Interaction of Apo-cytochrome b_5 with Cytochromes P4503A4 and P45017A: Relevance of Heme Transfer Reactions†

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ABSTRACT: Maximal activity of CYP3A4 is obtained using a reconstitution system consisting of NADPH−P450 reductase (CPR), dioleoylphosphatidylcholine (DOPC), an ionic detergent, and cytochrome b_5 (b_5). The mechanism by which b_5 stimulates the catalytic activity of CYP3A4 is controversial. Recent data report that apo-cytochrome b_5 (apo-b_5) can substitute for holo-b_5 by serving as an allosteric effector. These authors concluded that b_5 is not directly involved in electron transfer reactions to CYP3A4. We have studied the effect of apo-b_5 on the ability of purified CYP3A4 to catalyze the 6β-hydroxylation of testosterone and horse CYP17A to catalyze the 17,20-lyase reaction. The high molecular weight form of holo-b_5 (HMW-holo-b_5) stimulates the 6β-hydroxylation of testosterone while the low molecular weight (truncated) form of holo-b_5 (LMW-holo-b_5) does not. When added to the reconstituted system, HMW-apo-b_5 stimulates the activity of CYP3A4 to a level 50−60% of that obtained with HMW-holo-b_5. A similar stimulation of 17α-hydroxyprogesterone metabolism is seen when studying the CYP17A-catalyzed reaction. Neither LMW-holo-b_5 nor LMW-apo-b_5 stimulates the activity of CYP3A4 or CYP17A. CYP3A4 forms a complex during affinity chromatography with immobilized HMW-holo-b_5 but not with immobilized HMW-apo-b_5. Incubation of apo-b_5 with CYP3A4, using conditions required for reconstitution of enzymatic activities, results in the transfer of heme from the CYP3A4 preparation to apo-b_5, thereby forming holo-b_5. The separation of heme proteins by thiol−disulfide exchange chromatography confirms the formation of holo-b_5. A His67Ala mutant of HMW-b_5 as well as the Zn-substituted protoporphyrin derivative of HMW-b_5 do not stimulate the activity of either CYP3A4 or CYP17A. These data show that the mechanism of stimulation of CYP3A4 and CYP17A activities by apo-b_5 results from the formation of holo-b_5 by a heme transfer reaction.

Cytochrome P450 enzymes (P450 or CYP) represent an extremely large, versatile, and important group of naturally occurring monooxygenases. Many P450s catalyze the oxidative biotransformation of a wide variety of chemical compounds of different structure and size. These include endogenous and exogenous compounds such as steroids, fatty acids, prostaglandins, aromatic hydrocarbons, pesticides, and drugs, to name but a few (*1*, *2*). The P450s form the largest superfamily of structurally related proteins currently characterized. Those P450s with a greater than 40% similarity of amino acid sequence have been divided into 71 subfamilies (*3*). In general P450s from different subfamilies display different substrate specificities and profiles of catalytic activity.

In mammals, many P450s are associated with the endoplasmic reticulum of a cell, where the P450s function with an electron transfer flavoprotein, NADPH−cytochrome P450 reductase (CPR). This flavoprotein catalyzes the reduction of P450 as well as cytochrome b_5 (b_5). In a number of instances, in vitro studies have shown that b_5 can modify the rate of a P450-catalyzed reaction (*4*). However, the mechanism by which b_5 influences these P450 reactions is still not clear (*5*).

CYP3A4 plays a critical role in the biotransformation of different types of xenobiotics including orally administered drugs. This versatility has led to the hypothesis that the active site of CYP3A4 is very large in size and probably capable of simultaneously binding more than one molecule of chemicals at a time (*6*). A second characteristic of CYP3A4 is its inactivation during catalysis (*7*, *8*) and the stimulation of its activities with some flavonoids (*9*, *10*). The mechanism of the stimulatory effect of flavonoids is not clear, but it has been suggested that both substrate and effector molecules interact within the same active site and the activation effect depends on the proximity of substrate and effector molecules (*6*, *11*). A third characteristic of many CYP3A4-catalyzed reactions is the stimulation of the reaction by b_5. Why b_5 stimulates some reactions and not others is unclear. However, the stimulatory effect of b_5 on CYP3A4-catalyzed reactions remains an important consideration when evaluating rates of drug clearance and the extent of drug−drug interactions.

Recombinant CYP3A4 has been recently expressed in yeast (*12*), *E. coli* (*13*−*15*), COS cells (*16*), the baculovirus

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system (*17*), and mammalian cells (*18*) and successfully used to study the metabolism of many different chemicals. A fusion protein consisting of CYP3A4 and CPR has been expressed in *E. coli* and is also active in catalyzing the metabolism of many steroids and drugs (*19*).

The maximal catalytic activity of purified, recombinant CYP3A4 is obtained when the P450 is reconstituted with CPR and b_5 in the presence of phospholipids (*20*−*22*) and ionic detergents (*22*). It was suggested that phospholipids and detergents promote the interaction of CPR and CYP3A4 (*22*). The effect b_5 exerts on CYP3A4-catalyzed reactions is complicated and highly dependent on the reconstitution conditions used and the type of substrate oxidized. There are three hypotheses to explain the stimulatory effect of b_5 on some P450-catalyzed reactions. One hypothesis proposes that b_5 is directly involved in electron transfer reactions during the cyclic function of P450. This "electron transfer hypothesis" proposes that reduced b_5 donates the second electron required for the conversion of the ferrous−dioxygen complex of P450 for the generation of "active oxygen" (*23*, *24*). This hypothesis was supported by results showing electron transfer from reduced b_5 to P450 (*24*, *25*) and the ability of b_5 to decrease the amount of "uncoupling" of CYP3A4 reactions (*26*).

A second hypothesis proposes the formation of a complex of b_5 with P450. This complex is proposed to accept two electrons from CPR (*27*). The two-electron-reduced complex then can provide the two electrons required by P450-catalyzed reactions. A third hypothesis has recently been proposed to explain the stimulatory effect of b_5 on CYP3A4-catalyzed reactions. This hypothesis suggests that b_5 causes a conformational change of the P450 protein which influences the redox state of P450, thereby facilitating the interaction of P450 with substrate (*28*, *29*) or the electron donor flavoprotein, CPR (*30*). Recent data showing that apo-b_5 (heme-free b_5) stimulates the CYP3A4-catalyzed oxidation of testosterone and nifedipine support this hypothesis (*31*). In this case, it was shown that apo-b_5 stimulates electron flow from CPR to CYP3A4. Further evidence supporting the "conformational change hypothesis" are recent results showing that the addition of holo- or apo-b_5 to isolated yeast microsomes containing human recombinant CYP17A and CPR results in a stimulation of CYP17A activities (*32*).

The present paper reports the results of experiments designed to better understand the role that b_5 plays in P450-catalyzed reactions. We report here confirmation of the effect of apo-b_5 on the ability of purified recombinant CYP3A4 to catalyze the 6β-hydroxylation of testosterone and the ability of horse CYP17A to catalyze the 17,20-lyase reaction for the metabolism of 17α-hydroxyprogesterone to androstenedione. The HMW-apo-b_5 stimulates the activity of CYP3A4 similar to HMW-holo-b_5, reaching a level of stimulation that is 50−60% of that obtained with HMW-holo-b_5. Neither LMW-holo- nor LMW-apo-b_5 can stimulate CYP3A4 activity. The present studies also show that CYP3A4 forms a tight complex with HMW-holo-b_5 as determined by measuring optical absorbance changes associated with the reaction. These optical changes are similar to the spin-shift absorbance changes seen when substrate binds to P450. The binding of b_5 to CYP3A4 has also been shown by affinity chromatography using HMW-holo-b_5 immobilized on Sepharose 4B.

Central to the interpretation of our results is the observation that incubation of CYP3A4 with apo-b_5, under conditions used for reconstitution of enzymatic activities, results in the transfer of heme from the CYP3A4 preparation to apo-b_5, thereby forming holo-b_5. Formation of holo-b_5 has been confirmed by spectrophotometrically measuring the formation of holo-b_5 from apo-b_5 during the preincubation of apo-b_5 with CYP3A4 and the separation of heme proteins using thiol−disulfide exchange chromatography. These results suggest that the mechanism of stimulation of CYP3A4 activity by apo-b_5 may be attributed to the formation of holo-b_5, which can then function in electron transfer reactions.

MATERIALS AND METHODS

The chemicals used in the present study were purchased from the following sources: testosterone, 6β-hydroxytestosterone, 17α-hydroxyprogesterone, sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS),[1] SDS, NADPH, phenylmethylsulfonyl fluoride (PMFS), Coomassie R-250, hemin, Zn-protoporphyrin, and myoglobin (Sigma Chemical Co.); CNBr-activated Sepharose 4B (Pharmacia); Ni-NTA-Agarose (Qiagen); Emulgen 913 (Kao Atlas, Japan); agarose, low-temperature melting agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol (BRL); Bacto-Tryptone, Bacto-Peptone, and Bacto-Yeast extract (Difco Laboratories); [^{3}H]-17α-hydroxyprogesterone (NEN). Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs; prestained protein molecular weight standards were purchased from Bio-Rad. The DNA-modifying enzymes and chemicals for PCR were obtained from Promega, BRL, NEB, and Boehringer. The *E. coli* DH5α competent cells were purchased from Promega. The kit for site-directed mutagenesis (Quik Change) was obtained from Stratagene.

Expression of CYP3A4 in E. coli. *E. coli* were transformed with the plasmid pCWhum3A4HT and were plated and grown overnight. High-producing clones were selected by inoculating 10 separate colonies into 7 mL of LB for growth overnight at 37 °C with shaking. A 3 mL aliquot from each probe was used for inoculation of 1 L of TB containing thiamin (1 mM), 100 mM potassium phosphate buffer, pH 7.6, rare salt solution, and ampicillin (100 μg/mL). Cells were grown in 2.8 L Fernbach flasks maintained at 37 °C in a rotary shaker set at 185 rpm until an $OD_{600} = 0.4$ was obtained (about 5 h), after which IPTG (0.5 mM), δ-aminolevulinic acid (0.1 mM), and ampicillin (100 μg/mL) were added. The cells were then grown at 27 °C in a rotary shaker set at 125 rpm. After 40 h of growth, the cells were cooled on ice for 1 h and harvested by centrifugation at 3000*g* for 10 min.

