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P450 reductase and cytochrome *b*₅ interactions with cytochrome P450: Effects on house fly CYP6A1 catalysis

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Abstract

The interactions of protein components of the xenobiotic-metabolizing cytochrome P450 system, CYP6A1, P450 reductase, and cytochrome *b*₅ from the house fly (*Musca domestica*) have been characterized. CYP6A1 activity is determined by the concentration of the CYP6A1-P450 reductase complex, regardless of which protein is present in excess. Both holo- and apo-*b*₅ stimulated CYP6A1 heptachlor epoxidase and steroid hydroxylase activities and influenced the regioselectivity of testosterone hydroxylation. The conversion of CYP6A1 to its P420 form was decreased by the addition of apo-*b*₅. The effects of cytochrome *b*₅ may involve allosteric modification of the P450 enzyme that modify the conformation of the active site. The overall stoichiometry of the P450 reaction was substrate-dependent. High uncoupling of CYP6A1 was observed with generation of hydrogen peroxide, in excess over the concomitant testosterone hydroxylation or heptachlor epoxidation. Inclusion of cytochrome *b*₅ in the reconstituted system improved efficiency of oxygen consumption and electron utilization from NADPH, or coupling of the P450 reaction. Depending on the reconstitution conditions, coupling efficiency varied from 8 to 25% for heptachlor epoxidation, and from 11 to 70% for testosterone hydroxylation. Because CYP6A1 is a P450 involved in insecticide resistance, this suggests that xenobiotic metabolism by constitutively overexpressed P450s may be linked to significant oxidative stress in the cell that may carry a fitness cost.

Keywords

P450 reductase; cytochrome *b*₅; stoichiometry; uncoupling; xenobiotic metabolism; reactive oxygen species

1. Introduction

The P450 enzymes are heme-thiolate proteins that utilize iron-porphyrin complexes to activate the dioxygen molecule into various forms of highly reactive oxygen intermediates capable of oxidation of even the least reactive groups in organic compounds. These enzymes play crucial roles in the biosynthesis and metabolism of a number of endogenous substrates (steroid hormones, lipophilic signal molecules, etc.) and in the activation or detoxification of a vast

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variety of xenobiotics (Werck-Reichhart and Feyereisen, 2000). Because of their ability to oxidize compounds that are otherwise extremely stable under normal conditions, P450 enzymes have been studied extensively with respect to possible application in bioremediation or biocatalytic synthesis of biologically active compounds (Bernhardt, 2006). In insects, particular attention has been focused on the role of P450 enzymes in insecticide resistance and in the adaptation to host plants (Feyereisen, 2005; Li et al., 2007).

Activation of the oxygen molecule by a P450 enzyme and completion of the catalytic cycle requires the delivery of two electrons. In the endoplasmic reticulum, NADPH:cytochrome P450 reductase (EC 1.6.2.4; hereafter “P450 reductase”) provides electrons required for functioning of the cytochrome P450 enzymes (review in Murataliev et al., 2004). Another redox partner involved in cytochrome P450 function is cytochrome *b*₅, a small membrane-bound electron carrier hemoprotein (see Porter, 2002; Schenkman and Jansson 2003 for reviews). Cytochrome *b*₅ (hereafter *b*₅) is generally considered capable of providing the second electron in the catalytic cycle of P450. The reduction potential of *b*₅ (-26 mV for house fly *b*₅, Guzov et al., 1996) is indeed too positive for the first electron transfer to the ferric forms of P450. The effect of *b*₅ on P450 function can vary widely, and it can be required for catalysis or it can determine the fate of substrates by influencing reaction rate and/or the type of reaction catalyzed. The effect of *b*₅ varies with different cytochromes P450, and, for a given P450, depends on the substrate that is oxidized (Auchus et al., 1998; Loughran et al. 2001; Yamazaki et al., 2002; Zhang et al., 2008).

A number of studies have suggested that the interactions between the component proteins of a P450 system is not limited to electron transfer, but also include allosteric interactions that might be essential for the modulation of redox and catalytic properties of the heme iron. For example, P450 reductase binding to cytochromes P450 can be revealed by a type I spectral shift of the heme (corresponding to a low to high spin state transition) (French et al., 1980), suggesting that conformational changes can occur at the heme catalytic site upon interaction of the two proteins. As mentioned above, holo-*b*₅, a physiological redox partner of the P450 systems, modulates the catalytic properties of many P450 enzymes. Interestingly, redox inert apo-*b*₅ or Mn-*b*₅ are also known to alter catalytic properties of P450 enzymes (Yamazaki et al. 2001, 2002; Zhang et al., 2008). In other words, protein-protein interaction between P450 enzymes and their redox partners, such as P450 reductase and cytochrome *b*₅, can influence the properties of P450 without involving electron transfer.

These aspects of P450 function have not been investigated in detail for any insect P450 system, even though the role of P450 reductase and *b*₅ in P450 reactions is well established in insects (review in Feyereisen, 2005). Moreover, although the absolute requirement for NADPH and O₂ is known, no study has documented the stoichiometry of a P450 reaction in insects. Formally, one molecule of oxygen and two electrons from NADPH are needed to add one atom of oxygen to the substrate. When the ratio of NADPH or oxygen to substrate is greater than one, the P450 reaction is “uncoupled”, reflecting failed monooxygenation with the generation of superoxide, hydrogen peroxide or water (Ortiz de Montellano and De Voss, 2005). This potentially harmful (Bast, 1986) production of reactive oxygen species in P450 reactions has received scant attention in insect toxicology.

