of Rat Holocytochrome bs in S. cerevisiae—In order to determine the optimal time to harvest yeast expressing cytochrome bs, the concentration of functional heme-containing cytochrome bs was measured spectrophotometrically at various time periods following induction by copper (Fig. 4). To our knowledge, copper does not interfere with either heme or heme protein synthesis. In the absence of added heme the concentration of membrane-bound cytochrome bs was at a maximum between 24 and 48 h with peak levels of 3 x 10⁶ molecules/cell. Thereafter, the amount of holocytochrome bs slowly declined, decreasing by about 35% by 72 h. The soluble form of cytochrome bs was maximally expressed at 4-6 x 10⁶ molecules/cell and exhibited a similar time course (data not shown). The discrepancy between the amount of apocytochrome bs (1.8 x 10⁶ molecules/cell) and the holoprotein (3 x 10⁶ molecules/cell) indicates that approximately 80% of the expressed protein is apoprotein in the absence of added heme. Since addition of hemin (final concentration in growth medium 13 µg/ml) increased the concentration of holocytochrome bs by an estimated 20-50%, it was routinely added to the culture medium. The effect of the hemin addition could not be more precisely determined because excess hemin interferes with the spectroscopic determination of cytochrome bs.

Intracellular Localization and Concentration of Expressed Cytochrome bs—Yeast cells were fractionated into cytosolic and microsomal membrane fractions and examined by polyacrylamide gel electrophoresis. As expected, the soluble form of cytochrome bs localized to the cytosol while the amphipathic molecule was found in the microsomal membrane fraction (Fig. 5, lanes 3 and 7, respectively). The spectrophotometrically measured concentration of membrane-bound cytochrome bs in the microsomal membrane fraction was 1.0-1.5 nmol/mg microsomal protein, as determined by dithionite reduction. This is the level achieved in mammalian liver microsomes. However when NADH was used as the reductant, only 0.4-0.7 nmol of cytochrome bs/mg of microsomal protein was observed. Previous investigators (Ishidate et al., 1969) have also noted that NADH only partially reduces the cytochrome bs in yeast microsomal membranes. The control microsomes from strain inv memb-bs, 20B-12 contained less than 15% (determined by dithionite) of the amount of heme protein found in the strain expressing cytochrome bs.

The concentration of cytochrome bs in yeast microsomes expressing the membrane-bound cytochrome bs was determined by Western blot (Fig. 6) to be 1.3 nmol/mg microsomal protein. This is the same amount which was determined spectrophotometrically using dithionite as the reductant indicating that most of the cytochrome bs in the microsomal function is in the holomembrane form. Lanes 1 and 2 of Fig. 6 contain...
brane-bound cytochrome b. Nonetheless, each strain exhibits a faint
protein. The Ala-116 Pro mutant is less immunoreactive
Pro-Mutants—These mutant proteins were expressed, purified, and characterized to investigate both the topology of the membrane-anchoring domain of cytochrome b₅ in the endoplasmic reticulum and the role of Pro-115 in the interaction of the protein with its membrane-bound electron transfer partners. The results are summarized in Table I. The Pro-115 → Ala and Ala-116 → Pro mutants, which are both expected to have an essentially helical linear membrane spanning domain (see Fig. 1 and "Discussion"), are expressed to the same extent as the wild type protein and targeted appropriately to the yeast endoplasmic reticulum. Fig. 7 (Pro-115 → Ala, lanes 4 and 5; Ala-116 → Pro, lanes 6 and 7) demonstrates that both proteins remain associated with microsomes even in the presence of high pH (0.1 M sodium carbonate, pH 11.5), which is consistent with the notion that they are integral membrane proteins. In the endoplasmic reticulum the proteins are reduced by NADH, presumably via the yeast cytochrome b₅ reductase to the same extent as the native protein. The fact that the mutants function in vivo in a manner indistinguishable from the wild type protein provides strong circumstantial evidence for the equivalence of their structures. The purified mutant proteins also functioned normally in vitro as assessed by their ability to support the cytochrome b₅-dependent cytochrome P-450-catalyzed metabolism of methoxyflurane. This latter result indicates that the mutant cytochrome b₅ molecules receive electrons from cytochrome P-450 reductase and in turn provide an electron to cytochrome P-450 at a rate which does not limit the speed of the overall reaction. Considered as a whole our findings indicate that neither the cis/trans isomerization of Pro-115 nor the presence of a kink at this position is important for membrane insertion or functioning in electron transfer reactions either in a membrane or a reconstituted system. In contrast to the normal reactivity of the mutant proteins in vivo and in vitro, their interaction on a Western blot with the monoclonal anti-cytochrome b₅ antibody was distinctly different from that of the wild type protein. The Ala-116 → Pro mutant is less immunoreactive whereas the Pro-115 → Ala protein reacts more strongly than the wild type protein, suggesting that Pro-115 and Ala-116 are determinants of the structure of the cytochrome b₅ epitope which binds to the monoclonal antibody (Table I).