The harvested cells from 1 L of growth media were washed by suspension in 100 mL of buffer A (50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 0.5 mM EDTA, and 0.5 mM PMSF). The washed cells were suspended in buffer A (minus EDTA) (1 volume of cells/4 volumes of buffer) and frozen at −70 °C.

[1] Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; TB-medium, terrific broth; PMSF, phenylmethylsulfonyl fluoride; DOPC, dioleoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Purification of Recombinant CYP3A4. Frozen cells were thawed, and membranes were prepared by sonication using a Fisher Scientific 550 sonic disintegrator fitted with a 1/4−20 stud (1/2 in. diameter) tip. Unbroken cells were removed by centrifugation at 3000*g* for 10 min. Membranes were harvested by centrifugation at 100000*g* for 60 min, and the membranes were suspended to a protein concentration of about 3−4 mg/mL with buffer B (50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 50 μM testosterone). Protein was solubilized from the membranes by the dropwise addition of a 10% solution of Emulgen 913 to give a final concentration of 1% w/v (i.e., approximately 3 mg of Emulgen 913 per milligram of membrane protein). The suspension was stirred for 1 h at 4 °C followed by centrifugation at 100000*g* for 30 min. The supernatant containing the solubilized membrane proteins was decanted and applied to a DE-52 column equilibrated with buffer B containing 0.2% Emulgen 913. The effluent was immediately applied to a Ni-NTA-Agarose column where CYP3A4 strongly binds. The Ni-NTA-Agarose column was washed with 10 volumes of buffer B containing 100 mM glycine, 0.3 M NaCl, 0.2% Emulgen 913, and eluted with the same buffer but containing 50 mM histidine.

Colored fractions containing CYP3A4 were diluted with 4 volumes of water containing 20% glycerol, 0.1 mM DTT, and 50 μM testosterone and applied to a hydroxyapatite column (1.5 × 10 cm). The column was washed with 5 volumes of buffer C (50 mM sodium phosphate, pH 7.2, 20% glycerol, 0.1 mM DTT), and CYP3A4 was eluted slowly with buffer C containing 300 mM sodium phosphate. Purified CYP3A4 was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 0.1 mM DTT. The purified protein was divided into aliquots and was stored frozen at −70 °C. Different preparations of purified CYP3A4 contained 14−17 nmol of P450/mg of protein and 17−23 nmol of heme/mg of protein.

Purification of Recombinant Horse CYP17A. A similar process was used for the purification of horse CYP17A except for the inclusion of 50 μM progesterone in solubilization buffer B and in the buffers used for elution of the purified protein from the Ni-NTA-Agarose column. Purified CYP17A was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 0.1 mM DTT prior to freezing for storage at −70 °C. The purified preparation of recombinant horse CYP17A contained 16.8 nmol of P450/mg of protein and 25.1 nmol of heme/mg of protein.

Purification of Recombinant Human and Rat HMW-holo-b_5. Recombinant human HMW-holo-b_5 and LMW-holo-b_5 contained a histidine tag at the N-terminal sequence and were purified as previously described (*33*, *34*).

Preparation of Apo-b_5. HMW-apo-b_5 was prepared by removal of heme by acid−acetone treatment. Highly purified human HMW-holo-b_5 or LMW-holo-b_5 (1000 nmol in 2 mL of 50 mM Tris-HCl buffer, pH 8.1) was added dropwise to 50 mL of cold acetone containing 0.2% HCl (−20 °C), and the mixture was stirred for 30 min at 0 °C. The white precipitate was recovered by centrifugation at 10000*g* for 15 min at 4 °C, dried under a stream of nitrogen gas, and homogenized in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 0.5% CHAPS, 1 mM EDTA, and 1 mM DTT. The sample was centrifuged following dialysis against three changes of 1000 mL of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% CHAPS, 1 mM EDTA, and 1 mM DTT. The HMW-apo-b_5 species showed a single band following SDS−PAGE and did not contain any heme absorbance in the Soret region following spectrophotometric analysis.

Preparation of Zn-Substituted b_5. Zn(II)-protoporphyrin was dissolved in a small volume of a 1:1 mixture of 0.5 M K_2CO_3 and methanol to give a 1 mM solution of protoporphyrin. The Zn(II)-protoporphyrin was added to a solution of HMW-apo-b_5 dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol and 0.1% CHAPS at a 1.2:1 ratio, and the mixture was incubated at 4 °C for 1 h. The mixture was dialyzed and subjected to gel-filtration using Sephadex G-25. The column (1.5 × 5 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol and 0.2% CHAPS. The fractions that eluted from the column in the void volume were then placed on a hydroxyapatite column (1.5 × 5 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol and 0.2% CHAPS. The column was washed with 20 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1% CHAPS, and the Zn-porphyrin-substituted HMW-b_5 was eluted from the column with 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1% CHAPS. The noncovalently bound Zn-metalloporphyrin, which remained bound to the hydroxyapatite column, was eluted with 400 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1% CHAPS.

Site-Directed Mutagenesis of HMW-b_5. A mutant of HMW-b_5 devoid of the heme group was prepared using site-directed mutagenesis. The heme-binding amino acid (His^{67} of rat b_5) was substituted by Ala. Site-directed mutagenesis was carried out using the Quik Change kit (Stratagene) and the following primers:

5′-CTTTGAGGACGTCGGGGCCTCTACGGATGC-3′

5′-GCATCCGTAGAGGCCCCGACGTCCTCAAAG-3′

E. coli JM109 cells were transformed with the plasmid pCWori+::RatCytb5(His67Ala). In contrast to bacterial cells expressing recombinant wild-type HMW-b_5, that are usually pink in color, cells expressing the His67Ala mutant of b_5 were colorless.

The His67Ala mutant of HMW-b_5 was purified from the membranes using the procedure described above. The final preparation of the His67Ala mutant of b_5 (about 1 mL) was dialyzed overnight against 2 L of 50 mM Tris-HCl buffer, pH 7.8, containing 20% glycerol and was stored at −70 °C.

Immunoblotting Analysis. Immunoblotting analysis was carried out as described previously (*34*), using antibodies against rat liver microsomal HMW-b_5. Immunoblotting analysis of the His67Ala mutant of b_5 was used to estimate the level of expression of the mutant protein in *E. coli* and for detection of the mutant b_5 during the purification procedure.

Spectral Studies of the Interaction of Different b_5 Species with CYP3A4. Spectrophotometric measurements were made using an Aminco DW2a spectrophotometer modified with the Olis RSM1000 Spectroscopy System. Tandem spectrophotometer cells were used to study the interaction between CYP3A4 and different types of b_5. Buffer containing CYP3A4 was placed in the first compartment of both tandem

spectrophotometer cells, and buffer was added to the other compartment. During the titration of CYP3A4, aliquots of concentrated b_5 solution were added to the CYP3A4-containing compartment of the sample cell and the buffer-containing compartment of the reference cuvette. Spectra were recorded after 5 min preincubation of the mixtures.

The equilibrium dissociation constants were calculated using nonlinear regression analysis to fit the experimentally observed absorbance changes using the equation (*35*):

$$\Delta A = \Delta A_{max}\left[\frac{K_d + [P_0] + [B_0]}{2} - \sqrt{\frac{K_d + [P_0] + [B_0]^2}{4} - [P_0][B_0]}\right]$$

where $[P_0]$ is the concentration of CYP3A4 and $[B_0]$ is the concentration of b_5 added to the reaction mixture. K_d is the equilibrium dissociation constant for the complex of b_5 and CYP3A4. ΔA is the observed absorbance change determined following the mixing of b_5 and CYP3A4. ΔA_{max} is the maximal absorbance change per unit of P450.

Stop-Flow Experiments. Stop-flow experiments were carried out using an Aminco-Morrow Stopped-Flow Apparatus attached to an Aminco DW2 spectrophotometer linked to an IBM computer by a Strawberry Tree I/O board. Components of the reactions were mixed, preincubated 10 min at 22 °C under an atmosphere of carbon monoxide, and placed in 5 mL plastic syringes. The final concentrations of components were 2 μM CYP3A4, 4 μM CPR, 2 μM b_5, 0.5 mg/mL CHAPS, and 0.3 mg/mL DOPC in one syringe and 1 mM NADPH in Tris-HCl buffer (pH 7.5) in the other syringe. Reduction kinetics of CYP3A4 were measured at 450 minus 490 nm using the extinction coefficient 91 mM^{-1} cm^{-1} (*36*).

The absorbance changes were fitted to either a one- (eq 1) or a two-exponential decay (eq 2):

$$A_t = A_0 + A_1(1 - e^{-k1t}) \quad (1)$$

$$A_t = A_0 + A_1(1 - e^{-k1t}) + A_2(1 - e^{-k2t}) \quad (2)$$

where $k1$ and $k2$ are the observed rate constants for the fast and slow phases, respectively. A_1 and A_2 are related to the initial absorbance, and A_t is the absorbance at time t. The k_{obs} for each reaction was determined as the average of at least three measurements. Nonlinear curve fitting of the data was performed with the Microcal Origin 6.0 computer program.

Studies of the Interaction of Heme with Apo-b_5. The kinetics of the interaction of heme with apo-b_5 were studied spectrophotometrically using the stopped-flow apparatus described above. The association rate constants (K_{on}) were measured by mixing 4 μM free heme in 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl in one syringe with 2.3 μM HMW-apo-b_5 dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mg/mL CHAPS and 0.3 mg/mL DOPC in the other syringe. The formation of holo-b_5 was measured at 413 nm at 25 °C. Heme dissociation from holo-b_5 (K_{off}) was measured by following the increase of absorbance at 406 nm following mixing of 10 μM apo-myoglobin with 2 μM HMW-holo-b_5. K_{eq} was calculated from the ratio k_{on}/k_{off} and is an index of the affinity of heme to apo-b_5.