In this study we address the role of protein-protein interactions of P450 reductase and cytochrome *b*₅ on the catalytic properties of CYP6A1, a xenobiotic-metabolizing P450 from the house fly (Andersen et al., 1994, 1997; Sabourault et al., 2001; Jacobsen et al., 2006). The CYP6 family of P450 enzymes is highly diversified and is phylogenetically close to CYP3 family of vertebrates that includes the well-described human liver CYP3A4 (Feyereisen, 2006). The effect of the [P450] / [P450 reductase] ratio on enzyme turnover and stoichiometry has been analyzed in reconstituted system. We also studied the effects of cytochrome *b*₅

(both holo- and apo-*b*₅) on CYP6A1 catalysis in reconstituted systems. The use of apo-*b*₅ was thought to allow a discrimination of the redox and allosteric effects of the protein (Yamazaki et al., 2001; 2002). Because the effect of holo- and apo-*b*₅ on catalysis varies with the substrate (Auchus et al., 1998; Yamazaki et al., 2001; 2002), we used two different xenobiotic substrates, heptachlor and testosterone; the former is bulky and subject to epoxidation at a single site (Andersen et al., 1994), while the latter is planar and offers several hydroxylation sites (Jacobsen et al., 2006).

2. Materials and methods

2.1 Purification of recombinant house fly CYP6A1, P450 reductase and cytochrome *b*₅

The membrane fraction from *E. coli* cells expressing CYP6A1 was obtained and solubilized as described previously (Andersen et al., 1994). The supernatant obtained after centrifugation of the CHAPS-treated membrane suspension at 100,000 g was loaded on a DEAE-Sepharose column (2.5 × 25 cm) equilibrated with 20 mM Tris-HCl, pH 7.6, containing 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (buffer A) and 0.1% Emulgen 911. The column was washed with this buffer and CYP6A1 was eluted with a 500 mL linear gradient of 0 to 0.3 M NaCl in buffer A at a flow rate of 20 mL/hr. The P450-containing fractions were pooled, diluted 4-fold with 0.1 M Na-phosphate buffer, pH 7.4, containing 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and loaded on an Octyl-Sepharose column (2.5 × 8 cm) equilibrated with the same buffer. The column was washed with loading buffer, then sequentially with buffer A, buffer A containing 0.4% Na-cholate, and buffer A containing 0.4% Na-cholate and 0.05% Emulgen 911. CYP6A1 was eluted with buffer A containing 0.4% Na-cholate and 0.2% Emulgen 911. To remove the detergent, the enzyme was loaded on a DEAE-Sepharose column (1.5 × 10 cm). The column was washed with 20 mL of buffer A, then with 20 mL of the same buffer containing 0.5% cholate, and with another 25 mL of buffer A. The enzyme was eluted with 0.3 M NaCl in buffer A. The enzyme was concentrated by ultrafiltration, an equal volume of glycerol was added, and aliquots were stored at -80° C. Recombinant house fly P450 reductase and cytochrome *b*₅ were expressed in *E. coli* and purified as described elsewhere (Guzov et al., 1996; Murataliev et al., 1999).

Apo-*b*₅ was prepared by acid acetone extraction (Cinti and Ozols, 1975). Briefly, 650 nmol of purified *b*₅ in 2 mL of 25 mM Tris-HCl, pH 7.7 buffer was added dropwise into 30 mL of ice cold acetone containing 0.2% of HCl, and the suspension was incubated at 4°C with continuous stirring for 15 min. The protein was precipitated by centrifugation for 15 min at 3000 g. The pellet was washed with 5 mL of acetone and, after centrifugation as above, the liquid phase was removed by aspiration, and the pellet was dried under a stream of nitrogen. The dry pellet was dissolved in 1 mL of 50 mM Tris-HCl, pH 7.7 buffer containing 0.5% CHAPS, and dialyzed against three changes of 25 mM Tris-HCl, pH 7.7 buffer, 1 L each. The difference absorbance spectrum of the final preparation after reduction with sodium dithionite revealed that apo-*b*₅ retained only 0.4% of the original heme.

2.2. Activity assays

All of the reactions were carried out at 25°C. Unless indicated otherwise, an NADPH regenerating system, consisting of 2.0 mM glucose-6-phosphate and 3.0 U/mL glucose-6-phosphate dehydrogenase was used. Reconstitution of the proteins was carried out as detailed earlier (Guzov et al., 1996), except that the concentrations of CYP6A1, P450 reductase, cytochrome *b*₅, and NADPH which were as specified in the figure legends. The heptachlor epoxidase activity assay followed the published procedure (Guzov et al., 1996), except that 0.1 M Tris-HCl pH 7.7 buffer was used. Heptachlor concentration was always 50 µM. The rates were calculated based on the amount of heptachlor epoxide formed.