Pro-115 → Stop Mutant—Several years ago Dailey and Strittmatter (1978, 1981) generated and studied a cytochrome b₅ molecule which had been shortened proteolytically by 18 amino acids at the COOH terminus resulting in a peptide with Pro-115 as its COOH-terminal residue. They were able to demonstrate that the shortened protein bound to phospholipid vesicles in a "loose" binding form but nonetheless was able to support the catalytic activity of stearyl-CoA desaturase. We have expressed, purified, and partially characterized...
a very similar protein (Pro-115 → Stop), which is shorter by 1 amino acid, in an attempt to define which residues on the membrane binding domain are determinants of its function. Results of the characterization are presented in Table I.

Expression of the mutant protein is intermediate between that of the soluble and amphipathic form of cytochrome b₅. Approximately 63% of the total amount of the expressed protein was found in the cytosol with the remainder associated with microsomes. Although the truncated protein occurred at 58% of the wild type level in yeast microsomes, it could not be reduced by cytochrome b₅ reductase in the presence of NADH. The protein also dissociated from the membrane in the presence of high pH (Fig. 7, lanes 13 and 14), indicating that the majority of the Pro-115 → Stop mutant protein is localized to the cytosol, while the remainder binds to the microsomes as a nonfunctional protein. In a purified reconstituted cytochrome P-450 system the mutant protein had minimal activity (20% of native protein), presumably because it was unable to bind to its electron transfer partners. It was also unreactive with the monoclonal antibody on a Western blot, suggesting that the distal nonpolar domain constitutes a portion of the protein epitope which reacts with the antibody.

**Table I**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Whole cell expression</th>
<th>Cellular localization</th>
<th>Function in reconstituted P-450 system</th>
<th>Reactivity with anti-b₅ monoclonal antibody</th>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<tr>
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<td>ND</td>
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</tr>
<tr>
<td>Ala-131 → Lys, Glu-132 → Lys</td>
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<td>121</td>
<td>0.56</td>
<td>0.08</td>
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<tr>
<td>Polyleucine</td>
<td>94</td>
<td>65</td>
<td>0.25</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Determined spectrophotometrically in cell suspension. 100% level in wild type is 3 × 10⁵ molecules/cell.

* The microsomes and cytosol were isolated as described under “Experimental Procedures.” The specific content of cytochrome b₅ in microsomes is 1.03 nmol of b₅/mg of microsomal protein (100% level reduced by dithionite) and 0.48 nmol of b₅/mg of microsomal protein (100% level, NADH reduced). The concentration of cytochrome b₅ in the cytosol (nmol of b₅/mg of cytosolic protein) was determined following reduction with dithionite.

* The ratio of the amount of cytochrome b₅ reduced by NADH versus dithionite.

* The ability of each form of cytochrome b₅ to support methoxyflurane metabolism in a reconstituted system containing cytochrome P-450 and cytochrome P-450 reductase was measured as described under “Experimental Procedures.” The 100% level of fluoride ion production in the presence of active cytochrome b₅ is 17.8 nmoles of F⁻ produced/nmol of cytochrome P-450/30 min.

* The Western blots were performed as described under “Experimental Procedures.” The reactivity was scored by visual inspection of the blot and is semiquantitative.

