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Role of Cytochrome *b*<sub>5</sub> in Modulating Peroxide-supported CYP3A4 Activity: Evidence  
for a Conformational Transition and P450 Heterogeneity

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**Running Title:** Modulation of CYP3A4 activity by cytochrome *b*<sub>5</sub>

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**Abbreviations:**

CYP, Cytochrome P450; 7-BQ, 7-Benzyloxyquinoline; 7-BFC, 7-Benzyloxy-4-(trifluoromethyl)coumarin; 7-EFC, 7-Ethoxy-4-(trifluoromethyl)coumarin;  $\alpha$ -NF,  $\alpha$ -Naphthoflavone; SRS, Substrate recognition site; CPR, NADPH-cytochrome P450 reductase; *b*<sub>5</sub>, Cytochrome *b*<sub>5</sub>; H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide; CuOOH, Cumene hydroperoxide

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### Abstract

The role of cytochrome  $b_5$  ( $b_5$ ) in the  $\alpha$ -naphthoflavone ( $\alpha$ -NF)-mediated inhibition of  $H_2O_2$ -supported 7-benzyloxyquinoline (7-BQ) debenzoylation by heterologously expressed and purified cytochrome P450 3A4 (CYP3A4) was studied. Although  $\alpha$ -NF showed negligible effect in an NADPH-dependent reconstituted system, inhibition of 7-BQ oxidation was observed in the  $H_2O_2$  system. Analysis of the effect of various constituents of a standard reconstituted system on  $H_2O_2$ -supported activity showed that  $b_5$  alone resulted in a 2.5-fold increase in the  $k_{cat}$  value and reversed the inhibitory effect of  $\alpha$ -NF. In addition, titration with  $b_5$  suggested that only 65% of the CYP3A4 participated in the interaction with  $b_5$ , consistent with P450 heterogeneity. Study of the influence of  $b_5$  on the kinetics of  $H_2O_2$ -dependent destruction of the P450 heme moiety suggested two distinct conformers of CYP3A4 with different sensitivity to heme loss. In the absence of  $b_5$ , 66% of the wild-type enzyme was bleached in the fast phase, while the addition of  $b_5$  decreased the fraction of the fast phase to 16%. Finally, to locate amino acids residues that might influence  $b_5$  action, several active site mutants were tested. Substitution of Ser-119, Ile-301, Ala-305, Ile-369, or Ala-370 with the larger Phe or Trp decreased or even abolished the activation by  $b_5$ . Ser-119 is in the B'-C loop, a predicted  $b_5$ -P450 interaction site, and Ile-301 and Ala-305 are closest to the heme. In conclusion, the interaction of  $b_5$  with P450 apparently leads to a conformational transition, which results in re-distribution of the CYP3A4 pool.

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Cytochrome P450 3A4 (CYP3A4) is the most abundant P450 enzyme in human liver and plays a significant role in the metabolism of a wide variety of drugs (Guengerich, 1999; Nebert and Russel, 2002). CYP3A4 demonstrates homotropic cooperativity (sigmoidal  $v$  versus  $S$  plot) with a number of substrates (Harlow and Halpert, 1998; Hutzler and Tracy, 2002; Ueng et al., 1997). The enzyme is also known to exhibit heterotropic cooperativity, which is characterized by increased oxidation of one substrate in the presence of an effector, such as  $\alpha$ -naphthoflavone ( $\alpha$ -NF), which may also serve as a substrate (Harlow and Halpert, 1998; He et al., 2003; Domanski et al., 2000, 2001). Interestingly, activation by  $\alpha$ -NF of CYP3A4 activity is reversed when the reaction is carried out using cumene hydroperoxide (CuOOH),  $H_2O_2$  or iodosobenzene (Ueng et al., 1997). In addition, CYP3A4 cooperativity may be influenced by the levels of the redox partners CPR and  $b_5$  relative to the P450. Therefore, it is likely that the different effects of  $\alpha$ -NF on CYP3A4 in the NADPH vs. peroxide-supported reactions reflect the reconstitution conditions, although in one study the removal of  $b_5$  from the standard reconstituted system did not prevent stimulation by  $\alpha$ -NF of carbamazepine 10, 11-epoxidation at a single concentration of substrate,  $\alpha$ -NF, and  $b_5$  (Ueng et al., 1997).

