Molecular Cloning, Overexpression in *Escherichia coli*, Structural and Functional Characterization of House Fly Cytochrome b₅*†

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A microsomal cytochrome b₅ cDNA from the house fly, *Musca domestica*, was cloned and sequenced. The deduced amino acid sequence of the full-length house fly cytochrome b₅ (134 residues) is 48% identical to that of rat microsomal cytochrome b₅. The house fly cytochrome b₅ protein was overexpressed in *Escherichia coli*, purified, and characterized. Absorption and EPR spectroscopy reveal properties very similar to cytochrome P₄₅₀.

The turnover of cytochrome P₄₅₀ depends on the concentration of cytochrome P₄₅₀ and on the rate of the second electron transfer to P₄₅₀, which is influenced by various factors such as the concentration of NADPH, the presence of inhibitors, and the nature of the substrate.

Insect P₄₅₀s have been extensively studied because of their crucial role in the biosynthesis of hormones regulating insect growth, development, and reproduction (ecdysteroids and juvenile hormones) and in the biotransformation of foreign compounds of synthetic (insecticides) or natural (plant and microbial toxins) origin (22). Metabolism of insecticides by P₄₅₀s is thought to be an adaptation to the hazards of herbivory (24). Both CYP6A1, an insect P₄₅₀, which is overproduced in insecticide-resistant strains of the house fly, and NADPH-dependent cytochrome P₄₅₀ reductase, which provides electrons to P₄₅₀s from NADPH, have been cloned from the house fly, *Musca domestica* (25, 26) and expressed in *E. coli* (27). We have found that epoxidation of the cyclodiene insecticide heptachlor by CYP6A1 is stimulated by rat microsomal cyt b₅ in a reconstituted system. Furthermore, immunological evidence for the involvement of cyt b₅ in several P₄₅₀-dependent monooxygenase activities in house fly microsomes has been reported (28).

Our concerted effort to clone, express, and reconstitute elements of the insect cytochrome P₄₅₀ system therefore led us to clone house fly cyt b₅ cDNA. In addition to a better understanding of the role of cyt b₅ in P₄₅₀-mediated reactions, it was felt that the characterization of house fly cyt b₅ would facilitate the study of the evolutionary conservation of structural and functional properties of this electron carrier. We report here that the cloned house fly cyt b₅ has been overexpressed in *E. coli*, purified to electrophoretic homogeneity, and extensively characterized both structurally by a number of spectroscopic techniques (absorption, NMR, EPR) and functionally (redox and electron-transfer properties). We reconstituted in vitro an in-

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The abbreviations used are: cyt, cytochrome; P₄₅₀, cytochrome P₄₅₀; CYP6A1, cytochrome P₄₅₀ 6A1; RT, reverse transcription; PCR, polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; SHE, standard hydrogen electrode.

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sect P450 system consisting of P450 reductase, CYP6A1, cyt \( b_5 \), and phospholipid. CYP6A1-catalyzed epoxidation of the insec-
ticide heptachlor is stimulated by cyt \( b_5 \) in this system. The mechanisms by which cyt \( b_5 \) stimulates P450 turnover are discussed.

**EXPERIMENTAL PROCEDURES**

**Amplification of a Partial cDNA Encoding House Fly Cytochrome \( b_5 \) by RT-PCR**—Four-day-old larvae of the diazinon-resistant strain "Rut-
gers" of the house fly, \( M. \) domestica, were used as the source of poly(A)-
RNA. About 1 \( \mu \)g of poly(A)-RNA was reverse transcribed using the Superscript RNase H- Reverse Transcriptase Kit (Life Technologies, Inc.) and an 18-mer oligo(dT) primer. The mRNA-cDNA duplex was used as template for PCR with AmpliTaq polymerase (Perkin Elmer) Inc.) and an 18-mer oligo(dT) primer. The mRNA-cDNA duplex was reverse transcribed using the Superscript RNase H- Reverse Transcriptase Kit (Life Technologies, Inc.) and an 18-mer oligo(dT) primer. The mRNA-cDNA duplex was used as template for PCR with AmpliTaq polymerase (Perkin Elmer) Inc.) and an 18-mer oligo(dT) primer. The mRNA-cDNA duplex was reverse transcribed using the Superscript RNase H- Reverse Transcriptase Kit (Life Technologies, Inc.) and an 18-mer oligo(dT) primer. The mRNA-cDNA duplex was reverse transcribed using the Superscript RNase H- Reverse Transcriptase Kit (Life Technologies, Inc.) and an 18-mer oligo(dT) primer.