* Not determined.

the intracellular processing and in vitro properties of the mutant protein.

A summary of our findings is presented in Table I. Briefly, the mutant protein is expressed to the same extent as the wild type protein and is appropriately targeted to the endoplasmic reticulum. Within the endoplasmic reticulum it functions with its native electron transfer partner and hence must have the unaltered native transmembrane orientation with the heme-containing domain in the cytosol. In the presence of alkaloids (Fig. 7, lanes 9 and 10), which transforms microsomes into open sheets and releases luminal and peripheral membrane proteins, the mutant behaves as an integral membrane protein. In vitro the mutant protein stimulates the activity of the mixed function oxidase system with methoxyflurane as the substrate to the same extent as the wild type protein. On a Western blot it reacts normally with the monoclonal antibody to cytochrome b₅.

**Polyleucine Mutant:** (Ser-104 to Met-125) → Leu, Tyr-126 → Ala — The hydrophobic part of the cytochrome b₅ molecule anchors the hydrophilic heme-containing catalytic domain of the protein to the membrane, thereby increasing its effective local concentration and enabling the protein to undergo translational diffusion in the plane of the membrane. This arrangement also results in optimal spatial orientation for interactions between the cytoplasmically located catalytic domains of cytochrome b₅ and its numerous electron transfer partners (Dailey and Strittmatter, 1978). It is possible that this is the sole function of the membrane anchor of cytochrome b₅. However, the remarkable conservation of the amino acid sequence in the membrane binding segment of cytochrome b₅ (Ozols, 1989) suggests that it may also participate in direct specific protein-protein interactions particularly with its integral membrane electron transfer partners such as cytochrome P-450 and stearoyl-CoA desaturase.

In order to determine whether the hydrophobic anchor of cytochrome b₅ is involved in amino acid-specific intramembrane protein-protein interactions, or merely provides a non-specific hydrophobic membrane binding segment, the putative transmembrane domain of cytochrome b₅ residues 104–125, was replaced by 22 consecutive leucine residues. In this poly-
the experimental error. In support of this conclusion is the polyleucine mutant and the wild type protein are similar. The presence of cytochrome bs in the microsomes was analyzed by SDS-polyacrylamide gel electrophoresis on a 13.5% gel. A volume of microsomal proteins (treated at pH 7.4 or 11.5) corresponding to an initial amount of 40 µg of protein was analyzed on the gel. Microsomes from wild type and mutant strains treated at pH 7.4 or 11.5 are juxtaposed: bs wild type (lane 2, pH 7.4; lane 3, pH 11.5); Pro-115 → Ala (lane 4, pH 7.4; lane 5, pH 11.5); Ala-116 → Pro (lane 6, pH 7.4; lane 7, pH 11.5); Ala-131 → Lys, Glu-132 → Lys (lane 9, pH 7.4; lane 10, pH 11.5); Pro-115 → Stop (lane 13, pH 7.4; lane 14, pH 11.5); polyleucine mutant (lane 15, pH 7.4; lane 16, pH 11.5). The arrows indicate the position of the wild type and mutant cytochrome bs. Lanes 1 and 8 contain molecular weight markers (see Fig. 5).

leucine mutant Tyr-126 was changed to an alanine in the course of generating an XhoI restriction site where the DNA coding for polyleucine could be inserted. Our findings are summarized in Table I. The total cellular expression of the polyleucine mutant protein could not be detected spectrophotometrically in the cytosol. Only 25% of the polyleucine cytochrome bs is reduced by NADH in yeast microsomes (versus 50% for the wild type) suggesting a diminished capacity of this mutant protein to be reduced by its electron transfer partner cytochrome bs reductase (Table I). In the reconstituted mixed function oxidase system the polyleucine mutant protein is unable to support the oxidation of the anesthetic methoxyflurane. When microsomes containing the polyleucine mutant protein (Fig. 7, lanes 15 and 16) and the partially purified protein were loaded onto SDS-polyacrylamide gels the mutant protein migrated at an anomalously high molecular weight (22,500). The apparent high molecular weight of the mutant protein is likely to be an additional manifestation of the known anomalous behavior of the hydrophobic domain of the wild type protein with SDS (Robinson and Tanford, 1975). A second anomaly was observed with this mutant protein. When equal amounts of the wild type and polyleucine protein were loaded onto SDS-polyacrylamide gels, the intensity of the Coomassie Blue staining band of the polyleucine mutant protein was always less than that of the wild type protein and exhibited far greater variability. Whether this is due to a diminished staining and/or a decreased entry of the very hydrophobic protein into the gel is unclear. In spite of the anomalous behavior, the mutant protein could be detected in microsomal membranes treated at high pH (Fig. 7, lane 16), and we therefore conclude that the polyleucine mutant protein is an integral membrane protein (Spiess and Handschin, 1987).