Testosterone oxidation was carried out using [^{14}C]testosterone as described earlier (Jacobsen et al., 2006). Analysis of the dependence of CYP6A1 activity on testosterone concentration revealed that the enzyme activity was saturated at 80–100 μM testosterone with a half-saturating concentration of 15 μM or less (data not shown). Because of the relatively low sensitivity of our assay at low testosterone concentrations, accurate measurement of a K_m was not possible. A saturating concentration of 100 μM testosterone was used in all assays.

The reaction mixture (100 μL) contained 100 μM testosterone, 50 μM NADPH, NADPH-regenerating system, and 0.02 μCi of [^{14}C]testosterone. The concentration of [^{14}C]testosterone did not exceed 3% of the total testosterone concentration. The reactions were started by addition of the enzyme mixture, incubated for 5 min at 25°C, and the reactions were stopped by addition of 100 μL of MeOH containing 10% of 5 N HCl, and 100 μL was analyzed by HPLC on a Supelco C18 column equipped with a guard column at 1 mL/min flow rate. A gradient was developed as follows: 0–5 min 50% A, 50% B, 5–25 min a linear gradient of 50 to 100% B followed by 5 min 100% B, where solvent A – water, solvent B – MeCN/H₂O/acetic acid 50/50/0.05 (v/v/v). Chromatography was monitored by both absorption at 240 nm and by flow-through radioactivity detection. Peaks of the radioactivity profile were integrated, and the activity was calculated based on the fraction of the [^{14}C]testosterone converted. Similar procedures were used to measure oxidation of androstenedione and progesterone.

2.3 Coupling stoichiometries

Because of the relatively low sensitivity of the oxygen consumption and hydrogen peroxide assays, the concentrations of the proteins, CHAPS, and phospholipids were chosen relatively high to allow accurate measurements. However, these conditions are somewhat different from those required for maximal activities (Guzov et al., 1996). The reconstitution mixture contained 2.9 mg/mL dilauroylphosphatidyl choline, 0.43% CHAPS, 42.9 μM *b*₅ or apo-*b*₅, and CYP6A1 and P450 reductase at concentrations 30 times those in the assay. The mixture was incubated on ice for 10 min, and then an aliquot was diluted 30 times into the reaction mixture containing either 50 μM heptachlor or 100 μM testosterone as the substrate and 100 μM NADPH. No NADPH regeneration system has been used in these experiments. The reactions were carried out at 25°C.

Coupling stoichiometries were measured at three different concentrations of CYP6A1 and P450 reductase: 0.2 and 0.2; 0.075 and 0.67; and 0.67 and 0.075 μM , respectively. When used, *b*₅ and apo-*b*₅ were present at a 1.43 μM concentration. Because of the different CYP6A1 to P450 reductase ratio used and because the “true” concentration of CYP6A1-P450 reductase ($\pm b_5$) complex could not be estimated accurately, the reaction rates used in this study are expressed as $\mu\text{M}/\text{min}$. This also allows an easy comparison of the catalytic activities at different protein ratios. The experiments described in this study were repeated with several different preparations of CYP6A1 and P450 reductase, and the results varied within experimental error.

Oxygen consumption assay—Oxygen consumption was measured as described by Murataliev and Feyereisen (1996). The reactions were carried out at 25°C in a final volume of 1.5 mL. The initial oxygen concentration was calculated based on the atmospheric pressure. For instrument scale calibration, oxygen was removed after 5 min incubation by adding laurate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and cytochrome P450BM3 to the final concentrations of 0.5 mM, 2 mM, 1.0 U/mL, and 0.3 μM , respectively.

NADPH oxidation assay—Preliminary experiments demonstrated that absorbance changes of the cytochromes P450, *b*₅ and P450 reductase upon addition of NADPH interfered with the accurate determination of the NADPH oxidation rate. To avoid the interference, an alternative procedure was developed. The reactions (0.5 mL final volume) were started by the addition of

the enzyme, stopped by addition of an equal volume of 2% SDS solution to denature the proteins. Absorbance at 340 nm was then measured, and NADPH concentration was calculated using $\epsilon_{340}=6.2 \text{ mM}^{-1}\text{cm}^{-1}$. For “zero” time sample, the enzyme reconstitution mixture was added after the SDS solution.

Hydrogen peroxide assay—Hydrogen peroxide production was measured as described by Hildebrandt et al. (1978). Briefly, the reaction was stopped by the addition of 150 μL of 25% trichloroacetic acid to a 600 μL reaction mixture. Samples were centrifuged at 4000 g for 20 min, and 600 μL of the clear supernatant was transferred into a clean tube for the colorimetric assay. Colored product was formed after addition of 200 μL of 20 mM ferrous ammonium sulfate followed by 200 μL of 1.8 M KSCN. Absorbance at 480 nm was measured after 20 min incubation at 25°C, and concentration of H_2O_2 was calculated using a calibration curve. The concentration of H_2O_2 solution used for calibration was measured using $\epsilon_{240}=43.6 \text{ M}^{-1}\text{cm}^{-1}$. Addition of 90 U/mL of superoxide dismutase to the reaction mixture had no effect on enzyme activities and hydrogen peroxide formation.