The 6β-Hydroxylation of Testosterone by Purified Recombinant CYP3A4. Aliquots of recombinant CYP3A4 (25.5 μL of 39.3 μM) and recombinant rat CPR (65.8 μL of 30.4 μM) were added to DOPC (15 μL of 20 mg/mL) and CHAPS (10 μL of 50 mg/mL) followed by the addition of various amounts of recombinant human HMW-apo-b_5 or HMW-holo-b_5 (33.2 and 130 μM, respectively). The mixture was incubated 10 min at 37 °C with stirring, and the samples were diluted to a final volume of 2 mL with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, testosterone, [^{14}C]testosterone, and an NADPH regenerating system. Final concentrations in the reaction mixture were 0.5 μM CYP3A4, 1 μM CPR, 0.25 mg/mL CHAPS, 0.15 mg/mL DOPC, 100 μM testosterone, [^{14}C]testosterone (about 600 000 cpm), 8 mM sodium isocitrate, and 0.1 unit/mL isocitrate dehydrogenase.

Samples were incubated for 5 min at 37 °C, and the reaction was initiated by the addition of NADPH (1 mM final concentration). Aliquots (0.5 mL) were removed after 2, 5, and 8 min and rapidly mixed with 5 mL of methylene chloride. The organic layer was removed and dried under a stream of nitrogen. The residue was dissolved in 100 μL of methanol, and aliquots were injected into a Waters 840 HPLC instrument containing a 10 μm C_{18} μBondapak column (3.9 × 300 mm), a Spectraflow 757 absorbance detector, and an INUS β-RAM radioactive flow detector. Steroid metabolites were identified based on the retention times of known standards.

Determination of the 17,20-Lyase Activity of CYP17A. The hydroxylation of 17α-hydroxyprogesterone was catalyzed using recombinant horse CYP17A. An aliquot of concentrated CYP17A was mixed with aliquots of concentrated CPR and b_5 as described above for CYP3A4 (note: detergent and phospholipid were not used with CYP17A reactions). The preincubated samples were diluted after 10 min with 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. The final concentration of CYP17A was 0.5 μM and 1 μM CPR. Radioactive 17α-hydroxyprogesterone was dissolved in ethanol (25 mM stock solution) and used at a final concentration of 50 μM and with a specific radioactivity of about 100 000 cpm per 0.5 mL of reaction medium. The reaction was started by the addition of NADPH (final concentration 0.5 mM). Samples of 0.5 mL were removed from the incubation mixture at different time intervals and rapidly mixed with 5 mL of methylene chloride. The mixture was mixed vigorously, and the layers were separated by centrifugation. The methylene chloride layer was carefully removed and dried under a stream of nitrogen. The residue was dissolved in 100 μL of methanol, and aliquots were injected into the computerized Waters 840 HPLC instrument described above.

Immobilization of Recombinant Rat HMW-holo-b_5. Immobilization of HMW-holo-b_5 on CNBr-activated Sepharose 4B was achieved by adding a solution of HMW-holo-b_5 to the gel equilibrated with 0.1 sodium bicarbonate buffer, pH 8.3. The mixture was stirred at room temperature for 1 h and then overnight at 4 °C. Spectrophotometric analysis of the gel with HMW-holo-b_5 bound indicated approximately

100 nmol of b_5/mL. The nonreacted active groups of CNBr-activated Sepharose 4B were inactivated by washing with 0.1 M glycine followed by washing with 50 mM sodium phosphate buffer (pH 7.4) containing 1.0 M NaCl and 0.3% sodium cholate.

Preparation of Immobilized Recombinant HMW-apo-b_5. The HMW-holo-b_5−Sepharose 4B was added dropwise into a tube containing a large excess of cold acetone and 0.2% HCl cooled to −20 °C. The mixture was carefully stirred for 30 min at 0 °C and the supernatant containing the heme of b_5 removed by centrifugation at 5000*g* for 5 min. The gel was washed several times with 50 mM sodium phosphate buffer, pH 7.4, containing 1.0 M NaCl. Spectrophotometric measurements showed that essentially all the heme had been removed from immobilized HMW-holo-b_5.

Separation of CYP3A4 and b_5 by Thiol−Disulfide Exchange Chromatography. Thiopropyl-Sepharose 6B was used to separate CYP3A4 and the HMW-holo-b_5 formed from HMW-apo-b_5. Recombinant CYP3A4 (3 nmol) was mixed with 6 nmol of recombinant human HMW-apo-b_5 in 1 mL of 50 mM Tris-HCl buffer, pH 7.4. After 1 h incubation at 37 °C, the reaction mixture was applied to a Thiopropyl-Sepharose 6B column and the material washed with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$. To elute b_5, the Thiopropyl-Sepharose 6B column was washed with 0.2% sodium cholate and 0.5 M NaCl. The brown band of CYP3A4 bound to the Thiopropyl-Sepharose 6B remained on the top of the resin. To elute the bound CYP3A4, the Thiopropyl-Sepharose 6B column was washed with 0.2% sodium cholate containing 0.5 M NaCl and 20 mM DTT.

Analytical Methods. The concentration of CYP3A4 was determined spectrophotometrically using a molar extinction coefficient of 91 mM^{-1} cm^{-1} as described by Omura and Sato (*36*). The concentration of b_5 was determined from the absolute absorption spectrum of the oxidized heme protein using a molar absorbance coefficient of 117 mM^{-1} cm^{-1} at 413 nm (*37*).

Determination of the heme content of the CYP3A4 preparations was measured by the pyridine hemochromogen method. A sample of CYP3A4 was diluted with 0.2 M NaOH followed by pyridine (final concentration 20% pyridine). After reduction with sodium dithionite, the absorbance at 557 minus 575 nm was measured and the concentration of heme calculated using an extinction coefficient of 32.4 mM^{-1} cm^{-1} (*36*).

Protein concentrations were determined by the Micro BCA Protein Assay of Pierce.

SDS−PAGE was carried out using a Bio-Rad Protean II (Bio-Rad) apparatus (*38*).

RESULTS

Purification of Recombinant CYP3A4 and Spectral Properties of the Highly Purified Heme Protein. The cDNA for CYP3A4 was engineered into the pCWori$^+$HT vector as previously described for CYP17A (*39*). Expression in *E. coli* provided a high level of recombinant protein, which was purified using metallo-affinity chromatography. The yield of spectrally detectable CYP3A4 averaged 500−600 nmol of heme protein per liter of growth media. The details for purification of CYP3A4 are described under Materials and Methods.

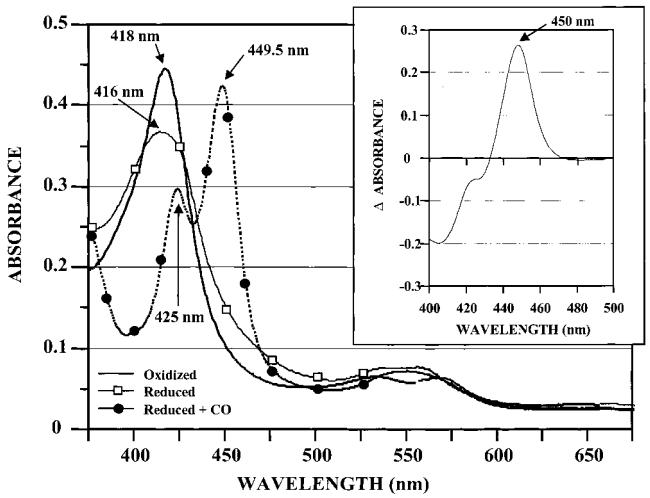

FIGURE 1: Absolute absorbance spectra of oxidized, reduced, and reduced plus CO of purified, recombinant CYP3A4. An aliquot of CYP3A4 (17.1 nmol of P450/mg of protein) was diluted to 3 μM in 3 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 50 μM testosterone and the absolute absorbance spectrum of the oxidized heme protein determined. The contents of the sample cuvette containing CYP3A4 were gassed with argon for about 10 min prior to the addition of a small amount of sodium dithionite. The spectrum of the reduced heme protein was then determined (solid line, open squares). The contents of the sample cuvette were then gassed with carbon monoxide, and the absolute spectrum of the CO-bound form of reduced CYP3A4 was recorded (dashed line with solid circles). (Inset) The difference spectrum for the CO-bound form of reduced CYP3A4 minus the absorbance of reduced CYP3A4. Experimental conditions are as described above.

Figure 1 shows the absolute absorbance spectra of purified CYP3A4 recorded in the presence of 50 μM testosterone (note: a small amount of testosterone was added during purification and recording of the spectra to stabilize the CYP3A4). The oxidize heme protein is mostly in the low-spin form with an absorbance maximum at 418 nm, similar to that seen with other recombinant P450s. The spectrum we obtain differs from that reported previously by Gilliam et al. (*40*), who reported spectral maxima at 412 nm for recombinant oxidized CYP3A4 and at 414 nm for recombinant oxidized CYP3A5. Analysis by SDS−PAGE confirms the high level of purity of our CYP3A4 preparation and shows there is only one protein band detectable by Coomassie Brilliant Blue staining in our preparation of the heme protein. Figure 1 also shows the absolute absorbance spectra of the sodium dithionite-reduced as well as the CO complex of reduced CYP3A4. Of interest is the presence (inset) of an absorbance band with a maximum at about 422 nm revealed in the difference spectrum in the presence of CO. This absorbance band is equivalent to the absorbance band with a maximum at 425 nm as seen in the absolute spectrum (Figure 1). Other experiments (not shown) provide evidence that the pigment with an absorbance at about 422 nm is slowly reduced enzymatically.