**Construction of the Vector for Expression of the House Fly Cyt \( b_5 \) in \( E. \) coli—Plasmid pCWori \( 1 \)** (kindly provided by the NCBi Blast Server (29).

**Isolation of the House Fly Cyt \( b_5 \) cDNA—**A house fly cDNA library in \( \lambda \) Zap (25) (approximately 1 \( \times \) 10\(^8\) plaques) was screened at high string-
genesis with the partial house fly cyt \( b_5 \) cDNA obtained by RT-PCR. The \( 3^{\prime} \)P-labeled probe was generated using Prim-It II kit (Stratagene) modified by replacing random primers with the degenerate primers used for RT-PCR (see above). Inserts were excised from purified phage, recircularized in pBluescript plasmids, and sequenced. Nucleotide sequences were analyzed with the BLAST program on the NCBI Blast Server.

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b5 is present in a reduced form in whole E. coli cells. Therefore, sodium dithionite and hydrogen peroxide were added to the sample and reference cuvettes, respectively, to determine cyt b5 by reduced versus oxidized difference spectra. Molar concentration of recombinant house fly P450 reductase was calculated based on protein content (36).

RESULTS

Molecular Cloning and Expression in E. coli

Cloning of House Fly Cyt b5—Conserved sequences around the two His ligands of the heme in known cyt b5s (Fig. 1) were used to design two oligonucleotide primers for RT-PCR from larval mRNA of the housefly. Cloning of the PCR products and sequencing of a few individual clones revealed the presence of two clones (1-2 and 1-7) containing cyt b5-like sequences (EE-TLDEVAGRD and EE-VLIEQAGKD, respectively). These short PCR products were 53% identical at the nucleotide level.

The inserts of clones 1-2 and 1-7 were used as probes to screen a house fly ZAP cDNA library, and six identical cDNA clones with inserts of approximately 1.35 kilobase pairs were isolated. One of these, clone 16-A2 was shown to contain an open reading frame of 402 base pairs encoding a protein of 134 amino acids (15.4 kDa) at the 5' end of the cDNA. The 5' end of clone 16-A2 containing the open reading frame was deposited in GenBank™ under the accession number L38464 (706 base pairs). Clone 16-A2 contained the exact sequence of the initial RT-PCR clone 1-7. No cDNA clone corresponding to the initial RT-PCR clone 1-2 was found.

Comparison of the coding region of the cDNA revealed a high similarity to known cyt b5s. The deduced protein sequence was 48% identical to rat microsomal cyt b5 and 46.5% identical to rat outer mitochondrial membrane cyt b5. It was only 27% identical to a portion of a Drosophila virilis sequence deposited in GenBank™ as cyt b5 (39).

Expression in E. coli and Purification of House Fly Cyt b5—A plasmid for expression in E. coli (Fig. 3) was constructed as described under “Experimental Procedures.” We obtained expression levels of 9 μmol/L culture in small scale cultures (50 ml) and 4–4.5 μmol/L culture in large scale cultures (500–750 ml). House fly cyt b5 was found in E. coli in the reduced Fe(II) form as was reported for rat and human cyt b5 expressed in E. coli (40), and the protein gradually oxidized during purifica-
tion. Table I shows purification steps of the recombinant house fly cyt b₅. After cell lysis by sonication, most of the cyt b₅ was found in the membrane fraction (Table I). CHAPS treatment solubilized about 65% of the cyt b₅. The solubilized protein was purified by a combination of ion-exchange and hydrophobic interaction chromatography to apparent electrophoretic homogeneity (Fig. 4) with a yield of 22% (Table I). The protein mobility under the conditions of SDS-PAGE corresponded to a polypeptide of about 19 kDa.