2.4 Other procedures

Concentrations of cytochrome b_5 and CYP6A1 were determined as described elsewhere (Guzov et al., 1996). Protein concentration was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

3. Results

3.1. P450 reductase interaction with P450 in CYP6A1 catalysis

Interaction of CYP6A1 (P450) and P450 reductase (CPR) results in formation of a complex [P450-CPR], and upon binding of NADPH, substrate, and oxygen, this complex undergoes the catalytic cycle to oxidize the substrate. The rate of the CYP6A1-catalyzed reaction is determined by the concentration of this complex, when NADPH, substrate and oxygen are not limiting:

$$v=[\text{P450} - \text{CPR}].k_{\text{cat}} \quad (1)$$

where v is the reaction rate, and k_{cat} is the catalytic constant specific for the individual P450, nature of the substrate, and reaction catalyzed. According to the mass action law, the equilibrium concentration of the [P450-CPR] complex formation will be determined as follows:

$$[\text{P450} - \text{CPR}]=K.[\text{P450}].[\text{CPR}] \quad (2)$$

where K is the equilibrium constant, [P450], [CPR], and [P450-CPR] are equilibrium concentrations of free cytochrome P450, P450 reductase and their complex, respectively. According to equation 2, the same concentration of the [P450-CPR] complex and hence, the same reaction rate should be achieved in the presence of molar excess of either P450 or P450 reductase.

We compared heptachlor epoxidation in the presence of a low concentration of CYP6A1 and increasing concentrations of P450 reductase and *vice versa*, while the concentration of holo- b_5 was maintained at 1 μM , the concentration producing near maximal activation (Guzov et al., 1996). The results (Figure 1) demonstrate that the same reaction rates can be obtained with

either CYP6A1 or P450 reductase present in excess, and half-maximal velocities were achieved at the same concentration of the varied protein, about 0.1 μM . Heptachlor epoxidase activity is thus determined by a steady-state concentration of the P450 reductase-CYP6A1 complex, and the same concentration of the complex is achieved in the presence of an excess of either P450 reductase or CYP6A1.

Similar results were obtained with testosterone as a substrate. Activities were 59.1 and 8.5 min^{-1} with and without holo- b_5 present, respectively, at 0.075 μM CYP6A1 and 0.67 μM P450 reductase. In the presence of 0.67 μM CYP6A1 and 0.075 μM P450 reductase the activities were 67.7 and 5.1 min^{-1} , with and without holo- b_5 present, respectively. These results demonstrate that as expected, CYP6A1 activity is determined by the concentration of the CYP6A1-P450 reductase complex, according to the mass action law.

3.2 Stimulation of CYP6A1 activity by cyt b_5

We showed previously that cyt b_5 stimulated catalytic activity of the enzyme toward heptachlor (Guzov et al., 1996). Here we show that hydroxylation of steroid substrates, such as testosterone, progesterone and androstenedione was stimulated 7-10 fold by holo- b_5 , compared to about 2-3 fold effect on heptachlor epoxidation (Table 1). Table 1 also shows the effect of apo- b_5 on CYP6A1 activity with the four substrates. About 2-fold stimulation of testosterone and androstenedione hydroxylation by CYP6A1 was observed in the presence of apo- b_5 , while heptachlor epoxidation and progesterone hydroxylation were stimulated by apo- b_5 by only 25-35% (Table 1).

The [^{14}C] elution profile of the testosterone hydroxylation reaction is shown in Figure 2. Incubation with the reconstituted CYP6A1 resulted in the formation of three major products, 15 β -hydroxytestosterone, 2 β -hydroxytestosterone, and a peak corresponding to the mixture of dihydroxylated derivatives, 2 β ,15 β -dihydroxytestosterone and 12 β ,15 β -dihydroxytestosterone (Jacobsen et al., 2006). Both apo- and holo- b_5 stimulated the catalytic activity of CYP6A1 without formation of any new reaction products.

3.3. Effect of P450 reductase and cytochrome b_5 on regiospecificity of testosterone hydroxylation by CYP6A1

To reveal the effect of protein-protein interactions on CYP6A1 catalysis, we studied the regiospecificity of testosterone hydroxylation at different CYP6A1/P450 reductase ratios and in the presence or absence of holo- or apo- b_5 . The ratio of 15 β :2 β monohydroxylated testosterone formed depended on the CYP6A1/P450 reductase ratio as well as on the presence or absence of holo- or apo- b_5 (Table 2). In other words, the relative rates of hydroxylation at 15 β and 2 β positions are modulated by the CYP6A1/P450 reductase ratio and by the interactions with holo- or apo- b_5 .

Hydroxylation at the 15 β position is faster than hydroxylation at the 2 β position under all conditions tested. In the presence of holo- b_5 production of 2 β -hydroxytestosterone increased compared to that of 15 β -hydroxytestosterone (Table 2) suggesting that in addition to stimulation of the hydroxylation rate, b_5 exerts some other effect on CYP6A1 catalysis, so that hydroxylation at the 2 β position is stimulated to a greater extent than that of the 15 β position. The uncoupling of the hydroxylation reactions (see below) leads to measurable levels of hydrogen peroxide being formed. This might serve in a “shunt” pathway bypassing the normal catalytic activation of oxygen in substrate hydroxylation (Ortiz de Montellano and De Voss, 2005). We have not verified whether testosterone can be hydroxylated by CYP6A1 in the presence of hydrogen peroxide.

