The Role of the Length and Sequence of the Linker Domain of Cytochrome \( b_5 \) in Stimulating Cytochrome P450 2B4 Catalysis*

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Cytochrome \( b_5 \) (cyt \( b_5 \)) is a 15-kDa amphipathic protein with a cytosolic amino-terminal catalytic heme domain, which is anchored to the microsomal membrane by a hydrophobic transmembrane \( \alpha \)-helix at its carboxyl terminus. These two domains are connected by an \( \sim 15 \)-amino acid linker domain, Ser\(^{30} \)–Asp\(^{104} \), which has been modified by site-directed mutagenesis to investigate whether the length or sequence of the linker influences the ability of cyt \( b_5 \) to bind ferric cytochrome P450 2B4 and donate an electron to oxyferrous (cyt P450 2B4), thereby stimulating catalysis. Because shortening the linker by 8 or more amino acids markedly inhibited the ability of cyt \( b_5 \) to bind cyt P450 2B4 and stimulate catalysis by this isozyme, it is postulated that 7 amino acids are sufficient to allow a productive interaction. All mutant cyts \( b_5 \) except the protein lacking the entire 15-amino acid linker inserted normally into the microsomal membrane. Alternatively, lengthening the linker by 16 amino acids, reversing the sequence of the amino acids in the linker, and mutating conserved linker residues did not significantly alter the ability of cyt \( b_5 \) to interact with cyt P450 2B4. A model for the membrane-bound cyt \( b_5 \)-cyt P450 complex is presented.

**Reaction 1**

\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ 
\]

Microsomal cytochrome \( b_5 \) (cyt \( b_5 \))\(^1 \) is an amphipathic electron transfer hemoprotein located in the membrane of the endoplasmic reticulum. It provides electrons for a broad range of reactions, including fatty acid desaturation, cholesterol biosynthesis, and a variety of cytochrome P450-dependent oxidation and hydroxylation reactions (1, 2).

Cytochrome P450 (cyt P450) is responsible for the oxidation of a large number of substrates. The overall stoichiometry of the reaction catalyzed by cyt P450 is shown in Reaction 1, where RH is the substrate (3).

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\(^1\) The abbreviations used are: cyt \( b_5 \), cytochrome \( b_5 \); cyt P450, cytochrome P450; RSL, reverse sequence linker; MALDI, matrix-assisted laser desorption/ionization; DLPC, dilauroyl phosphorylcholine; bp, base pair(s).

The cyt P450 catalytic reaction occurs via a reaction cycle that involves substrate binding, reduction of the ferric heme, \( \text{O}_2 \) binding, reduction of the oxyferrous heme (second electron transfer), substrate oxidation, and finally product dissociation (4). Both ferric and oxyferrous cyt P450 receive electrons from NADPH via its redox partner, NADPH-cytochrome P450 reductase (cyt P450 reductase), whereas cyt \( b_5 \) can donate the second electron. Because of the high redox potential of \( \approx 20 \text{ mV} \) for cyt \( b_5 \), the first electron required to reduce cyt P450 from the ferric to the ferrous state is always transferred from NADPH via cyt P450 reductase. However, as the redox potential of cyt P450 increases from \( \approx -230 \text{ mV} \) to \( +50 \text{ mV} \) on transition to the oxyferrous state, the second electron can be obtained from either cyt P450 reductase or cyt \( b_5 \) (5, 6).

Depending on the substrate, isozyme of cyt P450 and the experimental conditions cyt \( b_5 \) can stimulate, inhibit, or have no effect on cyt P450 activity (2). Studies of the stoichiometry of the metabolism of benzphetamine and methoxyflurane by cyt P450 2B4 have shown that cyt \( b_5 \) increases the efficiency of catalysis by cyt P450 2B4 primarily by decreasing the formation of the side-product superoxide. However, cyt \( b_5 \) can also inhibit the rate of oxidation catalyzed by cyt P450, by competing with cyt P450 reductase. This effect of cyt \( b_5 \) is observed as a decrease in the overall rate of NADPH consumption (7).

Microsomal cyt \( b_5 \) is a 15-kDa protein consisting of two domains. The larger, heme-containing domain is hydrophilic and contains the binding site for its redox partners. The membrane-binding domain at the COOH terminus consists of a hydrophobic \( \alpha \)-helix that anchors the protein to the lipid bilayer of the endoplasmic reticulum (8–10). The two domains are connected by a highly flexible peptide chain of \( \approx 15 \) amino acids, which is assumed to allow the heme domain enough flexibility to bind different electron partners while ensuring that the protein remains localized at the endoplasmic reticulum. Solubilization of the cyt \( b_5 \) heme domain by removal of the membrane anchor through protease cleavage of the linker region generates a protein that is capable of electron transfer to soluble electron acceptor proteins such as cytochrome \( c \), metmyoglobin, or met-hemoglobin, but incapable of electron transfer to microsomal cyt P450 (1). This dramatic change in the properties of cyt \( b_5 \) upon elimination of its transmembrane domain indicates that it plays an essential role in the function of cyt \( b_5 \).

The role of the linker sequence in facilitating the ability of cyt \( b_5 \) to reduce cyt P450 2B4 is explored by altering the length and sequence of the flexible linker region between the heme domain and membrane anchor of cyt \( b_5 \). Deletion of 8–10 residues from the linker region of cyt \( b_5 \) impaired the ability of cyt \( b_5 \) to associate with and transfer electrons to cyt P450, whereas addition of 16 alanine residues to, or complete reversal of, the linker region did not affect the ability to form an active complex.
with cyt P450. These findings demonstrate that, although the sequence of the linker region can be varied without significant effect on the properties of cyt b5, the linker region has a critical minimum length of 6–8 residues.

EXPERIMENTAL PROCEDURES

Materials—T-3-Dilauroyl phosphatidylcholine was purchased from Doosan Serdary Research Laboratories (Toronto, Canada), and methoxyflurane was from Abbott Laboratories (Abbott Park, IL). Pfu Turbo DNA polymerase and DpnI were purchased from Stratagene (La Jolla, CA). Restriction enzymes EcoRI, AflIII, and Ncol were from New England Biolabs (Beverly, MA). C41 and C43 cells were from Avidis (Saint-Beauzire, France). Benzyloxyflurane was from Sigma. All other chemicals were purchased from Fisher.

Delineation and Alignment of the Linker Region of Cyt b5—The strategy for delineating the position and length of the cyt b5 linker region was to identify both the COOH terminus of the heme domain and the NH2 terminus of the membrane binding domain and consider the linker region to be the polypeptide chain connecting the two domains. The full amino acid sequence of rabbit cyt b5 was aligned with 16 other cyt b5 sequences obtained from the National Center of Biotechnology Information (NCBI) nucleotide data base using BLAST (11, 12). The multiple-sequence alignment program ClustalW was used to align the heme domains and identify conserved residues (13). The COOH terminus was defined by the amino acid sequence as determined by Edman degradation and known crystal and NMR structures of the solubilized heme domains of bovine, rat microsomal, and outer mitochondrial membrane cyt b5, and the NMR structure of bovine microsomal cyt b5 (8, 14–18).