Figure 1 (inset) shows the difference spectrum of dithionite-reduced CYP3A4 treated with carbon monoxide minus reduced CYP3A4. Based on this difference spectrum, the purified preparation of CYP3A4 contains about 17 nmol of CYP3A4/mg of protein. A small shoulder of absorbance is present at about 420 nm in the final preparation of the heme protein, indicating a very low content of the CO-binding

P420 nm form of P450. Determination of the heme content of the CYP3A4 preparation by the pyridine−hemochromogen method indicated about 23 nmol of heme/mg of protein. This result shows that the presence of heme is significantly greater than the concentration of P450 determined from the CO-difference spectrum of reduced CYP3A4.

The Effect of Different Forms of b_5 on the Rate of 6β-Hydroxylation of Testosterone Catalyzed by CYP3A4. The presence of b_5 stimulates the rate of 6β-hydroxylation of testosterone as catalyzed by different preparations of CYP3A4 (*41*−*43*). For example, the presence of HMW-b_5 can exert as great as a 20-fold stimulation of the rate of testosterone metabolism when the reaction is catalyzed by a purified recombinant fusion protein containing CYP3A4 fused to CPR (*19*, *21*). A similar stimulation, albeit only 3−4-fold, is seen when HMW-holo-b_5 is added to purified recombinant CYP3A4 reconstituted with CPR, phospholipid, and detergent (*31*). Recently, it has been reported that HMW-apo-b_5 (the heme protein devoid of the heme group) is equally effective as HMW-holo-b_5 in stimulating the rate of 6β-hydroxylation of testosterone catalyzed by recombinant CYP3A4 reconstituted with CPR (*31*). Unexplained is the observation that maximal stimulation by HMW-apo-b_5 is observed at an apo-b_5:P450 ratio of 0.25:0.5.

To evaluate the effect of apo-b_5 on P450-catalyzed reactions, we studied two reactions known to be stimulated by HMW-holo-b_5: first, the 6β-hydroxylation of testosterone catalyzed by CYP3A4; and second, the 17,20-lyase reaction for the conversion of 17α-hydroxyprogesterone to androstenedione catalyzed by horse CYP17A. The effect of different forms of b_5 on the rate of 6β-hydroxylation of testosterone catalyzed by CYP3A4 is shown in Figure 2. In agreement with previous reports (*31*, *42*−*44*), HMW-holo-b_5 stimulates the rate of 6β-hydroxylation of testosterone about 2.5-fold. A maximal stimulation is reached at a ratio of b_5 to CYP3A4 of about 0.5 to 1.0.

Also shown in Figure 2, HMW-apo-b_5 is able to stimulate the 6β-hydroxylation of testosterone, but to lesser extent, i.e., about 50−60% of that obtained using HMW-holo-b_5. This result confirms recently published reports (*31*, *32*) showing a stimulatory effect of HMW-apo-b_5 on CYP3A4-catalyzed reactions. We also observed that the stimulatory effect of HMW-apo-b_5 occurs at a ratio of apo-b_5 to CYP3A4 of about 0.5.

The LMW form of b_5 (truncated form) failed to stimulate the 6β-hydroxylation of testosterone, showing that the membrane-binding domain of HMW-b_5 plays an extremely important role in facilitating the effect of b_5 on CYP3A4-catalyzed reactions.

Spectral Studies on the Interaction of b_5 with CYP3A4. To understand the mechanism by which HMW-apo-b_5 stimulates CYP3A4-catalyzed reactions, we studied the interaction of different forms of b_5 with CYP3A4 using a spectral titration method (*45*). Figure 3A shows the concentration-dependent spectral changes generated during the titration of CYP3A4 with HMW-holo-b_5. These spectral changes can be interpreted to indicate that the two heme proteins form a complex with a $K_d = 0.34$ μM (Table 1). The addition of HMW-holo-b_5 causes absorbance changes of the CYP3A4 similar to those seen when there is a shift of the spin state equilibrium of oxidized P450 following the addition of substrate. Human LMW-holo-b_5 (devoid of the membrane-binding segment) does not cause a significant spectral change when mixed with CYP3A4 (Figure 3B). This result indicates that the truncated form of b_5 reacts poorly with CYP3A4 or there is weak physical interaction that does not result in a spectral change of CYP3A4. The dissociation constant for the interaction of CYP3A4 with human LMW-b_5 is 1 order of magnitude higher when compared to HMW-holo-b_5 (Table 1). These data are in accordance with the inability of LMW-b_5 to stimulate the rate of testosterone 6β-hydroxylation catalyzed by CYP3A4.

The presence of testosterone enhances the interaction of HMW-holo-b_5 with CYP3A4. Spectral titrations show that the amplitude of spectral changes observed during the interaction of CYP3A4 with b_5 is not significantly changed in the presence of testosterone while the dissociation constant ($K_d = 0.08$ μM) is diminished by 5-fold (Table 1). This is in agreement with previously published data indicating that

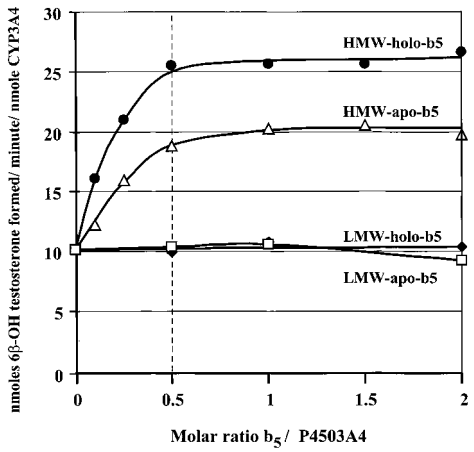

FIGURE 2: Effect of different forms of b_5 on the rate of 6β-hydroxylation of testosterone following reconstitution of CYP3A4 with CPR. An aliquot of 55 μM purified CYP3A4 was added to a mixture of CHAPS and DOPC together with an aliquot of 145 μM CPR. Aliquots of concentrated solutions of the different forms of b_5 were then added as indicated. After 10 min incubation at 37 °C, each sample of mixed concentrated proteins was diluted to 3 mL with a buffer mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, sodium isocitrate, and isocitrate dehydrogenase. The final concentrations of CYP3A4 and CPR were 0.5 and 1.0 μM, respectively. The final concentrations of the different forms of b_5 were as indicated. Testosterone (100 μM final concentration) was added followed by NADPH (1.0 mM final concentration) to initiate the reaction. The reaction samples were incubated at 37 °C, and 0.5 mL aliquots were removed at 1, 2.5, 5, and 10 min and added to 5 mL of methylene chloride with rapid mixing. Samples were prepared for HPLC analysis as described under Materials and Methods.

Table 1: Equilibrium Constants for the Interaction of Different Types of b_5 with CYP3A4[a]

type of b_5	additional system components	K_d, μM
HMW-holo-b_5	−	0.34
HMW-holo-b_5	50 μM testosterone	0.08
HMW-holo-b_5	2 μM CPR	0.02
LMW-holo-b_5	−	5.55

[a] K_d was calculated from the magnitude of spectral changes observed when human HMW-holo-b_5 interacts with CYP3A4 (Figure 4). An aliquot of CYP3A4 was diluted to a final concentration of 1 μM with 50 mM potassium phosphate buffer (pH 7.5), containing 20% glycerol, and 0.1% CHAPS.

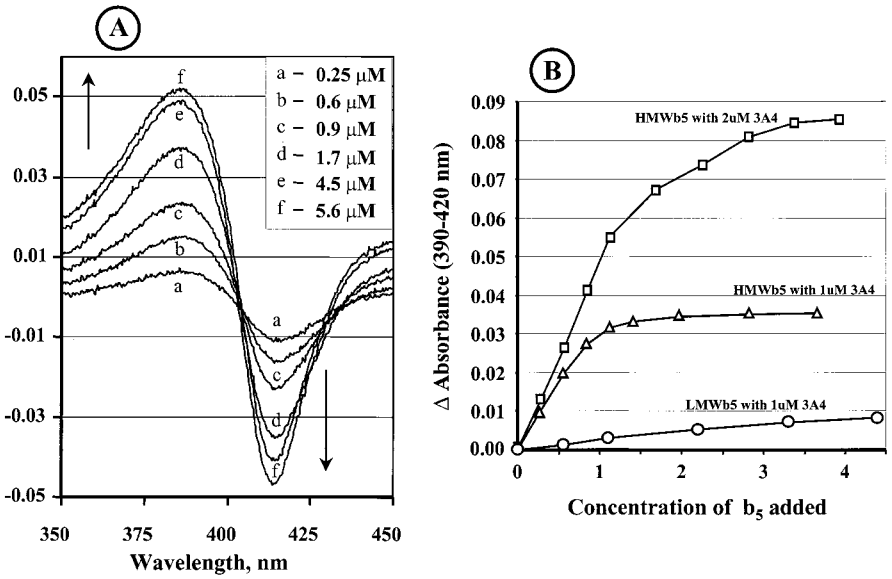

FIGURE 3: Spectral changes associated with the interaction of HMW-holo-b_5 with CYP3A4. (A) Purified CYP3A4 was diluted to 2 μM in a buffer containing 50 mM potassium phosphate (pH 7.5), 20% glycerol, and 0.1% CHAPS, and 1.4 mL aliquots were added to one compartment of each pair of "tandem cuvettes". A 1.4 mL aliquot of the buffer mixture was added to the other compartments of the cuvettes. One microliter aliquots of purified HMW-holo-b_5 were added to the compartment of the sample cuvette containing CYP3A4 and to the compartment of the reference cuvette containing buffer. The change in absorbance for the difference spectrum was then recorded. Increasing concentrations of HMW-holo-b_5 were added as indicated, and the change in absorbance at 390 nm minus 420 nm was determined. The optical path length of each compartment of the tandem cuvettes is 0.4375 cm. (B) A series of spectral titration experiments were carried out as described in (A) using 1 or 2 μM CYP3A4 and titration with HMW-holo-b_5 or LMW-holo-b_5. The magnitude of the absorbance change at 390 minus 420 nm was measured following each addition of b_5. Temperature, 22 °C.