3. 4 Effect of apo-*b*₅ on CYP6A1 thiolate stability

The results presented above showed that both holo- and apo-*b*₅ increase CYP6A1 turnover (Table 1), and alter the regiospecificity of testosterone hydroxylation (Table 2). Because apo-*b*₅ is inactive as an electron carrier, the observed effects should be attributed to some conformational changes of CYP6A1 due to the interactions of the proteins. The experiments described below provide some insight into the mechanism of the apo-*b*₅ effect.

When the content of catalytically active P450 in the reconstituted system was measured by monitoring the P450-CO complex, a slow decrease of the P450 content with time was observed (Figure 3). The absorbance maximum at 448 nm decreased with time and the peak at 420 nm increased, indicative of the P450 inactivation to P420 due to the loss of the thiolate ligand. However, in the presence of apo-*b*₅, the conversion of P450 to the P420 form was significantly slower (Figure 3).

Figure 4A compares the rates of P450 inactivation measured by decrease of the absorbance at 448 nm. Clearly, the conversion of P450 to P420 was slower when CYP6A1 was reconstituted with apo-*b*₅. The rate constants of P450 inactivation were 0.10 ± 0.04 and $0.16 \pm 0.02 \text{ min}^{-1}$ with and without apo-*b*₅ present, respectively. Similarly, formation of the P420 form was also slower in the presence of apo-*b*₅ (Figure 4B). The rate constants of 0.12 ± 0.02 and $0.19 \pm 0.02 \text{ min}^{-1}$ with and without apo-*b*₅ present, respectively, were obtained for the increase of P420, in a good agreement with the values obtained for P450 inactivation. Qualitatively the same results were obtained with holo-*b*₅ (data not shown). However, the absorbance of holo-*b*₅ present in relatively large concentration interfered with the accurate measurement of the rate constants. This stabilization of the ferrous carbonyl form of CYP6A1 by apo-*b*₅ was observed with sodium dithionite or NADPH as a reducing agent and with or without P450 reductase present. However, accurate measurement could not be carried out in the presence of P450 reductase due to the slow reduction of the P450 reductase flavins, which interfered with the assay. We checked whether *b*₅ induced any detectable change in the CYP6A1 spectral properties and we observed no Type I spectral changes of CYP6A1 upon addition of holo- or apo-*b*₅ (data not shown).

3.5 Stoichiometry of heptachlor epoxidation, testosterone hydroxylation, and effect of cytochrome *b*₅

We measured the consumption of oxygen and NADPH and the production of metabolites (heptachlor epoxide or hydroxytestosterones) as well as of hydrogen peroxide at three different ratios of CYP6A1 to P450 reductase and in the presence or absence of holo- and apo-*b*₅. The results are presented in Supplemental tables 1 and 2, and Table 3 summarizes the stoichiometries of the heptachlor epoxidation reaction and the effect of holo- and apo-*b*₅ under conditions of $[\text{P450 reductase}] > [\text{CYP6A1}]$. In all our experiments *b*₅ was used at concentrations well above the apparent K_d for P450 (Guzov et al., 1996; Noble et al., 2007; Zhang et al., 2008) and was therefore kept saturating despite the variation of the P450 to P450 reductase ratio. In the absence of *b*₅ only 9% of the reducing equivalents derived from NADPH oxidation were coupled to oxidation of the substrate, heptachlor. The rest of the electrons was wasted in side reactions leading to hydrogen peroxide. Addition of superoxide dismutase had no effect on measured rates or hydrogen peroxide levels, but we cannot rule out superoxide production. We assume that the balance of the electrons consumed are leading to the production of water with CYP6A1 functioning as terminal oxidase although this was not measured experimentally. Both apo- and holo-*b*₅ improved the efficiency of NADPH utilization for heptachlor oxidation to 13% and 18 %, respectively. In the case of testosterone hydroxylation, coupling of the reaction was higher than with heptachlor, reaching 54 to 70% in the presence of holo-*b*₅. The improvements resulted from an increased rate of heptachlor epoxidation as well as reduced rates of NADPH and oxygen consumption. Even though the efficiency of

electron utilization improved, most of the reducing equivalents was still consumed in side reactions. Electrons wasted in the production of water represented 20-30% of the NADPH consumed in the case of heptachlor epoxidation, whereas we calculated that little or no excess water was produced during testosterone hydroxylations. Holo-*b*₅ was most effective in reducing hydrogen peroxide production for both substrates. Noticeably, the ratio of oxygen and NADPH consumption was close to 1 in all cases, demonstrating that all of the reducing equivalents are accounted for. Measurements of coupling stoichiometries in the reconstituted system with different [CYP6A1]/[P450 reductase] ratios (Suppl. Tables 1 and 2) revealed that the most efficient utilization of reducing equivalents occurred in the presence of CYP6A1 excess.