To determine the NH2 terminus of the membrane binding domain, the program MPeX was used to predict the amino acids of the cyt b5 transmembrane helix (19–21). Using a sliding window of 19 amino acids, MPeX calculates the amount of free energy (\( \Delta G_{\text{Wocx}} \)) required to transfer the selected amino acid sequence from water into octanol. A negative value of \( \Delta G_{\text{Wocx}} \) indicates that the amino acid sequence will be stable in the =30-A hydrocarbon core of the lipid bilayer. After identifying the amino acids that are predicted to form the hydrophobic center of the transmembrane helix, the total length of the transmembrane helix was estimated by calculating the number of residues likely to interact with the interfacial regions of the membrane bilayer. The COOH terminus of the linker region was assumed to be in the ordered region of the heme domain. The NMR structure of bovine microsomal cyt b5 was used as a template to generate the deletion mutants 95–104, 93–104, and 90–104. This new plasmid contained the last 10 residues of the cyt b5 wild type linker region in reverse order. In the final step, a primer containing the DNA for the amino acid sequence Lys-Ser-Leu-Lys was inserted into plasmid pLW01-b5mem 91–101 to ultimately construct pLW01-b5mem 91–104 RSL, a plasmid containing the 14 residues of the wild type linker region in reverse order. The primers for each mutant are shown in Table I and had lengths of 41–44 bases.

Expression and Purification of Cyt P450 Reductase, Cyt P450 2B4, and Wild Type and Mutant Cyt b5—Cyt b5 was expressed and purified as previously described (23). Briefly, cultures of competent Escherichia coli C41 or C43 cells were prepared and the required plpW01 plasmid was inserted according to standard procedures (22, 24). Colonies were grown on LB agar plates containing 100 mg of cyt b5, the linker region Lys91–Asp104 reversed. Because a primer longer than 45 bp results in a decrease in the efficiency of annealing and in a lower rate of successful mutation, insertion of the 45 bp encoding the 15 amino acids of the reversed linker sequence was performed in three steps, where each step resulted in the sequential addition of 4–6 amino acids. The suffix RSL is used to denote a plasmid encoding the reverse sequence of the linker region between 91 and 104.

In the first step, the DNA primer containing the codons for the amino acid sequence Ser-Asp-Val-Thr-Thr-Ile was inserted into the plasmid pLW01-b5mem 90–104. This plasmid had been mutated to remove the entire linker region. Addition of six amino acids produced plasmid pLW01-b5mem 91–96RSL, \( \Delta G_{97–104} \) that contained a reinserted Ser90 in addition to the last 5 residues of the wild type linker region in reverse order. In the second step, the primer containing the codons for the amino acid sequence Leu-Thr-Glu-Met-Pro was inserted into the plasmid pLW01-b5mem 95–96RSL, yielding the plasmid pLW01-b5mem 91–100 RSL, \( \Delta G_{101–104} \). This new plasmid contained the last 10 residues of the cyt b5 wild type linker region in reverse order.

Expression and Purification of the Cyt b5 Reversal Mutant—The pLW01-b5mem 103–104 linker region was assumed to be in the ordered region of the heme domain. The NMR structure of bovine microsomal cyt b5 was used as a template to generate the deletion mutants 95–104, 93–104, and 90–104. To verify the primer had been successfully inserted without ancillary errors, the entire gene was sequenced by the DNA sequencing facility at the University of Michigan.

Constitution of Deletion and Substitution Mutants—The cDNA for wild type cyt b5 was used to generate the following substitution and deletion mutants: P95A and K94A, \( \Delta G_{95–104} \) and \( \Delta G_{90–104} \) mutant cyt b5. To check for the incorporation of the mutated primers in the purified plasmids of these three mutants, the EcoRI site was removed from the primer. Thus, when the plasmid was digested with EcoRI and AflIII, only the linearized DNA was observed instead of the two fragments observed with the cDNA of \( \Delta G_{97–104} \). To verify the primer had been successfully inserted without ancillary errors, the entire gene was sequenced by the DNA sequencing facility at the University of Michigan.

Constitution of a Mutant Cyt b5, in Which the Amino Acid Sequence of the Linker Region Has Been Reversed—To determine whether the sequence of the linker region was important for the function of cyt b5, a mutant cyt b5 was constructed that had the amino acid sequence of the cyt b5 linker region Lys91–Asp104 reversed. Additional alanines were then inserted after Leu92 at the N terminus of the cyt b5 linker region. An additional alanine was inserted into the linker region of the cyt b5 linker region.

Construction of Polyalanine Insertion Mutants—A total of 16 alanines were inserted into wild type cyt b5 to investigate the effect of lengthening the cyt b5 linker region. Initially, the plpW01-b5mem plasmid pLW01-b5mem 90–95 was used to prepare the plasmid pLW01-b5mem 90–99, where either 2 or 4 alanines were inserted after Val95 at the C terminus of the linker. The plasmid, pLW01-b5mem 103 + 4A, was used to construct pLW01-b5mem 103 + 8A. The plasmid pLW01-b5mem 102 + 8A was then used to construct the more stable pLW01-b5mem 103 + 9A plasmid.

Additional alanines were then inserted after Leu92 at the N terminus of the linker. The plasmid pLW01-b5mem 103 + 9A was used as a template to construct pLW01-b5mem 103 + 9A,92 + 3A, resulting in a plasmid containing the cDNA for cyt b5 with 12 inserted alanines. This plasmid was then used to add a further 4 alanines after Leu92 to ultimately yield a plasmid pLW01-b5mem 103 + 9A,92 + 7A containing the cDNA for cyt b5. DpnI was used to remove the 45 bp encoding the 15 amino acids of the reversed linker sequence.

Expression and Purification of Cyt P450 Reductase, Cyt P450 2B4, and Wild Type and Mutant Cyt b5—Cyt b5 was expressed and purified as previously described (23). Briefly, cultures of competent Escherichia coli C41 or C43 cells were prepared and the required plpW01 plasmid was inserted according to standard procedures (22, 24). Colonies were grown on LB agar plates containing 100 mg of cyt b5. The plasmids were extracted and purified using a Mini-prep plasmid extraction kit (Qiagen, Chatsworth, CA) and concentrated using vacuum centrifugation. A silent Ncol site was introduced into the primer used to prepare the cDNA of the \( \Delta G_{92–93} \) deletion mutant cyt b5. Because the 3.4 kbp plpW01-b5mem plasmid contains a second Ncol site \( \sim 3000 \) bp downstream of the \( \Delta G_{92–93} \) deletion, the purified plasmid was digested with Ncol and Pvu II to remove DNA fragments of \( > 200 \) bp on a 0.8% agarose gel (Invitrogen). A unique EcoRI site was introduced into the primers used to prepare the cDNA for the \( \Delta G_{103–104} \), \( \Delta G_{101–104} \), \( \Delta G_{99–104} \), and \( \Delta G_{97–104} \) mutant cyt b5. When the purified plasmids were digested with EcoRI and AflIII, two DNA fragments of \( \sim 200 \) bp were observed on a 0.8% agarose gel. In the absence of the EcoRI site, only a single band of linearized plasmid DNA was observed. The cDNA of \( \Delta G_{97–104} \) was used to construct the \( \Delta G_{95–104} \), \( \Delta G_{93–104} \), and \( \Delta G_{90–104} \) mutant cyt b5. To check for the incorporation of the mutated primers in the purified plasmids of these three mutants, the EcoRI site was removed from the primer. Thus, when the plasmid was digested with EcoRI and AflIII, only the linearized DNA was observed instead of the two fragments observed with the cDNA of \( \Delta G_{97–104} \).
Amphicath Cytochrome b5 Requires a 6–8-amino Acid Linker Domain