The role of CPR as a supplier of electron(s) for P450-mediated oxidations is well studied. However, the mode of action of  $b_5$  remains controversial (reviewed in Schenkman and Jansson, 2003). Although the role of  $b_5$  as a source of electrons for P450 is well known (Yamazaki et al., 2001; Guryev et al., 2001; Schenkman and Jansson, 1999), increasing evidence points to an allosteric effect of  $b_5$  mediated in part by an effect on the P450 spin state (Tamburini and Gibson, 1993; Jansson et al., 1985; Reed and Holenberg, 2003). The modulatory effect of  $b_5$  is further supported by the fact that  $b_5$  not

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only increases P450 activity but in some cases also inhibits activity (Yamaori et al., 2003; Reed and Hollenberg, 2003). For instance,  $b_5$  activates oxidation of several drugs by CYP3A4 and 3A5, while inhibiting the activity of CYP3A7 (Yamaori et al., 2003). In addition, the interactions with cytochrome  $b_5$  may affect the degree of oligomerization of cytochrome P450 in the membranes, as demonstrated for P450 1A2 by rotational diffusion measurements (Yamada et al., 1995). High pressure spectroscopic studies with CYP3A4 and CYP2B4 in solution and in the microsomes have revealed the presence of two conformers with different positions of spin equilibrium and different barotropic properties (Davydov et al., 1995, 2003). It is likely that the heterogeneity of the P450 pool arises from protein-protein interactions between different P450 molecules and between P450 and its redox partners.

More recently an allosteric effect of redox partners on CYP3A4 was demonstrated by changes in *N*-protoporphyrin regioisomer formation from phenyldiazene (Yamaguchi et al., 2003). Substitution of certain active site residues as in S119W, T309A, and A370F also caused a significant change in regioisomer formation and amplified the effects of the redox partners. This study strongly suggested that a conformational change occurs due to protein-protein interactions between CYP3A4 and  $b_5$  and modulates active site topology. The present study investigates the role of  $b_5$  in H<sub>2</sub>O<sub>2</sub>-supported 7-benzyloxyquinoline (7-BQ) debenzoylation by CYP3A4. The results suggest an important mechanism by which  $b_5$  modulates CYP3A4 activity and provide further evidence for P450 heterogeneity.

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## Material and Methods

**Materials.** 7-Benzyloxy-4-(trifluoromethyl)coumarin (7-BFC) and 7-BQ were purchased from Gentest (Woburn, MA), and 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was purchased from Molecular Probes, Inc. (Eugene, OR).  $\alpha$ -NF was from Sigma Chemical Co. (St. Louis, MO). Recombinant CPR and  $b_5$  from rat liver were prepared as described previously (Harlow and Halpert, 1997). All other chemicals were of the highest grade available from standard commercial sources.

**Expression and purification of CYP3A4 and mutants.** CYP3A4 wild type and mutants were expressed as His-tagged proteins in *Escherichia coli* TOPP3 and purified using Talon metal affinity resin (CLONTECH, Palo Alto, CA), as described previously (Harlow and Halpert, 1998; Domanski et al., 2001). P450 contents were determined by measuring reduced carbon monoxide difference spectra. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and bovine serum albumin as a standard. The specific content of CYP3A4 wild type was 15 nmol P450 per mg protein, and of mutants 3-12 nmol/mg protein (Table 2).