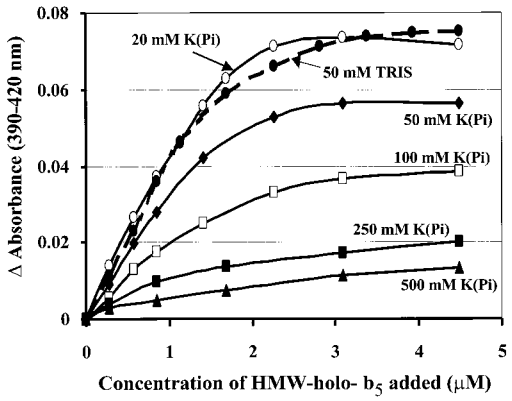

FIGURE 4: Effect of potassium phosphate buffer concentration on the binding of b_5 to CYP3A4 as measured by spectral titration experiments. A series of spectral titration experiments were carried out as described in Figure 3 using 2 μM CYP3A4 diluted in different concentrations of potassium phosphate buffer (pH 7.4). The absorbance change at 390 minus 420 nm was measured following each addition of HMW-holo-b_5. Comparison with the magnitude of spectral changes observed using 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ is shown.

substrates facilitate the interaction of b_5 with P450 (*28*, *29*, *46*).

The interaction of b_5 with P450 is thought to occur via electrostatic interactions between the negatively charged surface residues of b_5 with the positively charged residues of P450 (*47*). To check the role of electrostatic interactions in this reaction, we carried out a series of titration experiments at different ionic strengths. It was found that the interaction between the two heme proteins is dependent on the concentration of potassium phosphate buffer used (Figure 4). An increase in the concentration of potassium phosphate buffer from 20 to 100 mM results in a 2-fold decrease in the extent of spectral changes observed. A further increase of potassium phosphate buffer concentration to 500 mM essentially prevents the interaction of the two heme proteins as judged by the absence of any spectral changes.

Incubation of CYP3A4 with CPR does not dramatically influence the extent of interaction of HMW-holo-b_5 with CYP3A4. In the presence of CPR, the spectral response at higher b_5 concentrations is slightly less than that seen in the absence of flavoprotein. However, the dissociation constant for the complex of CYP3A4 and HMW-holo-b_5 is decreased significantly (Table 1), indicating that CPR does not compete but instead promotes the interaction of b_5 with CYP3A4. These data support the hypothesis that b_5 and CPR are interacting at different binding sites on the surface of CYP3A4.

Spectral Studies of the Interaction of CPR with CYP3A4. Recent reports conclude that the stimulatory effect of b_5 on CYP3A4 activities is mainly due to an increase in the rate of CYP3A4 reduction by CPR (*30*, *31*). To evaluate this hypothesis, we carried out a series of spectral titration studies to measure absorbance changes associated with CPR binding to CYP 3A4 in the presence and absence of b_5. Figure 5A shows that the addition of HMW-CPR to CYP3A4 causes absorbance changes of CYP3A4 similar to those described above for the interaction of CYP3A4 with HMW-holo-b_5. The dissociation constant of the HMW-CPR and CYP3A4 complex, calculated from the spectral changes seen at different concentrations of HMW-CPR, equals 1.58 μM. This value is significantly higher than that calculated for the binding of HMW-holo-b_5 to CYP3A4 (Table 1). This indicates that the affinity of HMW-holo-b_5 for CYP3A4 is significantly higher than the affinity of HMW-CPR for CYP3A4. In agreement with previously published data, the truncated form of CPR (LMW-CPR) does not cause a significant spectral change when added to CYP3A4 (Figure

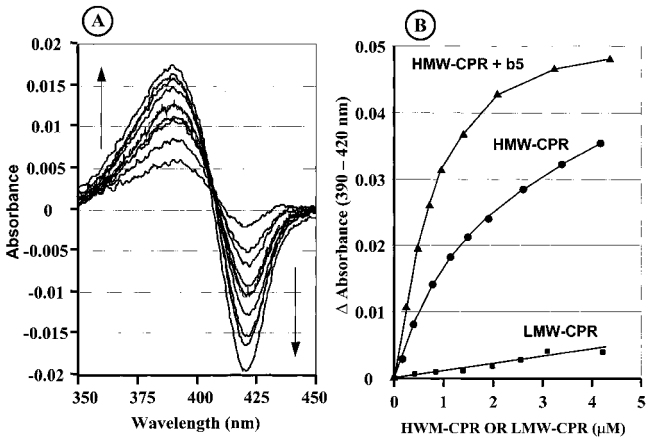

FIGURE 5: Spectral changes associated with the interaction of HMW-CPR with CYP3A4. (A) Purified CYP3A4 was diluted to 2 μM in a buffer containing 50 mM potassium phosphate (pH 7.5), 20% glycerol, and 0.1% CHAPS, and 1.4 mL aliquots were added to one compartment of each pair of "tandem cuvettes". A 1.4 mL aliquot of the buffer mixture was added to the other compartments of the cuvettes as described in Figure 3. One microliter aliquots of purified HMW-CPR were added to the compartment of the sample cuvette containing CYP3A4 and to the compartment of the reference cuvette containing buffer. The change in absorbance for the difference spectrum was then recorded. Increasing concentrations of HMW-CPR were added as indicated, and the change in absorbance at 390 nm minus 420 nm was determined. The optical path length of each compartment of the tandem cuvettes is 0.4375 cm. (B) A series of spectral titration experiments were carried out as described in (A) using 2 μM CYP3A4 and titration with HMW-CPR or LMW-CPR. The magnitude of the absorbance change at 390 minus 420 nm was measured following each addition of CPR. The experiment was repeated with 2 μM HMW-holo-b_5 included in the reaction medium containing CYP3A4.

Table 2: Equilibrium Constants for the Interaction of CPR with CYP3A4[a]

type of ligand	system components	K_d, μM
HMW-CPR	−	1.58
HMW-CPR	2 μM HMW-holo-b_5	0.92
LMW-CPR	−	ND[b]

[a] K_d was calculated from the magnitude of spectral changes observed when human HMW-CPR interacts with CYP3A4 (Figure 5). An aliquot of CYP3A4 was diluted to a final concentration 2 μM with 50 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, and 0.1% CHAPS. [b] ND: no spectral changes observed.

5B). Further, the interaction of HMW-CPR and CYP3A4 does not depend on the phosphate buffer concentration (ionic strength) of the reaction media.

The presence of HMW-holo-b_5 with CYP3A4 results in an increase in the affinity of HMW-CPR for CYP3A4. In this case, the dissociation constant for the complex of HMW-CPR with CYP3A4 (K_d) is decreased from 1.58 to 0.92 μM (Table 2). The presence of HMW-holo-b_5 has no effect on the maximal amplitude of spectral changes seen when HMW-CPR is added to CYP3A4, but HMW-holo-b_5 increases the affinity of HMW-CPR for CYP3A4. When these experiments were repeated using HMW-apo-b_5, a titration curve intermediate between those seen with HMW-CPR only and with HMW-CPR plus HMW-holo-b_5 was obtained. It appears that some HMW-apo-b_5 is converted to HMW-holo-b_5, resulting in a complex pattern of interaction. These data support the conclusion that HMW-holo-b_5 and HMW-CPR do not compete for a common binding site but interact with different sites on the surface of CYP3A4.

Table 3: First-Order Rate Constants for the Reduction of Recombinant CYP3A4[a]

system components	reducing agent	k_{fast}, min^{-1}	k_{slow}, min^{-1}
CYP3A4 + HMW-CPR	NADPH	27	6
CYP3A4 + HMW-CPR + HMW-b_5	NADPH	40	8
CYP3A4 + HMW-CPR	$Na_2S_2O_4$	17	11

[a] Aliquots of concentrated CYP3A4 and HMW-CPR were diluted with 5 mL of argon-gassed 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mg/mL CHAPS and 0.3 mg/mL DOPC. The mixture was then gassed for 2−3 min with carbon monoxide and the solution placed in one syringe of the stop-flow apparatus. When added, HMW-holo-b_5 was mixed with CYP3A4 and HMW-CPR before dilution. The final concentrations of the reactants were 2 μM CYP3A4, 4 μM HMW-CPR, and 2 μM HMW-holo-b_5. A second syringe contained the buffer mixture and 2 mM NADPH or a solution of sodium dithionite. The contents were gassed for about 5 min with CO. The reaction was initiated by mixing the contents of the two syringes using an Aminco Stopped-Flow apparatus and the absorbance change at 450 minus 490 nm recorded using an IBM computer linked to a Strawberry Tree I/O conversion board. Temperature = 22 °C.

Effect of b_5 on the Reduction Kinetics of CYP3A4. To confirm the report that both apo- and holo-b_5 facilitate the NADPH-dependent reduction of CYP3A4 by CPR, we did a series of stop-flow experiments. The reduction of CYP3A4 is rapid in both the presence and absence of HMW-holo-b_5, and in all cases the reaction is biphasic. The data for the time-dependent absorbance changes during reduction of CYP3A4 were fitted using two-exponential equations. The results of stop-flow experiments measuring the reduction of CYP3A4 are summarized in Table 3. The presence of HMW-holo-b_5 with CYP3A4 increases the rate of both the fast and slow phases of P450 reduction by HMW-CPR by about 1.5-fold. We noted that the total amount of CYP3A4 reduced by CPR in the presence of CO was significantly less than that seen following reduction with sodium dithionite. The enzymatic reduction of CYP3A4 by HMW-CPR is about 79% and 60% of that seen by chemical reduction in the presence and absence of b_5, respectively.

Affinity Chromatography of CYP3A4 on Immobilized HMW-b_5. To further prove the direct interaction of b_5 with CYP3A4, we measured the binding of CYP3A4 to immobilized HMW-holo-b_5. Figure 6 shows the results of two typical affinity chromatography experiments. The first experiment shows (Figure 6 curve with X−X) that CYP3A4 interacts tightly with the HMW-holo-b_5−Sepharose 4B affinity column. To dissociate the complex of the two heme proteins, we eluted CYP3A4 from the affinity column with a buffer containing 1 M NaCl and 0.5% CHAPS. This result indicates that both electrostatic and hydrophobic interactions appear to be involved in the formation of the complex of HMW-holo-b_5 with CYP3A4. In contrast, CYP3A4 applied to an HMW-apo-b_5−Sepharose 4B column does not bind and is eluted in the application volume. This result indicates that removal of heme from b_5 results in a loss of the ability to interact with CYP3A4.