After cell lysis and reconstitution of holo cyt b5 with hematin, the protein was purified to homogeneity using anion exchange chromatography on DEAE-cellulose (Whatman, Maidstone, United Kingdom) and size exclusion chromatography on Superdex G75 (Amersham Biosciences). The purity of all cyt b5 mutants was checked using SDS-PAGE. The majority of proteins showed a single band on acrylamide gels when stained with Coomassie Brilliant Blue G-250 (Bio-Rad), although proteins expressed at very low levels showed the presence of minor bands. The protein concentration was measured using a dye-reagent protein assay (Bio-Rad), and the specific content of cyt b5 ranged from 39 to 54 nmol of cyt b5/mg of protein.

Each purified mutant cyt b5 was analyzed by mass spectroscopy to confirm its molecular weight. Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy was performed on a TOF-Spectrometer (Micromass-Waters, Manchester, United Kingdom) equipped with a time-lag focusing source. Protein samples were run in the linear mode using sinapinic acid as a matrix. An external calibration curve was used to determine the molecular weight of each cyt b5 with an error of ±0.1%.

The observed molecular weight was compared with the theoretical average molecular mass using the software tool PEPTIDEMASS from the ExPASy world wide web proteome server (25) (Table II). cyt P450 reductase plus cyt P450 2B4 were purified as described previously (9).

Determination of the Spectral Dissociation Constant, Ks, of the Ferric Cyt P450 2B4-Ferric Cyt b5 Complex—The spectral dissociation constant (Ks) of the cyt P450 2B4-cyt b5 complex was determined by measuring the type 1 spectral change (increase in absorbance at 420 nm and decrease in absorbance at 385 nm) occurring when cyt b5 is added to a solution of 0.3 μM cyt P450 2B4, 0.05% methoxyflurane, and 12 μM dilauroyl phosphatidylcholine (DLPC). The Type-1 spectral change reflects an alteration in the spin state of the heme iron from a low spin hexacoordinate heme to high spin pentacoordinate heme. The change reflects an alteration in the spin state of the heme iron from a low spin hexacoordinate heme to high spin pentacoordinate heme. The procedure used to measure the Ks between cyt P450 2B4 and cyt b5 is a modification of previously described methods (9, 26).

Briefly, a saturated solution of methoxyfluran (0.1%) in 100 mM KPO4, pH 7.4, 20% glycerol, was prepared. The solution was vortexed vigorously for 30 s to ensure saturation of the buffer and then allowed to stand at room temperature for 15 min to permit excess methoxyflurane to coalesce into a separate phase and settle to the bottom of the tube. The reference and sample buffers were prepared by mixing the saturated solution of methoxyflurane with equal amounts of either 100 mM KPO4, pH 7.4, 20% glycerol to prepare sample buffer, or 100 mM KPO4, 20% glycerol, 24 μM DLPC to prepare reference buffer.

A mixture containing 6 μM cyt P450 2B4 in 100 mM KPO4, pH 7.4, 20% glycerol, and 240 μM DLPC was incubated at room temperature for 1 h, and subsequently diluted 20-fold with sample buffer to yield a final concentration of 0.3 μM cyt P450, 20% glycerol, and 12 μM DLPC immediately prior to use.

Absorbance measurements were performed on a Cary-3 dual beam spectrophotometer. A sample cuvette contained 1 ml of 100 mM KPO4, pH 7.4, 20% glycerol, 0.3 μM cyt P450 2B4, 12 μM DLPC, and the reference cell contained 1 ml of 100 mM KPO4, pH 7.4, 20% glycerol, 12 μM DLPC. The cuvettes were incubated for 5 min at 25 °C, and a base line was recorded. Cyt b5 (0.04–1.9 nmol) was carefully added to each cuvette. The cuvettes were then mixed by gentle inversion 6–10 times. After 2 min the spectrum was recorded between 400 and 550 nm. Although cyt b5 absorbs strongly at 412 nm with an ε412 of 117 mM−1 cm−1, the presence of an equal amount of cytochrome b5 in both the sample and reference cuvettes meant that only the cyt P450 2B4 spectrum was observed. The spectral changes were stable for 15 min, and the total absorbance change was determined by subtracting the absorbance change in nm from the absorbance increase at 855 nm.

Because protein precipitation in the cuvette and/or imperfect pipetting of cyt b5 may lead to significant error, it was necessary to examine the difference spectrum to ensure it reflected a type 1 spectral change and not precipitation or loss of cyt P450 2B4 or cyt b5. Data were considered acceptable if the absorbance decrease at 420 nm accounted for 75% or more of the total absorbance change between 420 and 450 nm and the isosbestic point of the spectral change was between 402 and 407 nm. Values that did not fall in this range were discarded. The spectral dissociation constant (Ks) was determined using Equation (1).

\[
\Delta A = \Delta A_{\text{max}} \left( \frac{[Ks] + [P2B4] + [b5]}{2} \right) - \Delta A_{\text{max}} \left( \frac{[Ks] + [P2B4] + [b5]}{4} \right) - [P2B4][b5] \\
\text{(Eq. 1)}
\]

In Equation 1 ΔA is the observed absorbance change; ΔAmax is the maximal absorbance change per unit of cyt P450; [P2B4] is the concentration of cyt P450; [b5] is the concentration of cyt b5, and Ks is the spectral dissociation constant of the cyt P450 2B4-cyt b5 complex.