The anti-cytochrome bs monoclonal antibody is completely unreactive with the protein on a Western blot. A monoclonal antibody against the polar heme containing domain is currently being isolated and may help solve some of the ambiguities.

**DISCUSSION**

Pro-115 → Ala and Ala-116 → Pro—What does the finding that the Pro-115 → Ala and Ala-116 → Pro mutants are indistinguishable from the wild type protein in all *in vivo* and *in vitro* variables investigated allow us to conclude about the topology of the membrane anchor of cytochrome bs? Basically, we want to know whether the membrane-anchoring domain spans the membrane or only goes half-way through and bends back on itself (see Fig. 1). Although thermodynamic considerations (Singer, 1971) lead to the conclusion that the hydrophobic domain of cytochrome bs should be incorporated as a single transmembrane α-helix, there is no compelling thermodynamic argument against the helix proceeding only one-half of the way through the bilayer and turning back on itself, provided too many hydrogen bonds are not broken. However, examples of membrane binding helices that loop back on themselves are only known from amphipathic helical presequences of mitochondrial proteins (Pfanner and Neupert, 1990). The membrane anchor of cytochrome bs is not amphipathic.

In the course of attempting to decipher the underlying principles and patterns of the conformation of proteins, investigators (Richardson and Richardson, 1989; Barlow and Thornton, 1988) observed that proline is the least frequently occurring amino acid in helices and that 10% of the time it occurs in the cis conformation which results in a turn in the protein. Thus it is rather surprising that prolines occur with relatively high frequency in the putatively α-helical transmembrane segments of many integral membrane proteins although its structural and functional role in the membrane is unknown (Williams and Deber, 1991).

By mutating Pro-115 to an alanine, a helix-forming amino acid, it is likely we generated a transmembrane helix. The Ala-116 → Pro mutation which gives rise to 2 prolines in a row should have an extended collagen-like conformation at these 2 residues which will interrupt the putative helix and lead to a minimally modified transmembrane α-helical anchor.
Mutants of the Membrane Anchor of Cytochrome b₅

(Richardson and Richardson, 1989; Bennett and Steitz, 1980). Both of these mutants should be similar in structure and presumably function to a wild type protein with a trans proline peptide bond but very different in structure and function compared to a wild type protein with a cis-X-Pro peptide bond which has its carboxyl terminus in the cytosol. There is no known mutant which we could make which would definitely cause a hairpin loop in the membrane anchor.

Membrane insertion of cytochrome b₅ into microsomes occurs posttranslationally in a signal recognition particle-independent manner following synthesis on cytosolic ribosomes without a transient amino-terminal signal sequence (Anderson et al., 1983; Okada et al., 1982; Rachubinski et al., 1980). The exact mechanism of cytochrome b₅'s insertion into the endoplasmic reticulum in vivo is unknown. Since spontaneous insertion without the aid of a specific membrane receptor would lead to intracellular chaos, we suggest that this mode of insertion is unlikely to exist in vivo (Enoch et al., 1977, 1979; George et al., 1989; Anderson et al., 1983; Rachubinski et al., 1980). The authors hypothesize that cytochrome b₅ is inserted into the endoplasmic reticulum either via an unknown membrane receptor or via a mechanism which bypasses the signal recognition particle but nonetheless uses the same multiprotein complex by which signal and internal signal sequences are incorporated into membranes (Rapoport, 1991). Our results, which reveal normal intracellular targeting and levels of expression of Pro-115 → Ala and Ala-116 → Pro, indicate that the putative intracellular machinery for membrane insertion of cytochrome b₅ does not require either a hairpin loop generated by cis proline at position 115 or the kink generated by a trans proline.