**Preparation of CYP3A4 apo-protein.** Removal of the heme from CYP3A4 and preparation of the apo-protein was performed by treatment with H<sub>2</sub>O<sub>2</sub> (Uvarov, et al., 1990; Pikuleva et al 1992). We implemented very mild conditions of treatment to prevent any peroxidative damage of the protein. In brief, CYP3A4 was diluted to 50  $\mu$ M in 100 mM Na-phosphate buffer, pH 7.2, 20% glycerol (Buffer A). Catalase was added directly to this solution to a final concentration of 1.7 units/ml. The sample was then

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dialyzed in a Spectra/Por Type I molecular weight cut-off 6000-8000, 10 cm flat width dialysis bag (Spectrum Lab, CA) against 50 volumes of buffer A containing 100 mM H<sub>2</sub>O<sub>2</sub>. Dialysis was performed at 4°C with continuous stirring for 24 h. The sample was removed and was further dialyzed against 50 volumes of 100 mM Mops, pH 7.4, 10% glycerol, 1mM EDTA, 0.2 mM DTT. Protein concentration was determined using the Bradford protein assay kit (BioRad, Hercules, CA).

**7-BQ oxidation assay.** The enzyme assay was essentially done as described previously with P450eryF (Khan and Halpert, 2002) except for the use of a 300- $\mu$ l 96-well microtiter plate and a Biomek 2000 robotic sample handling system, as described previously (Kumar et al., 2005). In brief, a substrate mixture of different concentrations (0-200  $\mu$ M) in the absence and presence of  $\alpha$ -NF (as defined in the individual experiments) was prepared in 100 mM Hepes buffer, pH 7.4, with 2% as the final concentration of methanol. The substrate mixture was pre-incubated with 25 pmol CYP3A4 (in the absence and presence of various redox partners as defined in the individual experiments) for 5 min at room temperature in a 300- $\mu$ l 96-well microplate before initiation of the reaction with H<sub>2</sub>O<sub>2</sub> (10 mM final). The total reaction volume of the assay was 100  $\mu$ l. Thus, the concentration of CYP3A4 in the reaction mixture was equal to 0.25  $\mu$ M, if not otherwise indicated. After 5 min of incubation, the reactions were stopped by adding 340 units (50  $\mu$ l) of catalase. Subsequently, 50  $\mu$ l of 100 mM Hepes buffer, pH 7.4, was added before recording the fluorescence intensity at  $\lambda_{\text{ex}} = 405$  and  $\lambda_{\text{em}} = 510$  nm using a fluorescence plate reader (Ascent Fluoroscanner, Ramsey, MN). In all the cases, the final activity was calculated by comparison to a standard curve. The  $k_{\text{cat}}$ ,  $S_{50}$ , and Hill

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coefficient ( $n$ ) were determined by fitting the data to the Hill equation using the SpectraLab software package (Davydov et al., 1995).

**P450 heme-depletion assay.** Determination of the kinetics of CYP3A4 heme depletion in the presence of H<sub>2</sub>O<sub>2</sub> was done using conditions similar to those previously described (Uvarov, 1990; Pikuleva et al., 1992). The reaction was carried out at 25°C in 100 mM Hepes buffer, pH 7.4, in 1 ml semi-micro spectrophotometric cell with constant stirring. The reaction mixture contained 1 μM protein and 60 mM H<sub>2</sub>O<sub>2</sub>. Bleaching of the hemoprotein was followed by measuring a series of absorbance spectra in the 340-700 nm range at 2 - 5 min time intervals over a 30 - 60 min incubation time. Each series contained at least 10 spectra. The measurements were done with a Shimadzu-2600 spectrophotometer. Determination of the total concentration of the heme protein was done by linear least square approximation of the spectra by a linear combination of spectral standards of CYP3A4 low-spin, high-spin and P420-states (Davydov et al., 2003). Fluctuations of the base line due to turbidity changes were compensated by polynomial correction in combination with principal component analysis (PCA) technique as previously described (Davydov et al., 1995, 2003; Renaud et al., 1996). All data treatment and fitting of the titration curves were performed with our SpectraLab software package (Davydov et al., 1995).