Spectral Characterization of Recombinant House Fly Cyt b₅

Absorption Spectroscopy—The absorbance spectra of oxidized and reduced recombinant house fly cyt b₅ (Fig. 5) show a wavelength maximum at 413 nm in the oxidized form and at 423, 526, and 556 nm in the reduced form. These characteristics are typical of cyt b₅ (40–43).

EPR and ¹H NMR Studies—The EPR spectrum of the recombinant cyt b₅ from house fly is shown in Fig. 6. The rhombic signal with three resolved g values (g₁ = 3.07, g₂ = 3.22, and g₃ = 1.35) is very similar to those of other cyt b₅’s, including bovine microsomal (44), bovine erythrocye (45), recombinant rat microsomal (46), and recombinant rat outer mitochondrial membrane (45) cyt b₅’s. The NMR spectrum of the recombinant house fly ferriytochrome b₅ is shown in Fig. 7, where the conditions of spectral acquisition were optimized for observing the hyperfine-shifted heme resonances rather than the protein resonances. From the number of resonances observed and their positions as compared to the NMR spectra of other cyt b₅’s (38, 43, 47, 48) summarized in Table II, it is clear that two forms, A and B, of the protein are present in approximately equal amounts. These forms result from the two possible ways that the noncovalently attached heme molecule can be found in the protein, which differ by a 180° rotation about the a,γ meso axis of the heme (38, 49–51). Thus, based on the results of absorption, EPR, and NMR spectroscopy we conclude that the gene cloned in this study encodes a typical cyt b₅.

Functional Characterization of Recombinant House Fly Cyt b₅

In order to investigate the role of house fly cyt b₅ in insect cytochrome P450-dependent monooxygenase systems we have characterized the electron-transfer properties of the protein, i.e. redox potential and reduction by NADPH-dependent cytochrome P450 reductase. We have also studied the effect of house fly cyt b₅ on epoxidation of the cyclodiene insecticide heptachlor catalyzed by CYP6A1 in a reconstituted system.

| Fraction                  | Total protein | Cyt b₅ specific content | Cyt b₅ total content | Purification | Recovery |
|---------------------------|---------------|-------------------------|----------------------|--------------|----------|
| Whole cells               | 3160          | 4.1                     | 13,100               | —            | 100      |
| 100,000 g centrifugation  | —             | —                       | —                    | —            | —        |
| Supernatant               | —             | —                       | —                    | —            | —        |
| Pellet                    | 1395          | 6.1                     | 8,500                | 1.5          | 65       |
| Solubilized membranes     | 752           | 7.6                     | 5,700                | 1.9          | 43.5     |
| 1st DEAE-Sepharose eluate| 106           | 50                      | 5,300                | 12.2         | 40       |
| Phenyl-Sepharose eluate   | 54            | 67                      | 3,700                | 16.3         | 28       |
| 2nd DEAE-Sepharose eluate, concentrated | 44 | 68 | 3,000 | 16.6 | 23 |

**Fig. 4.** SDS-electrophoresis in 15% polyacrylamide gel. 1, lysate of BL21 (pCWori+); 2, lysate of BL21 (pCb5); 3, purified cyt b₅.

**Fig. 5.** Absorbance spectra of purified recombinant house fly cyt b₅. Solid line, oxidized form; dashed line, sodium dithionite-reduced form.