Stimulation of Cyt P450 2B4-catalyzed Metabolism of Methoxyflurane by Cyt b5—The method used to measure the rate of methoxyflurane metabolism by cyt P450 2B4 in the presence and absence of cyt b5 is a modification of a previously described procedure (7). The reaction components were added to a reaction mixture in a specific sequence: 50 mM KPO4, pH 7.4, 10 μM cyt P450 2B4, 10 μM cyt P450 reductase, 0–90 μM cyt b5, and 1.9 mM DLPC. The mixtures were incubated for 1 h at room temperature before aliquots were added to assay tubes containing glucose 6-phosphate and glucose-6-phosphate dehydrogenase. The final volume of the reaction mixtures was 450 μl and contained 50 mM KPO4 buffer, pH 7.4, 1 μM cyt P450 2B4, 1 μM cyt P450 reductase, 0–9 μM cyt b5, 190 μM DLPC, 5 mM glucose 6-phosphate, and 5 μL/min glucose-6-phosphate dehydrogenase. The reaction mixtures were pre-incubated for 5 min at 37 °C before addition of NADPH to a final concentration of 66 μM. The reaction mixtures were agitated at 37 °C and 120 rpm in an Innova 4200 orbital incubator shaker (New Brunswick Scientific, Edison, NJ) for 30 min. The reaction was stopped by immersion in a 70 °C water bath for 2 min. 22 μL of acetic buffer, containing 2.5 mM NaOH and 5 mM acetic acid, were added to each tube, and the fluoride concentration of a 300-μl aliquot was measured using a fluoride ion electrode (Orion Research Inc., Beverly, MA). The concentration of fluoride in each sample was determined by comparison to a standard curve of 1–100 μM sodium fluoride.

The apparent Ks ([KsP]c) of cyt b5 for cyt P450 2B4 was determined by fitting the rate of methoxyflurane metabolism at different [cyt b5] to Equation 2.

\[ v = v_0 \frac{V_{\text{max}}[b5]}{K_m + [b5]} \]

(Eq. 2)

v and Vmax are the observed and maximal rate of fluoride formation, respectively; v0 is the rate of fluoride ion formation in the absence of cyt b5; and [b5] is the concentration of cyt b5.

Incorporation of Exogenous Cyt b5 into Microsomes—Heaptic microsomes from rabbits pretreated with phenobarbital were incubated with varying concentrations (0–10 nmol/mg of microsomal protein) of purified wild type and mutant cyt b5s at 37 °C for 30 min with shaking. The endogenous cyt b5 and cyt P450 content of the microsomes was 1.2 and 2.3 nmol/mg of microsomal protein, respectively. The microsomes were a gift from Drs. Paul Hollenberg and Ute Kent, University of Michigan.

The final microsomal protein concentration was 2 mg/ml in 50 mM KPO4 buffer, pH 7.4. After 30 min Na2CO3, pH 11.5, was added to a final concentration of 0.1 M (27). Incubation was continued for an additional 30 min at 37 °C. The reaction mixture was centrifuged at 100,000g for 30 min to pellet the membranes. The supernatant was determined by comparison to a standard curve of 1–100 μM sodium fluoride.

The method used to measure the rate of methoxyflurane metabolism at different [cyt b5] is the observed and maximal rate of fluoride formation, respectively; v0 is the rate of fluoride ion formation in the absence of cyt b5; and [b5] is the concentration of cyt b5.

RESULTS AND DISCUSSION

Identification and Alignment of the Linker Region of Rabbit Cytocrome b5—Cyt b5 is an amphipathic protein, which contains an NH2-terminal heme domain anchored to the membrane by a COOH-terminal transmembrane α-helix. The two domains are connected by ~15 amino acids, which are referred to as the linker domain. To gain an understanding of the role of the length and sequence of the linker region of cyt b5 in orienting the heme domain for its interaction with its redox partners, the length and sequence of the cyt b5 linker domain were altered using site-directed mutagenesis. The length and sequence of the putative cyt b5 linker region vary depending on the species. It was therefore necessary to define the borders of the linker region by identifying both the COOH terminus of the heme domain and the NH2 terminus of the membrane domain. The amino acid sequence alignment of cyt b5 from mammals, invertebrates, and plants (Fig. 1) demonstrates that the heme domain is highly conserved (40–95%).

The crystal structures for the soluble domains of both bovine cyt b5 (1CYO) (7) and rat outer mitochondrial membrane cyt b5...
have been solved and show that the root mean square deviation of the backbone atoms of the heme domain differ by only 0.6 Å. However, it was noted that the secondary structure of the COOH-terminal region of the two cys b₅ was significantly different. In the crystal structure of bovine cyt b₅ residues Pro86–Arg89 form an /H9251-helix, whereas in the rat outer mitochondrial membrane cyt b₅ these residues are part of a random coil. In addition, the NMR-determined structure of bovine cyt b₅ showed that the secondary structure became increasingly disordered after Pro 86.

Examination and comparison of the crystallographically and NMR-determined structures of the similar but nonidentical solubilized heme domains of bovine, rabbit, rat microsomal, and rat outer mitochondrial cyt b₅ indicated that observable secondary structure in the heme domain ends near Arg 89, which is 7 amino acids further along from the invariant Gly 80. Thus, the NH₂ terminus of the linker region was considered to be Ser90. As the amino acid sequence alignment of cyt b₅ from mammals, invertebrates, and plants (Fig. 1) demonstrates that the heme domain is highly conserved (40–95%), it was assumed that the NH₂ terminus of the linker region would begin in the same position in other cyt b₅ sequences.

Previous studies have shown that, in rabbit cyt b₅, the residues from 5 to 88 and from 104 to 127 are relatively resistant to cleavage by proteases (30). Residues 5–88 correspond to the heme domain, which is consistent with the sequence alignment shown in Fig. 1, where the heme domain comprises residues 1–89 of rabbit cyt b₅. The protease-resistant region of 103–127 was also similar to the region predicted by MPEX (106–131) to be the membrane anchor of cyt b₅. The protease-sensitive region was between 90–104, and was therefore identical to the linker region shown in Fig. 1, which was determined by prediction of the heme and membrane binding domains. The se-

FIG. 1. Sequence alignments of amphipathic cytochromes b₅. The sequences were aligned as described under “Experimental Procedures.” The sequences and accession numbers obtained from the NCBI nucleotide data bank are rabbit (P00169), chicken (P00174), cow (P00171), horse (P00170), human (P00167), mouse (P56395), pig (P00172), rat (P00173), rat outer mitochondrial membrane (P04168), rice (546307), Arabidopsis thaliana (AAM61380), tobacco (P49098), Saccharomyces cerevisiae (P40312), cotton bullworm (AAC3371), housefly (P49096), and Rhizopus stolonifer (Q5HPU1). Identical residues are shown in gray, i.e. isoleucine to valine. Identical residues conserved in all 16 cyt b₅ sequences are marked with an asterisk (*).
Amphiphilic Cyt b<sub>5</sub> Requires a 6–8- amino Acid Linker Domain