Our experiments also demonstrate that both mutant proteins were reduced in yeast microsomes by NADH to the same extent as the native protein. This requires normal diffusion in the plane of the membrane, proper orientation of the catalytic site of the electron donor and recipient at the membrane aqueous interface and perhaps direct protein-protein interactions between the nonpolar segment of cytochrome b₅ and a nonpolar region of the reductase as well. These results indicate that a loop (cis-X-Pro) or kink (trans-X-Pro) at Pro-115 is not necessary for the apparently normal functioning of the cytochrome b₅ membrane anchor during electron transfer. Studies of the kinetics of the reactions of the mutant cytochrome b₅ molecules with their electron transfer partners are being conducted to determine if subtle differences in electron transfer capabilities exist between the mutant and native proteins. Thus, our findings suggest that the membrane binding domain of cytochrome b₅ spans the membrane.

Pro-115 → Stop—Dailey and Strittmatter (1978, 1981) demonstrated that cytochrome b₅, lacking 6 and 18 of its COOH-terminal amino acids, respectively, was only able to bind to phospholipid vesicles and microsomes in a loose binding form in contrast to the tight binding exhibited by whole cytochrome b₅. Certainly, our results, which demonstrate that the Pro-115 → Stop protein is only peripherally associated with microsomes in vivo, are consistent with their findings. An interesting difference between their results and ours is that in vitro the protein truncated by 18 amino acids exchanges electrons with its physiological electron transfer partners cytochrome b₅ reductase and stearyl-CoA desaturase. In contrast Pro-115 → Stop is virtually inactive in a reconstituted system with its alternate electron transfer partner cytochrome P-450. It is conceivable that the different results are simply due to the fact that the different systems (desaturase and cytochrome P-450) used to assay cytochrome b₅ activity have unique structural requirements. An alternative explanation is that Pro-115 provides the critical hydrophobic binding energy necessary for interaction with the components of the desaturase system (Eisenberg et al., 1984). In addition, our previous results with the Pro-115 → Ala mutant, whose behavior is similar to that of the native protein, eliminate the possibility that Pro-115 provided a unique structural or chemical function with cytochrome P-450 and cytochrome P-450 reductase.

Ala-131 → Lys, Glu-132 → Lys Double Mutant—Although cytochrome b₅ does not require signal recognition particle for its insertion into the microsomal membrane (Okada et al., 1982; Rachubinski et al., 1980), its COOH terminus may nonetheless play a role in determining the topology and/or stability of the binding of the protein to the membrane (Daily and Strittmatter, 1981). The previously described results indicate that the protein of the double mutant, Ala-131 → Lys, Glu-132 → Lys, is incorporated into microsomes in the correct transmembrane orientation as an integral membrane protein, consistent with the conclusions of the recently published survey of Spiess and his colleagues (Beltzer et al., 1991). They concluded that although the transmembrane orientation of a protein is primarily determined by the charges flanking the transmembrane domain, it could not be reversed by changing only 2 charged residues at the carboxyl terminus of the transmembrane spanning segment. Our experiments indicate that in vivo Glu-132 is not necessary for incorporation of cytochrome b₅ into microsomes as an integral membrane protein. The exact relationship between our test of integral membrane incorporation, resistance to extraction from membrane at high pH, and Strittmatter's, namely ability of cytochrome b₅ to transfer from one membrane to phospholipid vesicles, is unknown (Dailey and Strittmatter, 1981). Thus their finding that derivatives of cytochrome b₅, shortened by 6 carboxyl-terminal amino acids or chemically modified to remove negative charges were bound in a loose form and could transfer to phospholipid vesicles and our results are not directly comparable, because we are not measuring the same process. The ability of the Ala-131 → Lys, Glu-132 → Lys protein to support in vitro cytochrome P-450-catalyzed oxidation and of the protein lacking 6 amino acids to support fatty acid desaturation (Dailey and Strittmatter, 1978) indicates that the anionic residues of the carboxyl terminus are not important for interacting with their electron transfer partners.