Conversion of HMW-apo-b_5 to HMW-holo-b_5 during Incubation with CYP3A4. Since HMW-apo-b_5 stimulates the CYP3A4-catalyzed 6β-hydroxylation of testosterone, even though CYP3A4 does not interact with immobilized HMW-apo-b_5, we considered the hypothesis that the apo-b_5 (b_5

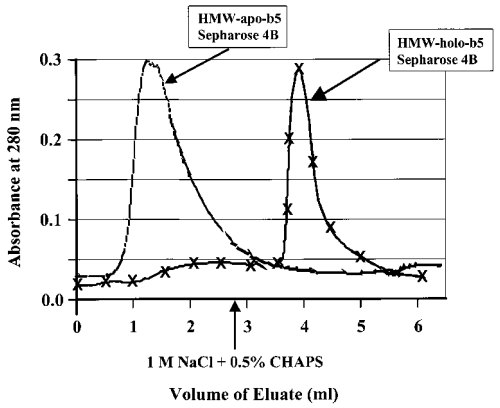

FIGURE 6: Binding of CYP3A4 to HMW-holo-b_5−Sepharose 4B or HMW-apo-b_5−Sepharose 4B. A 0.2 mL sample of CYP3A4 containing 14.6 nmol of purified protein was added to a column containing about 1.4 mL of Sepharose 4B to which either HMW-holo-b_5 or HMW-apo-b_5 had been immobilized as described under Materials and Methods. The columns were washed with 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol and 0.1% CHAPS. The arrow indicates the time when a solution of buffer containing 1.0 M NaCl plus 0.5% CHAPS was added. The absorbance at 280 nm of the effluent was measured using a Pharmacia FPLC System with a Single Path Monitor UV-1. Temperature, 4 °C.

devoid of the heme) when added to the reaction mixture may be altered during the preincubation period of the reaction, a step that is important for optimal reconstitution of CYP3A4 activities. We considered that heme, either from CYP3A4 or from another heme-containing species present in the CYP3A4 preparation, could be transferred to apo-b_5, thus generating holo-b_5. The latter is then able to efficiently stimulate the catalytic activity of CYP3A4.

To evaluate this hypothesis, we carried out a series of experiments to determine if any spectral changes occur when HMW-apo-b_5 is mixed with CYP3A4. Our first experiment (Figure 7A) showed that there was an increase in absorbance at 413 nm and loss of absorbance at 436 nm when HMW-apo-b_5 was mixed with CYP3A4. However, the conditions used for this experiment were not the optimal conditions required for reconstitution of enzymatic activities. Further, the spectral changes observed are small and difficult to interpret since they represent at least two reactions that probably are occurring simultaneously (i.e., the conversion of HMW-apo-b_5 to HMW-holo-b_5 and the binding of HMW-holo-b_5 to oxidized CYP3A4). We repeated this experiment using the conditions employed for measuring the 6β-hydroxylation of testosterone (i.e., the presence of HMW-CPR, substrate, phospholipid, detergent, and NADPH), including a preincubation for 10 min of the CYP3A4 with HMW-apo-b_5. When we scanned the difference spectrum of the mixture in the visible region of the spectrum (note: light scattering caused by the presence of phospholipid prevented an accurate measurement in the Soret region of the spectrum), we observed the absorbance spectrum of reduced holo-b_5 (Figure 7B). A second experiment was carried out where concentrated samples of proteins (including HMW-apo-b_5), phospholipid, and detergent were mixed and then immediately diluted and placed in the spectrophotometer.

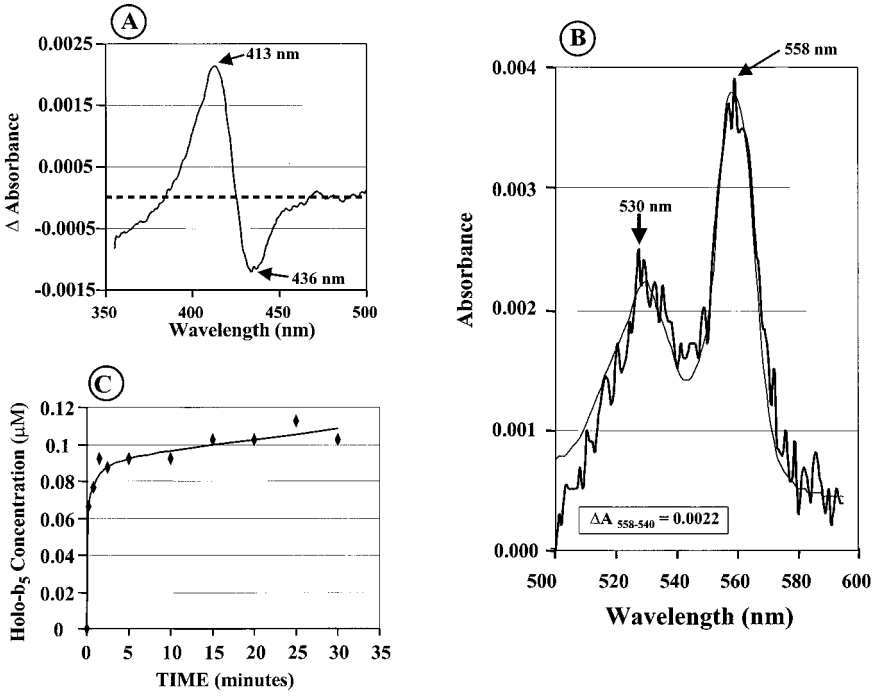

FIGURE 7: Spectral changes observed on adding HMW-apo-b_5 to CYP3A4. (A) An aliquot of CYP3A4 was diluted to a concentration of 1 μM in 6 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 0.1 mM DTT and divided equally into two cuvettes. A sample of HMW-apo-b_5 (2 μM final concentration) was added to the sample cuvette and the difference spectrum recorded after 30 min. (B) Aliquots of CYP3A4 and CPR were added to CHAPS and DOPC, preincubated about 5 min, and diluted with 50 mM Tris-HCl buffer (pH 7.4) containing sodium isocitrate, IDH, 1.0 mM NADPH, and 100 μM testosterone to give final concentrations of 0.5 μM CYP3A4 and 1.0 μM CPR as described under Materials and Methods. The diluted sample was divided equally into two cuvettes and an aliquot of HMW-apo-b_5 added to the contents of the sample cuvette to give a final concentration of 1.0 μM HMW-apo-b_5. The difference spectrum was recorded and compared with the absorbance spectrum of HMW-holo-b_5 (thin solid line curve). (C) The time course of absorbance change at 558 minus 540 nm was determined as described in (B) and the concentration of holo-b_5 formed calculated using an extinction coefficient of 20 mM^{-1} cm^{-1}. Time zero designates the time of dilution of the mix of concentrated proteins, phospholipid, detergent, and testosterone and the addition of NADPH.

Table 4: Kinetic and Equilibrium Constants for the Interaction of Heme with Apo-proteins

protein	k_{on}, $M^{-1}\,s^{-1}$ [a]	k_{off}, s^{-1} [b]	K_{eq}, M^{-1} [c]
HMW-apo-b_5	8.0×10^6	5.5×10^{-7}	1.5×10^{12}
apo-myoglobin[d]	7.0×10^7	8.4×10^{-7}	8.0×10^{13}
bovine serum albumin[d]	$\sim 5.0 \times 10^7$	1.1×10^{-2}	7.0×10^9

[a] Rate constants for the association of heme with apo-proteins were determined from stopped-flow absorbance measurements. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0) and 50 mM NaCl, at 25 °C. [b] The rate of heme dissociation from HMW-holo-b_5 was determined by mixing a 5-fold excess of apo-myoglobin with HMW-holo-b_5. The reaction mixture was 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, and 0.1 mM DTT, at 25 °C. [c] K_{eq} was calculated from the ratio k_{on}/k_{off} and is an estimate of the affinity of heme for HMW-apo-b_5. [d] Data taken from reference (*90*).

Following addition of NADPH, repetitive scanning of the spectrum showed that the conversion of apo-b_5 to form holo-b_5 is rather fast (Figure 7C). Of interest is the observation that the amount of holo-b_5 formed in this experiments approximates the amount of free heme calculated by comparing the concentration of CYP3A4, determined by the CO-difference spectrum, with the total amount of heme determined by the pyridine hemochromogen method (see above). These results show that apo-b_5, added to a reaction mixture containing CYP3A4 subjected to conditions used when measuring an enzymatic reaction, is converted to holo-b_5, which then can be reduced by CPR. This is direct evidence for the formation of holo-b_5 from apo-b_5 and shows the involvement of the formed holo-b_5 in electron transfer reactions in the reconstituted CYP3A4 system.

To directly prove the hypothesis that heme is transferred from the CYP3A4 preparation to apo-b_5, we used thiol−disulfide exchange chromatography to separate the b_5 and CYP3A4 after preincubation of the mixture as described above. This approach is based on the fact that b_5 does not contain cysteine residues and is not able to covalently interact with the Thiopropyl-Sepharose 6B affinity resin. In contrast, CYP3A4 efficiently interacts with this matrix, allowing a simple separation of the two proteins. Incubation of apo-b_5 with CYP3A4 followed by application to the thiol−disulfide column results in the retention of the proteins until the addition of an elution buffer mixture containing 0.5 M sodium chloride and 0.2% sodium cholate. The eluate was collected, and the contents were measured spectrophotometrically and by SDS−PAGE. One can detect the absorbance spectra of oxidized and reduced holo-b_5 (data not shown). We conclude that holo-b_5 is generated from apo-b_5 after incubation with CYP3A4, and the proteins can then be separated by thiol−disulfide exchange chromatography. The spectral properties of this newly formed holo-b_5 do not differ from those of native holo-b_5. The fact that apo-b_5 can accept heme from the CYP3A4 preparation and be converted to holo-b_5 provides an explanation for the way apo-b_5 stimulates P450-catalyzed reactions.