The DNA sequence of the 5′–3′ oligonucleotide primers used to mutate cytochrome b<sub>5</sub>

| Forward primer<sup>a</sup> | Template plasmid | Mutant |
|---------------------------|------------------|--------|
| GATGACAGATCAAAA–AGGCCcatggAaacctTatc | pLW01-b5mem | pLW01-A92–93 |
| CTCTATTACACC–TCggTACcccAGGCGTGTG | pLW01-b5mem | pLW01-A103–104 |
| CCTATGGAACCCTTATC–TCggTACcccAGGCGTGTG | pLW01-b5mem | pLW01-A105–1104 |
| GCAACCCCTTGAACACT–TCggAACcccAGGCGTGTG | pLW01-b5mem | pLW01-A99–104 |
| CAAAATGACAGCGCTATG–TCggTACcccAGGCGTGTGAC | pLW01-b5mem | pLW01-A97–104 |
| GATCACGAAAGACACACACCCAGGCGTGTG | pLW01-b5mem | pLW01-A97–104 |
| CGGATGACAGCACTACCAAGACCT–TCggTACcccAGGCGTGTG | pLW01-A97–104 |
| CGGATGACAGCACTACCAAGACCT–TCggTACcccAGGCGTGTG | pLW01-A90–104 |
| CCTTATGGAACCCTTATG–TCggTACcccAGGCGTGTGAC | pLW01-A90–104 |
| GTCACCAAGATCTTTACGGATACCTC–AAATCTCCGTCG | pLW01-91–96RSL | pLW01-91–100RSL |
| CTTATGGAACCCTTATG–TCggTACcccAGGCGTGTGAC | pLW01-91–100RSL | pLW01-91–104RSL |
| CGGATGACAGCACTACCAAGACCT–TCggTACcccAGGCGTGTG | pLW01-94–95del |
| CAGTACAAATTGCAACGCTATG–AAATCTCCGTCG | pLW01-b5mem | pLW01-P95A |
| GACAGTACAAATTGCAACGCTATG–AAATCTCCGTCG | pLW01-K94A |
| CCTTACCAAGATCTTTACGGATACCTC–AAATCTCCGTCG | pLW01-b5mem | pLW01-W103 + 2A |
| GACAGTACAAATTGCAACGCTATG–AAATCTCCGTCG | pLW01-W103 + 4A |
| CAGTACAAATTGCAACGCTATG–AAATCTCCGTCG | pLW01-W103 + 8A |
| CGGATGACAGCACTACCAAGACCT–TCggTACcccAGGCGTGTG | pLW01-W103 + 9A |
| CGGATGACAGCACTACCAAGACCT–TCggTACcccAGGCGTGTG | pLW01-W103 + 9A,92 + 3A |

<sup>a</sup> QuikChange mutagenesis kits require complementary forward and reverse primers. For simplicity, only the 5′–3′ primer is shown.

<sup>b</sup> RSL is used to denote cyt b<sub>5</sub> DNA used to construct a cyt b<sub>5</sub> mutant with the linker region residues 91–104 in reverse sequence order of Asp<sub>91</sub>, Val<sub>92</sub>, Thr<sub>93</sub>, Ile<sub>94</sub>, LEu<sub>95</sub>, Thr<sub>96</sub>, Thr<sub>97</sub>, Glu<sub>98</sub>, Met<sub>99</sub>, Pro<sub>100</sub>, Lys<sub>101</sub>, Ser<sub>102</sub>, Leu<sub>103</sub>, and Lys<sub>104</sub>.

The primers shown in Table I were used to mutate the cDNA of wild type cyt b<sub>5</sub> and to construct insertion mutants 103 + 2A and 103 + 4A with 2 and 4 alanines inserted after Val<sub>103</sub>. The cyt b<sub>5</sub> mutant 103 + 8A was then prepared by addition of 4 alanine residues to the cDNA of cyt b<sub>5</sub> 103 + 4A.

Primers were used to add an extra 4 alanines to the cDNA sequence of cyt b<sub>5</sub> 102 + 8A to construct a 102 + 12A mutant. Surprisingly, the DNA sequence of the resulting plasmids revealed that the mutated sequence of cyt b<sub>5</sub> had only 9 extra alanines instead of the expected 12 alanines. The program Oligonucleotide Analyzer (www.rnature.com/oligonucleotide.html) predicts the formation and free energies of hairpin and dimer structures in primers and oligonucleotide sequences. The template DNA of cyt b<sub>5</sub> 102 + 8A was predicted to form a 9–10 bp hairpin with a free energy of −5.6 kcal mol<sup>−1</sup>. During the attempt to generate the 102 + 12A cyt b<sub>5</sub> mutant by PCR, presumably a 9-base pair hairpin formed in the template DNA and resulted in the removal of three alanine codons without causing a frameshift. The loss of 3 alanines from the template DNA and the insertion of the 4 alanines in the primer gave a total increase of 1 alanine. In view of the difficulty observed in mutating cyt b<sub>5</sub> 102 + 8A, it was decided to use cyt b<sub>5</sub> 103 + 9A instead to prepare further insertion mutants.

The amount of wild type and mutant cyt b<sub>5</sub> purified from 500 ml of cell culture is shown in column 3 of Table II. The difference in the yield is the result of variation in levels of cyt b<sub>5</sub> expressed in cells as approximately the same proportion of initial cyt b<sub>5</sub> was lost during the purification of each mutant. No differences were observed in the properties of the cyt b<sub>5</sub> mutants during the purification of the protein. The majority of the purified cyt b<sub>5</sub> mutants ran as a single band on SDS-PAGE. However, cyt b<sub>5</sub> mutants purified from cells with extremely low expression such as Asp<sub>91</sub>, Thr<sub>92</sub>, Thr<sub>93</sub>, Thr<sub>97</sub>, Glu<sub>98</sub>, Met<sub>99</sub>, Pro<sub>100</sub>, Lys<sub>101</sub>, Ser<sub>102</sub>, Leu<sub>103</sub>, and Lys<sub>104</sub>.

The molecular masses determined by MALDI mass spectrometry of the mutant cyt b<sub>5</sub> are shown in column 3 of Table II and confirm the identities of the proposed mutants. The molecular mass of cyt b<sub>5</sub> plus a proton ± 0.1%. The mass spectrum of each mutant showed two peaks, one corresponding to the singly charged cyt b<sub>5</sub> species and one corresponding to the doubly charged cyt b<sub>5</sub> species. In no instance did the observed mass differ from the predicted mass by more than 22
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TABLE II

| Mutant cyt b₅ | Expressed in E. coli strain | Amount of cyt b₅ purified from 500 ml of cell culture | Observed mass (M + H⁺)¹ |
|--------------|-----------------------------|-------------------------------------------------------|--------------------------|
| Wild type    | C41                         | 57                                                    | 15,213 ± 15              |
| Δ92–93       | C41                         | 51                                                    | 15,013 ± 15              |
| Δ103–104     | C41                         | 122                                                   | 14,989 ± 15              |
| Δ94–95       | C41                         | 4                                                     | 14,995 ± 15              |
| Δ92–93,103–104| C41                        | 16                                                    | 14,800 ± 15              |
| Δ101–104     | C41                         | 15                                                    | 14,801 ± 15              |
| Δ99–104      | C41                         | 70                                                    | 14,581 ± 15              |
| Δ97–104      | C41                         | 14                                                    | 14,350 ± 15              |
| Δ95–104      | C45                         | 18                                                    | 14,118 ± 15              |
| Δ93–104      | C45                         | 33                                                    | 13,907 ± 15              |
| Δ90–104      | C45                         | 12                                                    | 13,550 ± 15              |
| 91–104RS     | C41                         | 5                                                     | 15,221 ± 15              |
| P95A         | C41                         | 10                                                   | 15,193 ± 15              |
| K94A         | C41                         | 64                                                   | 15,156 ± 15              |
| 103 + 2A     | C41                         | 71                                                   | 15,361 ± 15              |
| 103 + 4A     | C41                         | 15                                                   | 15,491 ± 15              |
| 103 + 9A     | C45                         | 2                                                     | 15,859 ± 16              |
| 103 + 9A,92  + 3A | C43                        | 5                                                     | 16,049 ± 16              |
| 103 + 9A,92  + 7A | C43                       | 2                                                     | 16,355 ± 16              |

¹ The molecular masses of the proteins were determined using MALDI mass spectrometry as described under “Experimental Procedures.”