Polyleucine Mutant—The diminished capacity of the polyleucine mutant of cytochrome b₅ to be reduced in yeast microsomes and in a reconstituted in vitro mixed function oxidation system with cytochrome P-450 and cytochrome P-450 reductase suggests that the hydrophobic membrane anchor contains sequence-specific information which is necessary for interaction with its electron transfer partners. The caveat that must be applied to this conclusion is that the polyleucine protein tail may be so hydrophobic that the protein forms very tight aggregates and that the monomers of the aggregate are not really free to associate with other integral membrane proteins either in the lipophilic environment of the bilayer or in the reconstituted system in aqueous solution. The marked conservation of amino acid sequence in the hydrophobic transmembrane segment also supports the idea that individual amino acids function in specific protein-protein interactions. This is especially credible in light of the fact that in the photosynthetic reaction center amino acids exposed to the membrane bilayer are poorly conserved in contrast to the highly conserved residues involved in interprotein interactions (Yeates et al., 1987).

Acknowledgments—We thank Dr. Sligar for generously providing.
the synthetic genes for cytochrome b₅, Dr. Zoller for expertise in DNA manipulations, Dr. Vigne for preparing the monoclonal antibody against cytochrome b₅, and Ms. Wu for purifying NADPH-cytochrome P₄₅₀ reductase. We are grateful to Dr. Arnold Fallick of the University of California, San Francisco Mass Spectrometry Facility for obtaining the molecular weight of the expressed rat protein from an electrospray spectra using a FISONS/VG (Manchester, U.K.) Bio-Q mass spectrometer. The Mass Spectrometry Facility is supported by National Institutes of Health DRR Grant RR 01614 (to A. L. Burlingame, Principal Investigator). We also thank Corbin Krug for his expert editorial assistance in preparing this manuscript.

REFERENCES

Mutants of the Membrane Anchor of Cytochrome b

EXPERIMENTAL PROCEDURES

Materials

DNA restriction enzymes, plasmids, polymerases and ligases were from Bethesda Research Laboratories, Inc. (Gaithersburg, MD) or New England Biolabs, Inc. (Beverly, MA). Chemical reagents were purchased from Amresco, Inc. (Solon, OH). Chemicals and reagents for cell culture were purchased from Sigma Chemical Co. (St. Louis, MO). The plasmid, pJCS21, the helper plasmid, pMC190, and the E. coli strains 85717 and 85718 were obtained from Dr. Maxi Orzechowski, Centre d'Etudes Nucleaires, Grenoble, France. The E. coli strains were grown on Luria-Bertani (LB) plates or in LB medium at 37°C. The plasmid, pJCS21, was derived from pJCS121 (Kuwabara et al., 1989) modified in the polylinker and containing an MluI site of origin of replication at the same site. The nuclease domains of the DNA containing the seventh, thirteenth, and thirteenth amino acids of the cytochrome b5 were replaced, respectively, with the MluI and HindIII sites of the pJCS21 plasmid containing the cytochrome b5 gene. The mutated plasmids were then ligated into the polylinker and the unique origin is in a unique site and SPPSCTGGAGCT (M 100) universal sequencing primer. The ampicillin concentration was 50 mg/ml and 500 μl of ampicillin stock solution was added to 50 ml of Luria-Bertani (LB) medium. The bacterial culture was grown in a shaking incubator at 37°C in a dark incubator at 140 rpm. The bacterial culture was harvested by centrifugation at 10,000 g for 15 min and the cell pellets were suspended in PBS containing 0.1% Triton X-100. The cell suspensions were cleared by centrifugation at 10,000 g for 15 min and the cell pellets were suspended in PBS containing 0.1% Triton X-100. The cell suspensions were cleared by centrifugation at 10,000 g for 15 min and the cell pellets were resuspended in PBS containing 0.1% Triton X-100. The cell suspensions were cleared by centrifugation at 10,000 g for 15 min and the cell pellets were resuspended in PBS containing 0.1% Triton X-100. The cell suspensions were clear...