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## Results and Discussion

**Hydrogen peroxide-supported 7-BQ debenzilation by CYP3A4.** Previous studies showed that the activity of CYP3A4 and P450eryF in peroxide-supported substrate oxidations is inhibited by  $\alpha$ -NF in contrast to the activation observed in NADPH-dependent reactions (Ueng et al., 1997; Khan et al., 2003). To elucidate the mechanistic basis of this apparent paradox, we established a sensitive high throughput assay of peroxide-supported CYP3A4 activity. The rates of  $\text{H}_2\text{O}_2$ - and  $\text{CuOOH}$ -dependent oxidation of a series of fluorogenic substrates, such as 7-BQ, 7-BFC, and 7-EFC, by CYP3A4 were measured in several buffer systems as described previously (Khan and Halpert, 2002). The enzyme showed maximal activity in  $\text{H}_2\text{O}_2$ -supported oxidation of 7-BQ, while the rates of oxidation of 7-BFC and 7-EFC were negligible (data not shown). Furthermore, incubation with 10 mM  $\text{H}_2\text{O}_2$  in 100 mM HEPES buffer, pH 7.4, gave a linear rate of reaction for up to 5 min with CYP3A4 wild-type and the mutants in the absence and in the presence of  $b_5$  (data not shown). Under these conditions, the turnover in  $\text{H}_2\text{O}_2$ -supported debenzilation of 7-BQ by purified CYP3A4 reached about 1/15 of the value obtained for the NADPH-dependent reaction in a standard reconstituted system (data not shown).

**Effect of the constituents of the reconstituted system on  $\text{H}_2\text{O}_2$ -supported oxidation of 7-BQ and inhibition by  $\alpha$ -NF.** Figure 1 illustrates the effect of  $\alpha$ -NF on CYP3A4-catalyzed  $\text{H}_2\text{O}_2$ -dependent debenzilation of 7-BQ. In the absence of  $\alpha$ -NF the dependence of  $v$  on  $[S]$  was given by an S-shaped curve obeying the Hill equation with  $k_{\text{cat}}$ ,  $S_{50}$ , and  $n$  values of  $1.4 \text{ min}^{-1}$ ,  $72 \text{ }\mu\text{M}$ , and 1.8, respectively. Addition of  $25 \text{ }\mu\text{M}$   $\alpha$ -

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NF decreased the value of  $k_{\text{cat}}$  to  $0.76 \text{ min}^{-1}$ , with no effect on  $S_{50}$  ( $79 \text{ }\mu\text{M}$ ) or  $n$  (1.9). Non-competitive inhibition by effectors of CYP3A4 in an NADPH-supported system has previously been suggested to result from competition for protein-bound reactive oxygen, leading to a decrease in the rate of substrate oxidation. (Ueng et al., 1997; Shou et al., 1994; Tracy et al., 2003). A similar explanation may be valid in the case of the  $\text{H}_2\text{O}_2$  system.

To test the hypothesis that the inhibition by  $\alpha$ -NF in the  $\text{H}_2\text{O}_2$ -dependent reaction reflects the lack of interactions of CYP3A4 with other components of a standard reconstituted system, namely CPR,  $b_5$  or phospholipids, the effect of these constituents on  $\text{H}_2\text{O}_2$ -supported oxidation of 7-BQ was assessed (Fig. 1). Addition of CPR,  $b_5$ , and phospholipid considerably increased the values of  $k_{\text{cat}}$ ,  $S_{50}$ , and Hill coefficient to  $1.9 \text{ min}^{-1}$ ,  $134 \text{ }\mu\text{M}$ , and 2.4, respectively. Importantly, in this case the values of  $k_{\text{cat}}$ ,  $S_{50}$ , and  $n$  observed in the presence of  $\alpha$ -NF ( $1.8 \text{ min}^{-1}$ ,  $129 \text{ }\mu\text{M}$ , and 2.4, respectively) revealed no inhibition by the compound (Fig. 1). Examination of the individual constituents (Table 1) revealed that the most dramatic effect was exerted by addition of  $b_5$ , which increased the value of  $k_{\text{cat}}$  >2.5-fold with a significant decrease in  $S_{50}$  (Table 1). The increase in  $k_{\text{cat}}$  caused by  $b_5$  was also observed in the presence of  $\alpha$ -NF, although in this case an increase in  $S_{50}$  and a decrease in the  $n$  value were also observed (Table 1). Overall the results strongly support an allosteric effect of  $b_5$  on CYP3A4. In contrast,  $\alpha$ -NF showed negligible effect in an NADPH-dependent reconstituted system, and removal of  $b_5$  caused no significant change in the action of  $\alpha$ -NF (data not shown).