Da. It was possible to observe the expected changes not only in the value of the mass of the insertion and deletion mutant cyts b₅, but also of the substitution mutants P95A and K94A.

Determination of the K₅ of the Wild Type and Mutant Cyt b₅-Cyt P450 2B4 Complexes—The ability of the mutant cyts b₅ to form a complex with cyt P450 2B4 was assessed by determining the spectral dissociation constant of wild type and mutant cyts b₅. The type 1 spectral change caused by increasing concentrations of cyt b₅ was fitted to Equation 1. Fig. 2 illustrates the data obtained from a typical experiment with wild type, Δ99–104, and Δ97–104 cyt b₅. The observed ΔA₁₅₀₅ₕₐₐ₅ that occurs when wild type cyt b₅ is incubated with ferric cyt P450 2B4 is much greater than the change that occurs when mutant Δ99–104 is incubated with cyt P450 2B4, indicating it binds 4-fold less tightly than the native cyt b₅ (Fig. 2 and Table III). The difference in the length of the linker region between the wild type and mutant Δ99–104 cyt b₅ is 6 residues. Deletion of 8 amino acids (Δ97–104) results in a cyt b₅ that binds 30-fold less tightly to cyt P450 2B4 (Fig. 2 and Table III). There is a small but significant change in the ability of cyt b₅ to bind to ferric cyt P450 after deletion of 6 residues, whereas deletion of 8 residues from the linker region of wild type cyt b₅ yields a protein markedly impaired in its ability to bind cyt P450 2B4. Deletion of 10 or more residues from the linker region resulted in a mutant cyt b₅ that was incapable of forming a detectable complex with cyt P450 under our experimental conditions. As a result this mutant was concluded to have a K₅ of > 100 μM, the limit of the sensitivity of this method.

The dissociation constants for all the mutants are provided in Table III. As the number of residues deleted from the linker region increases, the affinity of the mutant cyt b₅ for cyt P450 decreases and consequently the K₅ increases. The ΔAₘₐₛₙₜₜ for the cyt P450 2B4-cyt b₅ complex, was the same for all mutated cyts b₅ and had a value of 0.074 ± 0.01 au/μM cyt P450, indicating the nature of the complexes was similar and that the alterations to the K₅ were a result of a diminished ability of cyt b₅ to form a complex with cyt P450.

Wild type cyt b₅ has a K₅ of 0.076 ± 0.02 μM, which compares favorably to the K₅ determined by other investigators. Previous studies in this laboratory found a K₅ of 0.2 μM for the dissociation constant of the cyt P450 2B4-cyt b₅ complex in the presence of a saturated solution of methoxyflurane, whereas other investigators obtained a value of 0.25 μM in the absence of substrate and 0.08 μM in the presence of benzphetamine (9, 26).

The method used by Schenkman and co-workers is similar to the one used here where cyt b₅ was added to both sample and reference cuvettes. In contrast, a dual chamber cuvette was used in the previous experiments in this laboratory.

To determine whether the position of the deletion could make a difference in the ability of cyt b₅ to form a complex with cyt P450 2B4, 2 residues were deleted from the NH₂ terminus of the linker region of cyt b₅ (Δ92–93) and 2 were removed from the COOH terminus of cyt b₅ (Δ103–104). Deletion of 2 amino acids from either the NH₂ (Δ92–93) or the COOH (Δ103–104) terminus of the linker region did not alter the affinity of the mutant proteins for cyt P450 2B4. In addition, the K₅ determined for mutants with residues Thr₁⁰¹–Asp₁⁰⁴ deleted, were not significantly different from each other or wild type cyt b₅. These results indicate that deletion of residues from opposite ends of the linker region has no significant effect on the K₅ of the complex.

Addition of 2, 4, 9, 12, or 16 alanines to the linker region of wild type cyt b₅ resulted in no detectable change in the ability of cyt b₅ to form a complex with cyt P450 2B4. The most conserved amino acids of the cyt b₅ linker region are Lys⁹⁸ and Pro⁹⁵. Three mutants were prepared to investigate the role of these residues: P95A and K94A, and Δ94–95, where both amino acids had been deleted. All three mutants exhibited a K₅ similar to wild type cyt b₅, demonstrating that these changes in the sequence do not affect the ability of cyt b₅ to form a complex.
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TABLE III
Characterization of the mutant cyts b₅

| Mutant cyt b₅ | Amino acids mutated | Kₘ ± S.D. (μM) | Rate of methoxyflurane metabolism + b₅ | Kₘ<sup>app</sup> (μM) |
|---------------|---------------------|----------------|-------------------------------------|---------------------|
| Wild type     | 0                   | 0.076 ± 0.02   | 1.23 ± 0.2                          | 0.35 ± 0.04         |
| ΔK2-93       | 2 deleted           | 0.035 ± 0.01   | 1.01 ± 0.1                          | 0.52 ± 0.4          |
| ΔK103-104    | 2 deleted           | 0.11 ± 0.04    | 0.89 ± 0.1                          | 0.65 ± 0.1          |
| ΔK94-95      | 2 deleted           | 0.068 ± 0.02   | 1.42 ± 0.1                          | 0.19 ± 0.1          |
| ΔK2-93,103-104| 4 deleted          | 0.19 ± 0.02    | 0.87 ± 0.1                          | 0.68 ± 0.3          |
| ΔK101-104    | 4 deleted           | 0.15 ± 0.07    | 0.80 ± 0.1                          | 0.96 ± 0.2          |
| ΔK99-104     | 6 deleted           | 0.32 ± 0.04    | 0.68 ± 0.1                          | 1.3 ± 0.3           |
| ΔK97-104     | 8 deleted           | 2.2 ± 1        | 0.44 ± 0.04                         | 1.3 ± 0.3           |
| ΔK95-104     | 10 deleted          | >100           | 0.28 ± 0.02                         | 10.8 ± 2            |
| ΔK93-104     | 12 deleted          | >100           | 0.29 ± 0.04                         | 5.7 ± 3             |
| ΔK90-104     | 15 deleted          | >100           | 0.31 ± 0.08                         | 11.2 ± 5            |
| 91-104RSL    | 14 reversed         | 0.029 ± 0.02   | 1.30 ± 0.1                          | 0.49 ± 0.2          |
| P95A         | 1 substituted       | 0.13 ± 0.03    | 1.32 ± 0.1                          | 0.21 ± 0.2          |
| K94A         | 1 substituted       | 0.090 ± 0.03   | 1.58 ± 0.2                          | 0.19 ± 0.08         |
| 103 + 2A     | 2 inserted          | 0.057 ± 0.1    | 1.32 ± 0.1                          | 0.21 ± 0.08         |
| 103 + 4A     | 4 inserted          | 0.019 ± 0.01   | 1.32 ± 0.1                          | 0.12 ± 0.05         |
| 103 + 9A     | 9 inserted          | 0.056 ± 0.03   | 0.95 ± 0.1                          | 0.11 ± 0.04         |
| 103 + 9A,92 + 3A| 12 inserted      | 0.15 ± 0.02    | 0.96 ± 0.1                          | 0.28 ± 0.05         |
| 103 + 9A,92 + 7A| 16 inserted       | 0.077 ± 0.03   | 0.89 ± 0.2                          | 0.27 ± 0.1          |