4. Discussion

4.1 [CYP6A1]/[P450 reductase] ratio

The molar ratio of total cytochrome P450 to P450 reductase in the microsomal membranes of vertebrate liver can be as high as 20:1 (Estabrook et al., 1971; Shepard et al., 1983). Similarly, insect microsomes contain cytochromes P450 in excess of P450 reductase (6-18:1; Feyereisen et al., 1990). In other words, P450 reductase *in vivo* functions in the presence of a molar excess of cytochromes P450, so that one reductase molecule serves several P450s. However, P450 monooxygenase activity in reconstituted systems is usually studied in the presence of a molar excess of P450 reductase. Thus, a characterization of the function of the CYP6A1 monooxygenase system with [CYP6A1] > [P450 reductase] was desirable. We show here that CYP6A1 catalysis rate in the reconstituted system is determined by the concentration of the CYP6A1-P450 reductase complex. Formation of this complex obeys bimolecular kinetics, and the same effective concentration of the binary complex can be achieved in the presence of an excess of either protein. This observation is in agreement with the results obtained with P450 enzymes from vertebrates (Miwa and Lu, 1984; Miwa et al., 1979; Kominami et al., 2001).

We showed previously in the reconstituted system with P450 reductase present in excess over CYP6A1 that the reaction rate for heptachlor epoxidation followed Michaelis-Menten kinetics with *K_m* values for P450 reductase of 0.14 and 0.5 μ M with and without cytochrome *b*₅ present, respectively (Guzov et al., 1996). These values agree well with our current results. In a different heterologous expression system, Dunkov et al. (1997) noted that the activity of baculovirus-expressed CYP6A2 from *Drosophila melanogaster* was significantly improved by the addition of purified house fly P450 reductase and cytochrome *b*₅ to the cell lysates. Wen et al. (2003) also found that the endogenous P450 reductase activity of Sf9 cells was insufficient to reveal the full activity of baculovirus-produced CYP6B1 from *Papilio polyxenes* when they reported that co-expression of the house fly P450 reductase with CYP6B1 greatly improved enzyme activity. Highest activity was obtained at equivalent viral concentrations for each recombinant protein construct, a result that we interpret as having led to the highest concentration of the P450 - P450 reductase complex in the transfected Sf9 cells.

4.2 Effect of cytochrome *b*₅

Our results clearly show that cytochrome *b*₅ has multiple effects on CYP6A1 function. The first one, stimulation of CYP6A1 catalytic activity (Table 1), is likely a result of the electron transfer properties of holo-*b*₅. In the reconstituted system *b*₅ stimulated CYP6A1 turnover with all substrates (Table 1, see also Guzov et al., 1996), but the extent of holo-*b*₅ effect on catalysis with different substrates varied significantly. In heptachlor epoxidation, cytochrome *b*₅ increased catalytic activity about 2.5 fold, about 7-fold stimulation was observed with testosterone and progesterone as substrates, while androstenedione hydroxylation was stimulated almost 10-fold (Table 1). Some stimulation of CYP6A1 turnover was also observed

with apo-*b*₅. Heme-free apo-*b*₅ is a partially folded protein (Falzone et al., 2001) and was shown to stimulate activities of various P450 enzymes (Yamazaki et al., 2001,2002).

Our experiments with apo-*b*₅ demonstrate that cytochrome *b*₅ has an allosteric effect on CYP6A1 in addition to its electron-transfer properties, as revealed by the two observations: First, apo-*b*₅ alters the ratio of products of testosterone hydroxylation (Table 2). Second, apo-*b*₅ stabilizes the P450 form of CYP6A1 and prevents conversion to the inactive P420 form (Figures 3, 4). Neither of these observations involve and/or can be explained by electron transfer. Stability of spectrally measurable CYP3A4 was also enhanced in *E. coli* when co-expressed with cytochrome *b*₅ (Voice et al., 1999). These observations on the interaction of CYP6A1 and *b*₅ provide new insights into the importance of protein-protein interactions in a P450 system. Because CO binding is a mimic for oxygen binding, by analogy with the stabilization of the carbonyl complex we infer that the interaction of some reduced intermediate (s) of P450 catalysis with *b*₅ can protect the enzyme from the loss of the activated oxygen and/or inactivation, thus acting as a physiological control for the formation of the reactive oxygen species, and improving efficiency of electron utilization. The measurements of the reaction stoichiometry with heptachlor as a substrate revealed that apo- and holo-*b*₅ improved the efficiency of electron utilization by a factor of about 1.5 and 2.0, respectively (Table 3). The difference between the effects of apo- and holo-*b*₅ suggests that cytochrome *b*₅ can both serve as an alternative donor of the reducing equivalent and alter the catalytic properties of the CYP6A1 through protein-protein interactions.

The effect of cytochrome *b*₅ on the ratio of 15 β - and 2 β -hydroxylations of testosterone (Table 2) can be rationalized as follows. The formation of different products results from the catalytic conversions of different enzyme-substrate complexes. CYP6A1 interaction with *b*₅ may alter the ratio of these enzyme-substrate complexes and/or the rates of their catalytic conversion. This could be achieved by changing the conformation of the P450 catalytic site upon *b*₅ binding. Indeed, conformational effects of (apo)-*b*₅ on the catalytic site of CYP6A1 are evidenced by the decreased rate of conversion to the P420 form by apo-*b*₅ (Figure 3, 4). Moreover, type I spectral changes induced by *b*₅ strongly suggest that such a conformational effect also occurs with other P450s, both mammalian P450s (Bosterling and Trudell, 1982; Tamburini and Gibson, 1983) and bacterial P450 BM3 (Noble et al., 2007). Such conformational effect of *b*₅ on the P450 catalytic site may occur with or without alteration of the spectral properties of a P450 enzyme. Indeed, no type I spectral changes upon interaction with *b*₅ could be detected with CYP6A1 (see above) or with CYP4A7 (Loughran et al., 2001).