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**Effect of  $b_5$  on  $H_2O_2$ -supported oxidation of 7-BQ.** The role of  $b_5$  in the stimulation of CYP3A4 activity was studied in more detail. The changes in the parameters of  $H_2O_2$ -supported oxidation of 7-BQ caused by the addition of increasing concentrations of  $b_5$  to 1  $\mu$ M CYP3A4 is illustrated in Figure 2. The initial addition of  $b_5$  resulted in a sharp decrease in the  $n$  value and an increase in  $S_{50}$ . Both changes were partially reversed upon further addition of  $b_5$ . Due to the phenomenological nature of the Hill equation, these dependencies are hard to interpret in mechanistic terms. In contrast, the increases in  $k_{cat}$  observed upon addition of  $b_5$  appear to be proportional to the amount of CYP3A4- $b_5$  complex formed (Fig. 2)<sup>c</sup>. Consequently, we attempted to fit the dependencies of  $k_{cat}$  on  $[b_5]$  with the following equation, which is derived from the canonical relationship for the equilibrium of binary complex formation at comparable concentrations of the interacting species (Segel, 1993; eq. II-53b).

$$k_{cat}^{eff} = k_{cat}^0(1 - F_{bound}) + k_{cat}^1 * F_{bound} = k_{cat}^0 + (k_{cat}^1 - k_{cat}^0) \cdot \frac{([P450] + [b_5] + K_d) - \sqrt{([P450] + [b_5] + K_d)^2 - 4 \cdot [P450] [b_5]}}{2 \cdot [P450]} \quad (1)$$

Here  $k_{cat}^0$  and  $k_{cat}^1$  represent  $k_{cat}$  values characteristic of  $b_5$ -free,  $b_5$ -bound CYP3A4 respectively, while  $k_{cat}^{eff}$  stays for the effective value of  $k_{cat}$  exerted at given concentrations of CYP3A4 ( $[P450]$ ) and  $b_5$ , whose complex is characterized by a dissociation constant  $K_d$ . Although the square correlation coefficient ( $\rho^2$ ) for these fits was higher than 0.97, they showed a considerable systematic deviation from the experimental data (Fig. 2, solid lines). Based on prior findings of heterogeneity of the CYP3A4 pool (Davydov et al., 2003; Davydov and Halpert, 2004), we hypothesized that the concentration of P450 participating in the interactions with  $b_5$  was lower than the total concentration of the enzyme. This hypothesis is consistent with an earlier report, in

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which the maximal stimulation of testosterone 6 $\beta$ -hydroxylation and nifedipine oxidation was observed at a  $b_5$ :CYP3A4 molar ratio of 0.5 (Yamazaki et al., 1996). To probe this suggestion the effective concentration of CYP3A4 was treated as one of the parameters of the optimization procedure, together with the values of  $K_d$ ,  $k_{cat}^0$ , and  $k_{cat}^1$ . Such optimization showed that the best quality of the fitting ( $\rho^2 > 0.996$ ) was obtained by assuming that only 65% of CYP3A4 participates in the interaction with  $b_5$  (Fig. 2, dashed lines). This observation strongly supports the suggestion of functional heterogeneity of the CYP3A4 pool. The fitting of the dependencies of  $k_{cat}^{eff}$  on the concentration of  $b_5$  yields  $k_{cat}^0 = 1.4 \pm 0.1 \text{ min}^{-1}$ ,  $k_{cat}^1 = 5.1 \pm 0.2 \text{ min}^{-1}$  and  $K_d = 0.042 \pm 0.02 \text{ }\mu\text{M}$ .