*The K<sub>app</sub> and rate of methoxyflurane metabolism were determined as described under “Experimental Procedures.”*

with cyt P450 2B4. In addition, the cyt b₅ mutant 91–104RSL in which the linker region has a length identical to the wild type but with the residues between 91–104 in reverse order was also shown to have a Kₘ similar to that of the wild type cyt b₅.

Considered as a whole these results demonstrate that cyt b₅ requires a linker region containing a minimum of 7 amino acids to be able to form a complex with cyt P450 2B4 in vitro and presumably in a membrane in vivo. The precise role of the linker is uncertain, but its putative function will be discussed below.

**Ability of Mutant Cyts b₅ to Stimulate the Metabolism of Methoxyflurane by Cyt P450 2B4**—The ability of the cyt b₅ mutants to stimulate cyt P450 2B4-catalyzed metabolism of methoxyflurane (CCl₂HCF₂OCH₃) was assessed by measuring the rate of F⁻ formation resulting from the O-demethylation of methoxyflurane. Wild type cyt b₅ caused a 6-fold stimulation of the rate of fluoride formation from 0.21 ± 0.03 nmol of F⁻ min⁻¹ nmol of cyt P450⁻¹ in the absence of cyt b₅ to 1.23 ± 0.2 nmol min⁻¹ nmol of cyt P450⁻¹ with cyt b₅. These results are similar to the rate of methoxyflurane metabolism observed in previous studies (7, 9). Replacing the conserved residues Pro<sup>95</sup> and Lys<sup>84</sup> with alanines and reversing the amino acid sequence of the linker region did not significantly alter the rate of F⁻ formation.

Deletion of residues from the linker region resulted in a gradual decrease in the stimulation of cyt P450 2B4 metabolism of methoxyflurane (Fig. 3). The decrease in stimulation of cyt P450 2B4-dependent methoxyflurane metabolism was approximately linear until 10 residues were deleted. Mutants with 10 or more residues deleted from the linker region only minimally but reproducibly enhanced the rate of methoxyflurane metabolism by ≈50%.

Addition of 9–16 alanines to the linker region of cyt b₅ resulted in a modest 23% decrease in the ability of cyt b₅ to stimulate methoxyflurane breakdown. This small but significant diminution in the activity suggests that the length of the linker region is optimized for binding to cyt P450 2B4.

The major route of cyt P450 2B4-catalyzed metabolism of methoxyflurane is via dechlorination to methoxydifluoroacetic acid and HCl. It accounts for 86% of the total metabolism of methoxyflurane. Methoxyflurane is also O-demethylated by cyt P450 2B4 to produce fluoride ion, formaldehyde, and dichloroacetic acid (7). It is unlikely that the mutation of the cyt b₅ linker region alters the relative formation of the two products, as recent modeling studies have shown that, in cyt P450 2E1, the preferential metabolism of methoxyflurane to methoxydifluoroacetic acid is the result of differences in the relative activation energy required for H atom extraction rather than favorable substrate binding conformations (31). In addition, the relative amounts of methoxyflurane products formed by cyt
P450 2B4 are the same in the presence and absence of cyt b5 (7). These studies indicate that the observed reduction in F-formation caused by mutation of the cyt b5 linker region is the result of an attenuation in the overall metabolism of methoxyflurane rather than a selective decrease in O-demethylation relative to dechlorination.

**Determination of the Apparent \( K_m \) of the Mutant Cyts b5 for Cytochrome P450 2B4**—Previous studies have estimated that the apparent dissociation constant of the cyt P450 2B4-cyt P450 reductase complex is \( 0.02 \pm 0.02 \mu M \) by assuming that the ability of cyt P450 2B4 to metabolize benzphetamine is directly proportional to the concentration of the catalytically competent cyt P450-reductase complex (9). However, it is not possible to determine the \( K_m \) of the oxyferrous cyt P450 2B4-cyt b5 complex using the same experimental conditions employed to measure the affinity of cyt P450 reductase for cyt P450 because the reductase is required to reduce ferric cyt P450 to a ferrous protein capable of binding oxygen. As a result cyt b5 and cyt P450 reductase will compete with one another for their distinct but overlapping binding sites on the proximal surface of cyt P450 2B4 where the heme comes closest to the surface. In view of these experimental limitations, the affinity of different mutant cyts b5 was evaluated by measuring the ability of cyt b5 to stimulate methoxyflurane metabolism in the presence of a constant concentration of cyt P450 2B4 and reductase. Methoxyflurane is a substrate whose metabolism by cyt P450 2B4 is markedly stimulated by cyt b5. The arbitrary value obtained for the affinity of cyt b5 under our experimental conditions is considered to be an apparent \( K_m \) (\( K_m^{app} \)).

As residues were deleted in 2-amino acid residue increments from the linker region, the \( K_m^{app} \) gradually increased from 0.35 to 1.33 ± 0.30 \( \mu M \) following removal of 8 residues. Upon deletion of 10 or more residues, a large increase in the \( K_m^{app} \) was observed (Fig. 3B). The \( \Delta 95–104 \) mutant cyt b5 was essentially unable to form a complex with cyt P450 2B4.

In contrast, insertion of 2, 4, 9, 12, or 16 alanines in the linker region, substitution of alanine for the conserved residues, Lys84 and Pro95, and reversal of the entire linker region sequence did not affect the \( K_m^{app} \) significantly. These results indicate a minimum of 7 amino acids is necessary to enable cyt b5 to form a functional complex with cyt P450 2B4 capable of electron transfer and catalysis.