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**TABLE I**

Purification of recombinant house fly cyt b₅ from a 3-liter culture

| Fraction                  | Total protein | Cyt b₅ specific content | Cyt b₅ total content | Purification | Recovery |
|---------------------------|---------------|-------------------------|----------------------|--------------|----------|
| Whole cells               | 3160          | 4.1                     | 13,100               | —            | 100      |
| 100,000 g centrifugation  | —             | —                       | —                    | —            | —        |
| Supernatant               | —             | —                       | —                    | —            | —        |
| Pellet                    | 1395          | 6.1                     | 8,500                | 1.5          | 65       |
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| Phenyl-Sepharose eluate   | 54            | 67                      | 3,700                | 16.3         | 28       |
| 2nd DEAE-Sepharose eluate, concentrated | 44 | 68 | 3,000 | 16.6 | 23 |
reduced by P450 reductase under these conditions, as shown in the inset (Fig. 9A). An increase of P450 reductase concentration did not increase the rate of cyt b5 reduction, indicating that the rate of 5.5 s−1 is the maximal rate of cyt b5 reduction by P450 reductase in our reconstituted system. CYP6A1 reduction by P450 reductase was measured at 448 nm as the CYP6A1 ferrous CO complex formation in the presence of heptachlor, a CYP6A1 substrate (Fig. 9B). Under these conditions, about 80% of CYP6A1 was reduced by P450 reductase with an apparent first-order rate constant of 3.0 s−1. In a separate experiment, flash-photolysis of the preformed ferrous CO complex showed that CO binding by the reduced CYP6A1 was completed within 10 ms. Thus, the rate constant of 3.0 s−1 obtained in the stopped-flow experiments represents the rate of electron transfer from P450 reductase to CYP6A1. Neither the rate nor the amount of CYP6A1 reduced changed when the P450 reductase concentration was increased 3-fold (data not shown), indicating that the rate of 3.0 s−1 for CYP6A1 reduction by P450 reductase (Fig. 9B) was the maximal rate of electron transfer in our reconstituted system.

The results of the stopped-flow experiments presented in Fig. 9 demonstrate that, under our reconstitution conditions, the three purified recombinant proteins from the house fly interact in a catalytically competent manner. We therefore used the reconstituted system as a model to study the effect of cyt b5 on the catalytic turnover of CYP6A1 supported by P450 reductase. Stimulation of CYP6A1 Turnover by Cyt b5—Prior incubation of P450 reductase and CYP6A1 at high concentrations in the presence of L-α-dilauroyl-sn-glycero-3-phosphocholine and detergent was required for high rates of heptachlor epoxidation by CYP6A1. Co-reconstitution of cyt b5 with P450 reductase and CYP6A1 resulted in a stimulation of the rate of heptachlor epoxidation at both limiting and high P450 reductase concentrations (Fig. 10). CYP6A1 stimulation by cyt b5 was more prominent at lower P450 reductase concentrations (Fig. 10, inset). The dependence of CYP6A1 turnover on the P450 reductase concentration in the absence and presence of 1.0 μM cyt b5 is shown in Fig. 11. In both cases, the heptachlor epoxidation rate increased as the concentration of P450 reductase increased. The reaction obeyed simple hyperbolic kinetics (Fig. 11B) and could be described by a set of Kₘ and Vₘax values. Addition of cyt b5 increased the Vₘax of the reaction from 24 min⁻¹ to 57 min⁻¹ while it decreased the Kₘ from 0.50 μM to 0.14 μM. Thus, cyt b5 makes P450 reductase a more effective electron donor in the presence of cyt b5 despite the fact that cyt b5 drains electrons from P450 reductase.

DISCUSSION

The house fly cyt b5 is similar to mammalian, avian, plant, and yeast cyt b5 in that it is a protein with a hydrophobic C terminus and a highly conserved pair of histidine residues at the core of the N-terminal heme binding site. The noncovalently bound heme interacts with 15 amino acid residues in

### Table II

Proton NMR shifts of the heme methyl resonances and A:B ratios of various cyt b5s

| Heme methyl  | Chemical shift | A:B ratio |
|--------------|----------------|-----------|
|              | ppm            |           |
| B3-Me        | 29.9           | 20:1      |
| B8-Me        | 27.5           | 9:1       |
| A5-Me        | 23.5           |           |
| A5-Me        | 13.2           |           |
| A1-Me        | 12.6           |           |
| A:B ratio    | 20:1           | 9:1       |

* Data taken from Lee et al. (38).
* Data taken from Burch et al. (47).
* Data taken from Lloyd et al. (48).
* Data taken from Rivera et al. (43).
* Approximate values taken from Lloyd et al. (48).
* Assignments not yet confirmed.