Evidence for allosteric interactions between different redox partners is also available for mammalian P450 systems. For example, reduced mammalian holo-*b*₅ increased by 7.5-fold the rate constant of P450_{LM2} autooxidation without significant oxidation of *b*₅ (Guengerich et al., 1976). However, Perret and Pompon (1998) working with membrane-bound CYP3A4 in yeast developed a model where *b*₅ works mainly in a capacitor mode, and they reported that the stimulatory effects of *b*₅ could be explained chiefly by redox effects and P450 reductase binding site overlap. More recently, Zhang et al. (2008) provided additional evidence for the binding site overlap of *b*₅ and P450 reductase in CYP2B4, resulting in an inhibition of CYP2B4 activity at high concentrations.

In this study we have not considered the role of NADH cytochrome *b*₅ reductase (EC 1.6.2.2), known to coexist with *b*₅ and P450 reductase in microsomal membranes. However, this enzyme does not increase either NADH or NADPH-dependent activities of CYP3A4 or CYP2E1 in reconstituted systems in the presence of P450 reductase and *b*₅ (Yamazaki et al., 1996a, b), and the rate of house fly *b*₅ reduction by house fly P450 reductase far exceeds the CYP6A1 catalytic rates (Guzov et al., 1996), so that the effects of *b*₅ cannot be limited by the absence of NADH cytochrome *b*₅ reductase in our experiments.

4.3 Stoichiometry of the CYP6A1 catalyzed reactions

Although the formal stoichiometry of a P450 reaction predicts that substrate, molecular oxygen and NADPH are consumed in a 1:1:1 ratio, some degree of “uncoupling” is considered more a rule than an exception. In the well known bacterial P450cam system, nearly quantitative coupling is achieved during oxidation of camphor, whereas 95% uncoupling occurs when the same enzyme (CYP101A1) oxidizes styrene (review in Ortiz de Montellano and De Voss, 2005). Here we show that the house fly enzyme CYP6A1 is considerably uncoupled for two different substrates, the insecticide heptachlor and the steroid testosterone, which, to the house fly, is a xenobiotic. The production of hydrogen peroxide was observed during oxidation of both substrates, but the balance of the stoichiometries suggested that heptachlor epoxidation was also leading to excess water production. This difference is probably attributable to subtle kinetic changes in the activation of oxygen in the active site in the presence of the two substrates. It has been well documented that cytochrome *b*₅ in other systems can improve coupling, i.e. the relative utilization of reducing equivalents with respect to monooxygenation in the overall reaction stoichiometry (review in Schenkman and Jansson, 2003). This is clearly verified in the case of heptachlor epoxidation and testosterone hydroxylation by CYP6A1. Under all our experimental conditions, the presence of holo-*b*₅ led to highest coupling and lowest production of hydrogen peroxide. The ratio of P450 to P450 reductase was also important, with the highest efficiency of electron utilization seen under conditions of [CYP6A1]>[P450 reductase] i.e. the conditions found in the microsomal membrane. However we cannot easily extrapolate in vitro results with physiological conditions where different P450s compete with each other for P450 reductase and in some cases for substrate as well. Uncoupling of P450 is nonetheless a physiological reality, and CYP2E1 induction by acetone in the rat can lead to sufficient oxidative stress to decrease survival of the treated animals (Tindberg and Ingelmann-Sundberg, 1989). Substrate-dependent uncoupling of the P450 enzymes that protect insects from their chemical environment is therefore a corollary of the detoxification process, and the toxicological as well as signaling effects of this oxidative stress generated by P450-dependent metabolism deserves further study. This would be particularly important in the case of P450-mediated insecticide resistance, when insects depend on increased metabolism of a toxic xenobiotic for survival. Oxidative stress caused by uncoupling of the constitutively overexpressed P450(s) may constitute a fitness cost of resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>b</i> ₅	cytochrome <i>b</i> ₅
apo- <i>b</i> ₅	cytochrome <i>b</i> ₅ without heme bound
CHAPS	(3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate
P450 reductase	NADPH-cytochrome P450 reductase

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Figure 1.

Heptachlor epoxidation rates with limited concentration of one protein and increasing concentration of the second one. Concentration of CYP6A1 (▽) or P450 reductase (Δ) were kept constant at 0.05 μM and the concentration of the second enzyme was increased as shown on the horizontal axis. Concentration of cytochrome b_5 was 1.0 μM. The reaction was carried out in the presence of 50 μM NADPH and 50 μM heptachlor.

Figure 2.

HPLC elution profile of the products of testosterone hydroxylation by CYP6A1. A. Reaction mixture before incubation with CYP6A1 showing the unmetabolized testosterone peak. B. Reaction mixture after incubation with complete reconstituted system. The reaction was carried out in the presence of 0.075 μM CYP6A1, 0.67 μM P450 reductase, 1.0 μM of holo- b_5 , 100 μM testosterone, 50 μM NADPH, and NADPH regenerating system.. See Jacobsen et al. (2005) for details on the identification of the metabolites (dihydroxytestosterones, 15 β , 12 β and 2 β testosterone).