Stop-Flow Studies Measuring the Interaction of Apo-b_5 with Heme. To understand the mechanism of interaction of HMW-apo-b_5 with free heme and evaluate the affinity of heme to the apo-protein, we did stop-flow experiments to assess the kinetic parameters of this interaction. These results are presented in Table 4 and indicate that HMW-apo-b_5 has an extremely high affinity for heme with a binding constant similar to that of apo-myoglobin.

Effects of a His67Ala Mutant Form of HMW-b_5 and a Zn-Substituted b_5 on CYP3A4-Catalyzed Reactions. The data described above provide direct evidence that apo-b_5 is rapidly converted to holo-b_5 during incubation with CYP3A4. We suggest that the holo-b_5 formed from the apo-b_5 is responsible for the stimulation of CYP3A4-catalyzed reactions. To further confirm this conclusion, we engineered the cDNA for HMW-b_5 to generate a mutant protein of HMW-b_5 by replacing one of the histidine residues (His^{67}) with Ala. This mutation destroys the heme-binding properties of b_5 since His^{67} is identified as the distal ligand of b_5.

Measurement of the absolute absorbance spectrum of the highly purified recombinant His67Ala mutant protein of b_5 showed that the His67Ala mutant protein does not have any absorbance in the Soret region of the spectrum, indicating the loss of the ability of the His67Ala mutant protein to bind heme. However, the His67Ala mutant of b_5 is recognized by antibodies against rat HMW-holo-b_5, showing that both forms of the heme proteins have similar antigenic properties. Treatment of the His67Ala mutant of b_5 protein with trypsin results in the removal of the C-terminal hydrophobic fragment to form a soluble hydrophilic domain of the molecule (not shown). Recording of the circular dichroism spectra in the UV region indicates that both the His67Ala mutant protein of b_5 and HMW-holo-b_5 have a very similar content of α-helical structure. These data allow us to conclude that the His67Ala mutant of b_5 (lacking the heme group) folds in a manner similar to HMW-holo-b_5.

The His67Ala mutant of HMW-b_5 does not stimulate the 6β-hydroxylation of testosterone as catalyzed by CYP3A4 (Figure 8A). Further, spectrophotometric titration experiments indicate that the His67Ala mutant of b_5 does not cause a significant spectral change when added to CYP3A4 (Figure 8B). The main difference between the His67Ala mutant of b_5 and HMW-apo-b_5 is the inability of the mutant to accept heme. We conclude that there is an obligatory role for the heme of b_5 in the interaction with CYP3A4 and the stimulation of a CYP3A4 reaction.

To further evaluate this hypothesis, we prepared the Zn-substituted protoporphyrin derivative of HMW-b_5. The Zn-substituted protoporphyrin derivative of b_5, when added to the reconstituted system containing CYP3A4, does not stimulate the catalytic activity of CYP3A4 (Figure 8A) nor does it show a significant interaction with CYP3A4 as detected by spectral binding experiments (Figure 8B).

Effect of Apo-myoglobin on the Ability of HMW-apo-b_5 To Stimulate the 6β-Hydroxylation of Testosterone As Catalyzed by CYP3A4. The results presented above support our hypothesis that HMW-apo-b_5, when added to a preparation of CYP3A4, can accept heme, thereby reconstituting HMW-holo-b_5. Since the amount of heme available in the CYP3A4 preparation for transfer to apo-b_5 is limiting, we carried out experiments where we included apo-myoglobin to compete with apo-b_5 for available heme. Apo-myoglobin is known to possess a very high affinity for heme (Table 4). In contrast to apo-b_5, apo-myoglobin does not stimulate the activity of CYP3A4 when included in the reconstituted assay system (Figure 9). When both apo-b_5 and apo-myoglobin are present at a 1:1 ratio, the stimulation by HMW-apo-b_5 of the 6β-hydroxylation of testosterone catalyzed by CYP3A4 is not observed. Since apo-myoglobin has a higher affinity for heme than apo-b_5, we conclude that apo-myoglobin

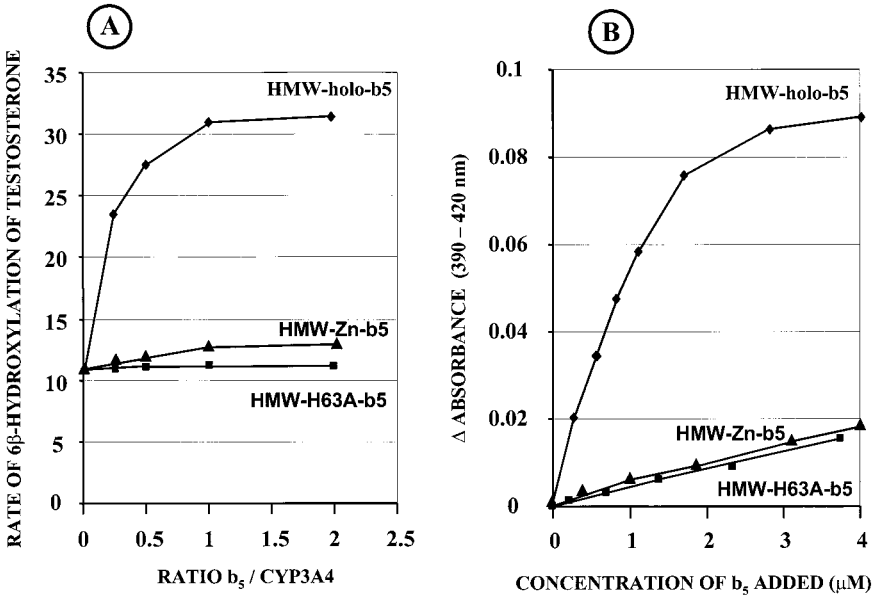

FIGURE 8: Effect of a mutant of HMW-b_5 and a Zn-porphyrin derivative of b_5 on CYP3A4 activity. A series of experiments were carried out as described in Figure 2. Aliquots of a purified mutant protein of b_5 (HMW-H67A-b_5) or the Zn-protoporphyrin derivative of HMW-apo-b_5 were added as indicated. (A) The effect on the rate of 6β-hydroxylation of testosterone. (B) Measurement of absorbance changes during spectral titration experiments.

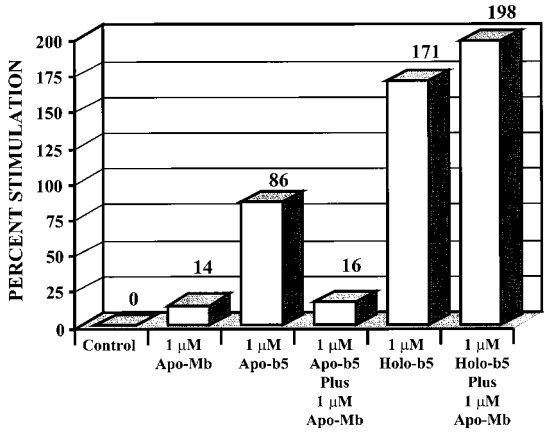

FIGURE 9: Influence of apo-myoglobin on the stimulation by HMW-apo-b_5 of the 6β-hydroxylation of testosterone catalyzed by CYP3A4. A series of experiments were carried out as described in the legend to Figure 2 using a final concentration of 0.5 μM CYP3A4 and 1 μM HMW-CPR. Where indicated, apo-myoglobin, HMW-apo-b_5, and HMW-holo-b_5 were included in the preincubation reaction media at 1 μM final concentrations. The initial concentration of testosterone was 100 μM, and the initial rate of 6β-hydroxylation in the control sample was 13 nmol min^{-1} (nmol of CYP3A4)$^{-1}$.

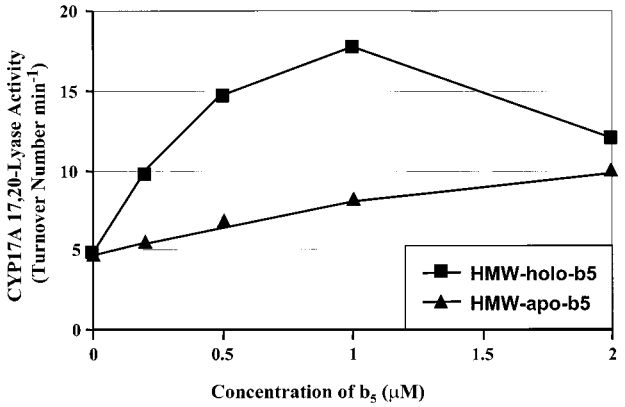

FIGURE 10: Effect of HMW-apo-b_5 on the rate of metabolism of 17α-hydroxyprogesterone by recombinant horse CYP17A. An aliquot of concentrated purified recombinant horse CYP17A was preincubated with purified recombinant HMW-CPR and the designated concentrations of HMW-holo-b_5 or HMW-apo-b_5 prior to dilution with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 50 μM 17α-hydroxyprogesterone. The final concentration of CYP17A and HMW-CPR was 1 and 2 μM, respectively. The reaction was started by the addition of 1 mM NADPH plus an NADPH generating system. Aliquots of 0.5 mL were removed at 1, 2, and 5 min and added to 5 mL of methylene chloride. After vigorous mixing, the samples were analyzed by HPLC as described under Materials and Methods.

accepts all available hemes, preventing the conversion of HMW-apo-b_5 to HMW-holo-b_5. Under the same conditions, apo-myoglobin does not affect the ability of HMW holo-b_5 to stimulate the 6β-hydroxylation of testosterone as catalyzed by CYP3A4.