FIG. 6. X-band EPR spectrum of recombinant house fly ferricytochrome b5 recorded at 4.3 K. The three g values are shown. The X marks an impurity signal arising from CuO in the cryostat.

FIG. 7. Proton NMR spectrum of a 1 mM solution of recombinant house fly ferri-cytochrome b5 recorded at 300 MHz in D₂O. The spectrum was recorded and plotted in such a way as to emphasize the resonances of the heme. These heme resonances are shifted outside of the normal diamagnetic window (~0–10 ppm) due to the unpaired electron on the iron(III) center. The three peaks at 30.8, 23.1, and 20.0 ppm are heme methyl resonances and the peaks at 14.9 and 14.8 ppm may also be due to heme methyls.

Chemical shift, ppm

| Chemical shift | ppm |
|----------------|-----|
| B3-Me          | 29.9|
| B8-Me          | 27.5|
| A5-Me          | 23.5|
| A5-Me          | 13.2|
| A1-Me          | 12.6|
| A:B ratio      | 20:1|

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Assignments not yet confirmed.
the crystal structure determined for the bovine enzyme (37, 38) (marked in Fig. 1). Of these, 14 are identical or highly conserved between the fly and the vertebrate proteins, and one (Ser-71) is replaced by Met in the fly protein. The "b5 fold" described by F. S. Mathews (11) has 13 invariant residues, all but one of which (Ala-34 in the house fly cyt b5, Fig. 1) are exactly conserved in the fly protein. Nonetheless, the overall amino acid positional identity of the fly and rat microsomal protein is only 48%. A partial amino acid sequence of the cyt b5 isolated from house fly microsomes3 is identical to the amino acid sequence deduced from our cDNA, thus confirming that our cDNA in fact codes for a microsomal cyt b5.

A phylogenetic analysis not only shows that all vertebrate cyt b5s cluster together, as was expected, but also reveals that the vertebrate outer mitochondrial cyt b5 is more closely related to vertebral microsomal cyt b5 than to fly, yeast, or plant cyt b5s (Fig. 2). Thus, outer mitochondrial membrane cyt b5 may have evolved from a common microsomal cyt b5 ancestor, perhaps after the deuterostome-protostome divergence. Our analysis also indicates that the sequence from D. viridis, highly similar (75% identity) to a Drosophila melanogaster cyt b5-like sequence (52) and reported earlier to be a cyt b5 (39), has some common features with cyt b5 sequences including the heme binding region, but must represent a cytochrome other than cyt b5. These Drosophila sequences are clearly distinct from cyt b5s and from proteins with a cyt b5-like core such as flavocytochrome b5 sulfite oxidase, and nitrate reductase.

Synthetic cyt b5 genes and natural cDNAs for cyt b5 have been expressed previously in E. coli, either constitutively (46, 53) or under the control of lacZ (54, 55) or T7 promoter (40, 43, 56). We have expressed the cDNA of house fly cyt b5 in the protease deficient E. coli strain BL21, under control of the double cassette of the strong synthetic isopropyl-β-D-thiogalactopyranoside-inducible Tac promoter. The high expression level of up to 9 μmol/L culture enabled us to purify the protein by a combination of conventional column chromatography techniques with good yield (20–25%) and without the help of an N-terminal His-tag as was done for the rat and human enzymes (40). Upon disruption of E. coli cells by sonication, most of the cyt b5 was found in the membrane fraction and could be solubilized by the detergent CHAPS, suggesting the presence of an intact C-terminal membrane-binding domain. This suggestion is supported by our NMR studies that show relatively broad proton resonances (Fig. 7). Because the widths of the peaks in NMR spectra are proportional to rotational correlation time of the tumbling species, which in turn is a function of its size (57), the broad peaks in the house fly cyt b5 NMR spectrum (Fig. 7) are likely to be due to cyt b5 aggregation, possibly caused by the hydrophobic C-terminal membrane-anchoring tail.