**Low Resolution Model of the Cyt P450-Cyt b5 Complex**—The structure of the heme domain of several microsomal cyts P450 including cyt P450 2B4 has recently been determined (32–36). Cyt P450 2C5 was used in our modeling rather than cyt P450 2B4 because cyt P450 2B4 crystallized in an extraordinarily open and presumably less physiological conformation than cyt P450 2C5. In addition to the NH2-terminal -helical transmembrane domain, a hydrophobic surface near Pro30 has been identified by x-ray crystallography and NMR (8, 16–18). If the backbones of the various forms of the soluble domains of cyt b5 are overlaid, they are very similar up to approximately Ser90. The similarity in structure up to Ser90 was a factor in considering Ser90 to be the first amino acid in the linker. At this point the COOH termini begin to show marked differences in conformation reflecting the intrinsic flexibility and dynamics of the linker sequence. A model is presented in Fig. 4 in which the hydrophobic surface of cyt P450 is positioned in the outer bilayer of a membrane (34). Cyt b5 has been manually docked with cyt P450 2C5 so that the cyt b5 residues Asp65 and Val66 are near residues Lys121 (at the beginning of the C helix) and Lys429 of cyt P450 2C5 (8, 31, 34). This orientation is illustrated because Lys121 and Lys429 of cyt P450 2C5 are homologous to cyt P450 2B4 residues Arg222 and Arg433, which have been implicated in interacting with Asp65 and Val66 of rabbit cyt b5 (2).

If the 15 amino acids of the linker were extended like a parallel \( \beta \)-sheet, its maximum length would be \( \approx 51 \) Å (3.5 Å/residue), whereas if arranged as an \( \alpha \)-helix, the 15 residues would span merely \( \approx 22.5 \) Å (1.5 Å/amino acid) (37). A 7-amino acid linker would therefore be predicted to span 24 Å if maximally extended but only 10.5 Å if folded as an \( \alpha \)-helix. The distance from Ser90 to the membrane is estimated to be \( \approx 15–20 \) Å and could easily be traversed by \( \approx 7 \) amino acids in an extended nonhelical conformation (Fig. 4).

Our data are consistent with the putative model of cyt P450 2C5 membrane binding proposed by Johnson and co-workers (34), which places the heme iron roughly 30 Å above the plane of the membrane. This orientation of cyt P450 enables its reduct partners to approach its unobstructed proximal surface, bind, transfer an electron, and subsequently dissociate. Docking the proteins with Asp65 and Val66 of cyt b5 and Lys121 and Lys429 of cyt P450 in contact positions Ser90 \( \approx 20 \) Å from the membrane surface, whereas rotating cyt b5 180° about the \( \alpha \)-\( \gamma \) axis of the heme would place Asp65 and Val66 and Ser90 roughly 40–45 Å above the plane of the membrane, probably too far for the 7-amino acid linker to extend. A caveat is that our experiments were not performed in a membrane bilayer but in a reconstituted system, which may not possess such steric constraints. Nevertheless, we postulate that the results are consistent with the notion that under the experimental conditions the hydrophobic domains of cyt b5 and cyt P450 bind to one another and the linker sequences restrict the orientation of the heme domains, thereby facilitating formation of a specific productive electron transfer complex. Interestingly, there is also a 15-amino acid linker (Arg84-Thr509) between the FMN domain of rat cyt P450 reductase and its membrane anchor. Moreover, the membrane anchor and linker are coded for by exon 2 of the cyt P450 reductase gene, which specifies amino acid residues

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1–59 and may constitute a structural and/or functional unit for the protein (38). It may not be a coincidence that the linker domains of cyt P450's two redox partners are of similar length.

**Mutant Cts b<sub>5</sub> Bind to Membrane—**To determine whether the cys b<sub>5</sub> with an altered linker length and sequence were able to incorporate into membranes, the purified mutant cys b<sub>5</sub> were tested for their ability to bind to microsomes in the presence of 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.5. All of the deletion and substitution mutant proteins and those with an extra 2 and 4 alanines were studied. Because they were as active with cyt P450 as the native cyt b<sub>5</sub>, the mutant proteins with the reversed sequence linker and a linker lengthened by more than 4 residues were not investigated. It was assumed they would react like the native cyt b<sub>5</sub>. Treatment of membranes with high pH distinguishes integral membrane proteins from peripheral membrane proteins. Integral membrane proteins remain bound to the membrane in the presence of high pH, whereas soluble and peripheral membrane proteins do not sediment with the membrane. The high pH carbonate procedure also forms microsomes to open membrane sheets, thereby releasing the contents of the microsomal vesicle (27).

Fig. 5 demonstrates that incubation of microsomes with wild type and mutant cys b<sub>5</sub> resulted in the incorporation of exogenous protein into the microsomal membrane which contained 1.2 nmol of endogenous cyt b<sub>5</sub>/mg of microsomal protein. All of the mutant cys b<sub>5</sub> with substitutions (K94A, P95A), an extra 2 and 4 alanines and shortened linkers, except the ∆90–104 cyt b<sub>5</sub> protein lacking 15 amino acids, inserted into the microsomal membranes like the wild type protein. The significantly (Student's t test) impaired incorporation of ∆90–104 cyt b<sub>5</sub> compared with native cyt b<sub>5</sub> is consistent with the proposed two-step process of spontaneous insertion of integral membrane proteins. The nonpolar segment initially binds parallel to the plane of the membrane at the interface between the hydrophobic core and the aqueous solvent. The second step is for the hydrophobic sequence to insert into the hydrophobic core of the membrane (20). It is suggested that the transmembrane domain of the ∆90–104 cyt b<sub>5</sub> is sterically hindered by the large heme domain from forming a tight complex with the interfacial surface of the membrane. The binding of the mutant and wild type cys b<sub>5</sub> was linear over the concentration range used in the experiments. Saturation of the microsomal membrane with cyt b<sub>5</sub> occurred when 15 nmol of cyt b<sub>5</sub> had been added to 1 mg of microsomal protein. At ≤15 nmol/mg of protein, the hemoprotein represents nearly 20% of the total protein of the preparation, which is similar to the amount of cyt b<sub>5</sub> previously reported to bind microsomes in a manner indistinguishable from that of endogenous cyt b<sub>5</sub> (39). Assuming the membrane surface area per mg of microsomal protein to be ~0.2 m<sup>2</sup> (40) and that a cyt b<sub>5</sub> molecule (heme domain radius 15Å) has a footprint of ~700 Å<sup>2</sup>, then a maximum of ~56 nmol of cyt b<sub>5</sub> could occupy this membrane surface in the absence of other proteins. There is a surprising good agreement between the theoretical calculation and the experimental observation given all the approximations. Control experiments demonstrated that the soluble heme-containing domain of cyt b<sub>5</sub> did not bind the microsomes under identical experimental conditions, implying that insertion requires the hydrophobic transmembrane domain. Nonspecific aggregation and sedimentation of the purified exogenous cyt b<sub>5</sub> was also ruled out by the observation that centrifugation of amphipathic cyt b<sub>5</sub> at pH 11.5 in the absence of microsomes did not result in its appearance in the pellet.

As further evidence that the mutant proteins can bind a membrane, it should also be noted that all of the mutant cts b<sub>5</sub> were isolated from the membrane fraction of *E. coli*. There is, therefore, in vivo and in vitro evidence that these mutant hemoproteins insert into membranes. Inability to bind to membranes cannot explain the diminished activity of cys b<sub>5</sub> with a linker region of less than 8 amino acids.

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