Effect of Apo-b_5 on the 17,20-Lyase Activity of CYP17A. The HMW-holo-b_5 is known to stimulate the 17,20-lyase reaction catalyzed by CYP17A. Horse CYP17A has the highest 17,20-lyase activity when tested with either 17α-hydroxyprogesterone or 17α-hydroxypregnenolone as substrates. These reactions are stimulated about 4-fold in the presence of HMW-holo-b_5 (*48*). Recently, it has been reported that the 17,20-lyase activity of recombinant human CYP17A, expressed in yeast, can also be stimulated by apo-b_5 when added to yeast microsomes (*32*). The effect of different types of b_5 in stimulating the 17,20-lyase activity of horse CYP17A is shown in Figure 10. At a ratio of HMW-holo-b_5 to horse CYP17A of 1:1, the HMW-holo-b_5 significantly stimulates the 17,20-lyase activity of horse CYP17A, while apo-b_5 increases this activity by only 40%. The His67Ala mutant of b_5 and the b_5-containing Zn-substituted protoporphyrin derivative of b_5 do not affect the 17,20-lyase activity of CYP17A, indicating that heme is an obligatory element for b_5 simulation of these reactions.

DISCUSSION

Cytochrome b_5 is a low molecular weight heme protein that is widely distributed in biology and is found in most

tissues of mammals. A number of functions are attributed to b_5 including its role: (a) in the transfer of electrons from NADH to stearoyl-CoA desaturase for the oxidation of fatty acids to form unsaturated fatty acids (*49*); (b) in the hydroxylation of CMP−*N*-acetylneuraminic acid (*50*−*52*); and (c) for the NADH-dependent reduction of methemoglobin to hemoglobin in erythrocytes (*53*). Most controversial and least understood is the mechanism by which b_5 stimulates some P450-catalyzed reactions but not others (5). The direct interaction of b_5 with CPR (*54*−*57*) and different forms of microsomal P450s (*58*−*65*), as well as with mitochondrial and bacterial types of P450, such as P450cam (*66*, *67*) and P450scc (*68*), has been shown.

The primary structure of b_5 has been determined by direct protein sequencing (*69*−*71*) and confirmed by the deduced amino acid sequence determined from the nucleotide sequence of cDNA (*72*, *73*). The full-length membrane-bound form of rat b_5 consists of 134 amino acid residues (HMW-holo-b_5). Treatment of HMW-holo-b_5 with proteolytic enzymes results in the removal of about 40 amino acid residues from the C-terminal sequence, forming the so-called "soluble" or "truncated" form of b_5 (LMW-holo-b_5) (*71*). The removal of the C-terminal segment of b_5 is accompanied by a loss in the ability of the heme protein to interact with CPR and P450 (*59*−*61*). The role of the hydrophobic C-terminal fragment of b_5 in stabilizing the tertiary structure of HMW-holo-b_5 is not clear since the three-dimensional structure of b_5 has been determined only for the LMW-b_5 form of the heme protein (*74*, *75*).

Recently, the heterologous expression of HMW-holo-b_5 in microorganisms has been achieved, allowing the production of large amounts of the different forms of recombinant b_5 (*33*, *34*, *76*−*83*). This approach has been used to study, for example, the role of specific amino acids of b_5 by site-directed mutagenesis (*84*, *85*), the mechanism of interaction of the C-terminal sequence of b_5 with membrane structures (*86*), and also the mechanism of electron transfer (*87*). Despite many elegant experiments, we still do not know the mechanism(s) by which b_5 influences some P450 reactions but not others.

The effect of b_5 on P450-catalyzed reactions depends on the type of P450 studied, the nature of substrate used, and the ratio of b_5 to P450. At least three hypotheses have been proposed to explain these results: The first hypothesis proposes (*23*) a role for reduced b_5 as an electron donor to "oxy-P450", thereby supplying the "second electron" required for the cyclic function of P450. This hypothesis was based on experiments where changes in the level of steady-state reduction of b_5 were measured during monooxygenation reactions catalyzed by P450 bound to rat liver microsomes (*23*). Further, this hypothesis has been extended to explain the role of b_5 in reducing the rate of hydrogen peroxide formation (uncoupling) during some P450-catalyzed reactions (*26*).

The second hypothesis is based on the observation that b_5 directly reacts with CPR as well as P450, forming a "salt sensitive" electrostatic complex (*88*). Extension of this hypothesis proposes a role for the complex of b_5 and P450 as an acceptor of two electrons from CPR (*27*). The direct formation of a complex between P450s and b_5 has been shown by different methods including phase-separation (*59*), spectrophotometric titration (*28*, *29*, *60*−*62*), chemical cross-linking (*56*), affinity chromatography (*63*, *89*), and site-directed mutagenesis (*84*). It has been shown that negatively charged amino acid residues of b_5 are important for the electrostatic interaction with P450 (*28*, *47*). Likewise, removal of positively charged amino acids from P450 deprive it of the ability to interact with b_5 (*24*, *35*). The presence of the hydrophobic domain at the C-terminus of the b_5 protein is an obligatory prerequisite for the interaction of P450 with b_5 (*59*, *62*).

A third hypothesis has been proposed, which envisages a nonspecific, conformational effect of b_5 on P450 without the direct involvement of b_5 in electron transfer reactions associated with P450 functions. It has been shown that the presence of b_5 significantly increases the affinity of P450 for the substrate (*46*). This observation led to the suggestion that b_5 causes a change in the conformation of P450, thus facilitating the oxidation of the substrate without an involvement of b_5 in electron transfer reactions. This hypothesis is also supported by the observation that b_5 stimulates the reduction of P450 by CPR (*30*). Recently Yamazaki et al. (*31*) published a paper titled "Lack of Electron Transfer from Cytochrome b_5 in Stimulation of Catalytic Activities of Cytochrome P450 3A4". The authors concluded that "b_5 can facilitate some P450 3A4 catalyzed oxidations by complexing with P450 3A4 and enhancing its reduction by NADPH−P450 reductase, without directly transferring electrons to P450". Central to the validity of these studies were a series of experiments showing that apo-b_5 (the protein without a heme prosthetic group) was as effective as holo-b_5 in modifying the activities of CYP3A4.

The studies reported in this paper were carried out in an attempt to better define the mechanism(s) by which apo-b_5 might cause conformational changes in P450, making reduction by CPR more preferable than in the absence of b_5. Our results (Figures 2 and 9) confirm the report that HMW-apo-b_5, when added to the reconstituted system containing CYP3A4, is able to stimulate the 6β-hydroxylation of testosterone. However, we provide evidence here that HMW-apo-b_5, when added to the assay mixture required for reconstitution of the optimal activity of CYP3A4 for the 6β-hydroxylation of testosterone, results in the conversion of the added HMW-apo-b_5 to HMW-holo-b_5. We have directly shown the formation of HMW-holo-b_5 from HMW-apo-b_5 by spectrophotometric methods, by affinity chromatography, and by competition with apo-myoglobin. These results lead us to the conclusion that the presence of the heme group in b_5 is essential for the stimulatory effect of b_5 on CYP3A4 reactions. Further, the membrane-binding segment at the C-terminus of b_5 plays a critical role in this stimulation process. Since HMW-apo- or HMW-holo-b_5 stimulates testosterone 6β-hydroxylation by CYP3A4 while LMW-apo- and LMW-holo-b_5 do not, we conclude that at least two important properties of the b_5 molecule—the heme group and the hydrophobic C-terminal sequence—are essential for the interaction of b_5 with CYP3A4.

The present study leaves unanswered the question of the mechanism(s) by which HMW-holo-b_5 influences some P450 reactions but not others. In addition, the present study has not defined the source of heme in the CYP3A4 preparation responsible for the conversion of HMW-apo-b_5 to HMW-holo-b_5. Pyridine hemochromogen measurements revealed the presence of "extra heme" in the CYP3A4 preparation.

This extra heme might be associated with the pigment having an absorbance at about 425 nm (Figure 1), which is slowly reduced enzymatically. The nature of this pigment and its relationship to CYP3A4 are unknown. Spectrophotometric measurements were carried out to determine whether there was a measurable change in the content of P450 during the preincubation of CYP3A4 with HMW-apo-b_5. No significant difference in the magnitude of the absorbance at 450 nm for the CO complex of reduced P450 could be detected. Perhaps, CYP3A4 represents a complex mixture of interchangeable forms of the P450 heme protein. It is tempting to speculate that only a part of CYP3A4 in the preparation is associated with the 6β-hydroxylation of the testosterone reaction and that the real concentration of multiple species of functionally active CYP3A4s in the incubation mixture is much less.

Also difficult to explain is the stoichiometry of about 0.5 for the maximal stimulatory effect of HMW-holo-b_5 and HMW-apo-b_5 on the CYP3A4-catalyzed reaction (*31*). Perhaps the mechanism of stimulation by b_5 of a CYP3A4-catalyzed reaction is more complicated than the formation of a stoichiometric complex with CYP3A4 followed by an intramolecular electron transfer. The observation that the same ratio is seen when using either holo- or apo-b_5 suggests that in both cases the active species is the same or very similar. Finally, it is difficult to interpret the biological relevance of the proposed phenomenon where apo-b_5 might stimulate the activity of CYP3A4 or any other P450.

The observation that neither LMW-holo-b_5 nor LMW-apo-b_5 has any effect on the CYP3A4-catalyzed reaction will be the subject of a subsequent paper (Gilep et al., in preparation). These results confirm the importance of the hydrophobic C-terminal sequence of b_5 for interaction with P450 and the resultant stimulation of its activity. This observation is supported by spectral binding experiments, which indicate that in contrast to the HMW forms of b_5, neither LMW-holo-b_5 (Figure 3) nor LMW (truncated) CPR (Figure 5) induces spectral changes in CYP3A4. The role of the hydrophobic membrane binding domains of b_5 and CPR will be the subject of a subsequent publication.

The data presented in this paper raise questions about the hypothesis that b_5 stimulates CYP3A4 reactions in the absence of a heme prosthetic group. Our demonstration of an active heme transfer reaction when using HMW-apo-b_5 as an affecter of the monooxygenase reaction results in the reconstitution of HMW-holo-b_5 which may be involved in electron transfer reactions associated with P450 functions.

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