Although the house fly cyt b5 cDNA encodes a protein of 15.4 kDa molecular mass, the purified protein has a decreased electrophoretic mobility under the conditions of SDS-PAGE and migrates as a protein of about 19 kDa (Fig. 4). A 19.7-kDa apparent molecular mass was estimated by SDS-PAGE for the cyt b5 purified from house fly microsomes (58).

The availability of purified house fly cyt b5 enabled us to investigate spectral and electron-transfer properties of the protein in detail. The spectroscopic properties of house fly cyt b5 (absorption, NMR, and EPR) indicate that this is a typical cyt b5 protein. The g values of the EPR spectrum of the house fly cyt b5 (Fig. 6) are characteristic of bis-histidine-coordinated heme centers (59) in which the histidine imidazole planes are approximately parallel in orientation (60). Thus, the EPR spectrum indicates that the heme binding site of house fly cyt b5 is
essentially identical, in the presentation of the protein’s histidine ligands to the metal, to those of other cyt \( b_5 \).

NMR spectroscopy is extremely sensitive to the protein environment of the heme, the orientation of the axial ligands, and the strength of the hydrogen bonds of the N–H protons of histidine ligands to protein backbone residues (61). Because of the unpaired electron of the low spin Fe(III) heme center, many of the resonances of the protons of the heme are shifted well outside the 0–10-ppm region of the protein NMR spectrum (38, 49–51, 61, 62). The similarity of the relative intensities and chemical shifts of the heme resonances of recombinant house fly cyt \( b_5 \) to those of other cyt \( b_5 \) (38, 43, 47, 48), summarized in Table II, is striking. This finding indicates that the shape of the heme pocket of recombinant house fly cyt \( b_5 \) is similar to that of other cyt \( b_5 \). The small differences in chemical shifts represent only very minor changes in the orientation of the heme group with respect to the planes of the histidine ligands (62). The NMR spectrum (Fig. 7) also reveals that heme is incorporated into house fly cyt \( b_5 \) with the two different orientations of the heme, both forms (A and B) being present equally. Protein residues that line the heme binding pocket create different shapes for the heme pockets of the cyt \( b_5 \) from various organisms. Each protein has a different relative stability for these two forms as shown in Table II. Thus, house fly cyt \( b_5 \) is similar to both rat cyt \( b_5 \) in the lack of specificity of heme orientation. Since the recombinant bovine microsomal (47, 53) and human erythrocyte (48) cyt \( b_5 \), both expressed in \( E. coli \), have approximately 9:1 ratios, we conclude, as did Lloyd et al. (48), that the nearly complete heterogeneity of heme orientation observed for two recombinant rat proteins and house fly cyt \( b_5 \) is clearly not a result of expression in \( E. coli \) but must be due to the sizes of the protein residues that line the heme pockets of each protein.

We have developed a modified electrode that allows rapid measurement of the midpoint potential by cyclic voltammetry (34). Using this method with 0.75 mM hexamminechromium(III) chloride as charge mediator, a reduction potential of \(-70 \text{ mV} \) for recombinant rat outer mitochondrial cyt \( b_5 \) was observed, while the more time-consuming spectroelectrochemical method yielded a potential of \(-102 \text{ mV} \) (34). Under the same conditions, trypsin-cleaved bovine cyt \( b_5 \) has a midpoint potential of 0 mV versus SHE (34), very similar to its potential measured by spectroelectrochemistry. In this work, we have measured the midpoint potential of the house fly cyt \( b_5 \) by cyclic voltammetry and found it to be \(-26 \text{ mV} \) versus SHE. These results show that the house fly cyt \( b_5 \) is more similar in midpoint potential to the bovine microsomal protein (and also the rat microsomal protein) (63) than it is to the rat outer mitochondrial membrane cyt \( b_5 \) (34).