Figure 3.

Stabilization effect of apo-*b*₅ on CYP6A1. CYP6A1 was reconstituted with DLPC and CHAPS, without (panel A) or with (panel B) apo-*b*₅, diluted with CO-saturated 0.1 M Tris-HCl, pH 7.7 buffer containing 50 mM heptachlor, and placed in a spectrophotometer cuvette. The reference cuvette contained buffer only. Background was corrected, CYP6A1 was reduced with sodium dithionite, and spectra were recorded with 1 min intervals. Spectra obtained at 0, 1, 2, 4, 6, and 8 min of incubation are shown (decreasing absorbance at 448 nm and increasing absorbance at 420 nm with time).

Figure 4.

Effect of apo-*b*₅ on the rate constants of CYP6A1 inactivation. The conditions were as described in the legend to Figure 3. CYP6A1 was reconstituted with (closed circles) or without (open circles) apo-*b*₅. The intensity of the peaks at 448 (panel A) and 420 nm (panel B) were measured in 1 min intervals.

Table 1**Stimulation of CYP6A1 activities by holo-*b*₅ and apo-*b*₅**

Activities were measured in the presence of 0.075 μ M CYP6A1, 0.67 μ M P450 reductase, and 1.0 μ M of holo- or apo-*b*₅, 50 μ M heptachlor or 100 μ M steroid, 50 μ M NADPH, and NADPH regenerating system. Results in turnovers/min are means $\pm$ SD of (n) measurements.

	No added <i>b</i> ₅	Holo- <i>b</i> ₅	Apo- <i>b</i> ₅
Heptachlor	11.8 $\pm$ 1.8 (4)	30.0 $\pm$ 2.1 (4)	15.9 $\pm$ 0.3 (4)
Testosterone	8.5 $\pm$ 1.2 (5)	59.1 $\pm$ 4.0 (5)	15.8 $\pm$ 1.2 (4)
Progesterone	8.2 $\pm$ 1.9 (4)	59.2 $\pm$ 3.6 (6)	10.1 $\pm$ 1.1 (3)
Androstenedione	4.3 $\pm$ 0.6 (4)	40.0 $\pm$ 1.9 (4)	9.5 $\pm$ 2.8 (5)

Table 2**Regiospecificity of testosterone hydroxylation by CYP6A1 at different [CYP6A1]/[P450 reductase] ratios and effect of holo- and apo-*b*₅**

Reaction products are designated as follows: di-OH-T: dihydroxy-testosterones; 15 β -OH-T :15 β -hydroxytestosterone, 2 β -OH-T :2 β -hydroxytestosterone. Product ratio was calculated taking 15 β -hydroxytestosterone as a reference. Reactions were carried out at three different concentrations of CYP6A1 and P450 reductase: 0.2 and 0.2; 0.075 and 0.67; and 0.67 and 0.075 μ M, respectively, 100 μ M testosterone, 50 μ M NADPH, and NADPH-regenerating system. Where indicated, holo- or apo-*b*₅ were present at 1.43 μ M.

	diOH-T :15 β -OH-T :2 β -OH-T product ratio		
	[CYP6A1]<[P450 Red]	[CYP6A1]=[P450 Red]	[CYP6A1]>[P450 Red]
CYP6A1 + P450 reductase	0.23 : 1 : 0.28	0.13 : 1 : 0.24	0.13 : 1 : 0.21
CYP6A1 + P450 reductase + apo- <i>b</i> ₅	0.42 : 1 : 0.41	0.39 : 1 : 0.38	0.19 : 1 : 0.32
CYP6A1 + P450 reductase + holo- <i>b</i> ₅	0.71 : 1 : 0.47	0.50 : 1 : 0.37	0.47 : 1 : 0.52

Stoichiometries of heptachlor epoxidation

Reactions were carried out at concentrations of CYP6A1 and P450 reductase of 0.075 and 0.67; respectively, 50 μ M heptachlor, and 100 μ M NADPH. Where indicated, holo- or apo-*b*₅ were present at 1.43 μ M. The rates of heptachlor epoxidized and of NADPH and oxygen consumption as well as H₂O₂ production are presented in μ M/min/ μ M CYP6A1) as mean of at least four measurements $\pm$ standard deviation. O₂/NADPH and heptachlor/NADPH are the ratios of reagents consumed.

	Heptachlor metabolized	NADPH consumed	O ₂ consumed	H ₂ O ₂ produced	O ₂ /NADPH ratio	Heptachlor /NADPH ratio
CYP6A1 + P450 reductase	11 $\pm$ 1.3	117 $\pm$ 8	108 $\pm$ 8	71 $\pm$ 8	1.08	0.09
CYP6A1 + P450 reductase + apo- <i>b</i> ₅	12 $\pm$ 0.4	91 $\pm$ 4	91 $\pm$ 9	63 $\pm$ 7	1.0	0.13
CYP6A1 + P450 reductase + holo- <i>b</i> ₅	17 $\pm$ 0.4	95 $\pm$ 1	91 $\pm$ 8	48 $\pm$ 4	1.04	0.18