The Fe\(^{III}/Fe^{II}\) midpoint potentials of the mammalian microsomal and erythrocyte proteins measured thus far span a range of \(-9 \text{ to } +5 \text{ mV} \) (48, 64–67), and those for microsomal mung bean and yeast cyt \( b_5 \)s are \(-30 \text{ mV} \) and \(-23 \text{ mV} \), respectively (68, 69). Values for proteolytically cleaved cyt \( b_5 \) purified from insect tissues are \(-57 \text{ mV} \) in case of \( Spodoptera eridania \) (70) and \(+6 \text{ mV} \) in case of \( M. domestica \) (71). The wide range of reported values may reflect different methods used to determine the midpoint potential. Changes in overall surface charge are known to cause shifts in reduction potential, with up to \(+8 \text{ to } +12 \text{ mV} \) shift in potential for every negative charge removed (63). However, the full-length house fly cyt \( b_5 \) has a net negative charge (\(-10 \) at neutral pH) close to that of outer mitochondrial membrane cyt \( b_5 \) (\(-9 \)) yet the midpoint potentials for

\footnote{M. Rivera, personal communication.}
these two proteins are significantly different (−26 and −70 mV, respectively, as measured by cyclic voltammetry). Thus, midpoint potential of cyt b₅₆₇ cannot be rationalized on the basis of net charge alone, and investigations of appropriately designed mutants using the same titration technique will be required in order to explain the wide range of midpoint potentials of cyt b₅₆₇.

Mammalian microsomal cyt b₅₆₇ are known to be involved in P₄₅₀-dependent monoxygenase reactions (2, 3), and studies with antibodies to house fly cyt b₅ show that this protein plays a similar role in insect P₄₅₀ systems (28). We have demonstrated here that recombiant house fly cyt b₅ can be efficiently reduced by house fly microsomal NADPH-dependent P₄₅₀ reductase, and is able to stimulate the activity of house fly microsomal CYP6A1 in a reconstituted system. It is well established that two electrons are required for a complete turnover of P₄₅₀ enzymes and that NADPH-dependent P₄₅₀ reductase can provide both electrons. It is widely held that reduced cyt b₅ can serve as an alternative donor of the second electron (12, 15, 16).

Under our experimental conditions, P₄₅₀ reductase is able to provide the first electron at a rate higher than the epoxidation rate catalyzed by CYP6A1. Indeed, the rate constant of CYP6A1 reduction by P₄₅₀ reductase is 3.0 s⁻¹ (Fig. 9B). With 1.0 μM P₄₅₀ reductase and 0.05 μM CYP6A1 present in the reaction mixture, the rate of heme reduction is 3.0 mμM/s, while the rate of heptachlor epoxidation does not exceed 0.05 μM/s even in the presence of 1.0 μM cyt b₅. Moreover, total CYP6A1 turnover including both productive (heptachlor epoxidation) and nonproductive ("uncoupled") use of molecular oxygen occurs at much lower rates than heme reduction. Therefore, CYP6A1 turnover is limited by a step following the first electron transfer from P₄₅₀ reductase. When reconstituted with 1.0 μM P₄₅₀ reductase, CYP6A1 turnover increases from about 15 min⁻¹ (without cyt b₅) to about 50 min⁻¹ with 1.0 μM cyt b₅ (Fig. 11). Because the redox potential of cyt b₅ (−26 mV) is not negative enough to allow efficient transfer of the first electron, and because cyt b₅ does not increase the rate of CYP6A1 reduction by P₄₅₀ reductase, the stimulation of CYP6A1 turnover by cyt b₅ (Figs. 10 and 11) appears to be a result of an increased rate of transfer of the second electron.

Analysis of heptachlor epoxidation as a function of P₄₅₀ reductase concentration showed that cyt b₅ lowers the apparent Kₘ of CYP6A1 for P₄₅₀ reductase 3-fold. This novel observation can reflect: (a) different affinities of CYP6A1 for P₄₅₀ reductase in the presence and absence of cyt b₅; (b) the formation of different P₄₅₀ reductase-CYP6A1 complexes for first and second electron transfer which are unmasked because cyt b₅ changes the rate-limiting step of epoxidation, and (c) another unrecognized property of the reconstituted system. Experiments designed to distinguish between these possibilities are currently under way.

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