**Effect of  $b_5$  on H<sub>2</sub>O<sub>2</sub>-dependent heme depletion.** The increased rate of H<sub>2</sub>O<sub>2</sub>-dependent substrate oxidation by CYP3A4 in the presence of  $b_5$  may be explained if the interactions of the two proteins results in increased accessibility of the heme moiety of CYP3A4 for H<sub>2</sub>O<sub>2</sub>. To probe this hypothesis we studied the effect of  $b_5$  on H<sub>2</sub>O<sub>2</sub>-dependent destruction of the P450 heme moiety of CYP3A4 (Fig. 3). Interestingly, the kinetics did not fit the equation of a simple pseudo-first order reaction but rather the equation of the sum of two exponents, as given below in equation 2.

$$[P450]_t = [P450]_0 \left( F_f e^{-k_f t} + [1 - F_f] e^{-k_s t} \right) \quad (2)$$

Here  $[P450]_t$  is the concentration of P450 at the time  $t$ ,  $[P450]_0$  is the total concentration of the enzyme,  $k_f$  and  $k_s$  are the kinetic constants of the fast and slow phases, respectively, and  $F_f$  is the fraction of the fast phase. The biphasicity of the kinetics of heme destruction was consistent with conformational heterogeneity of CYP3A4 and suggested

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the presence of two conformers of the enzyme with different accessibility of the heme moiety to H<sub>2</sub>O<sub>2</sub>. Sixty-six percent of the wild-type enzyme in the absence of *b*<sub>5</sub> was represented by the conformer bleached in the fast phase, while the addition of *b*<sub>5</sub> decreased the fraction of the fast phase to 16% (Fig. 3). Hence, *b*<sub>5</sub> increases the fraction of the slow phase from 34 to 84%, i.e. approximately 2.5 fold, which is similar to the increase in CYP3A4 activity in the presence of *b*<sub>5</sub>. Thus, the interaction of CYP3A4 with *b*<sub>5</sub> does not increase the accessibility of P450 heme moiety for H<sub>2</sub>O<sub>2</sub>, but rather displaces the partitioning of the enzyme towards the conformer with a slower rate of heme destruction. The results are reminiscent of the findings of Koley and co-authors, who first proposed conformational heterogeneity of CYP3A4 (Koley et al., 1995). That study showed that substrates have markedly variable effects on the CO binding kinetics of their target P450 3A4 conformers.

Additional evidence of conformational heterogeneity of CYP3A4 in solution and in microsomal membranes has been obtained recently in studies on pressure-induced transitions (Davydov et al., 2003). Only about 70% of CYP3A4 in solution and about 50% of the enzyme in microsomes of recombinant *Saccharomyces cerevisiae* was susceptible to a pressure-induced P450 → P420 transition. The results suggested that both in solution and in the membrane CYP3A4 is represented by two conformers with different positions of spin equilibrium and different barotropic properties. Interestingly, the estimate of 70% for the fraction of pressure-susceptible P450 is close to the value of 66% found for the rapid fraction of H<sub>2</sub>O<sub>2</sub>-dependent bleaching of CYP3A4. Importantly, the barotropic heterogeneity of CYP3A4 disappears upon monomerization of P450 oligomers (Davydov et al., 2003). We suggest, therefore, that the interactions of

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CYP3A4 with  $b_5$  affect the partitioning of CYP3A4 conformers by modulation of the architecture of P450 oligomers.

**Effect of substitutions of active site residues on  $b_5$ -activation.** To probe the structural basis of  $b_5$ -mediated allosteric modulation of H<sub>2</sub>O<sub>2</sub>-supported 7-BQ debenzoylation, mutants in SRSs 1, 4, and 5 were targeted because substitutions at these regions (S119W, T309A, and A370F) caused a significant change in regioisomer formation from phenyldiazene and amplified the effect of the redox partners (Yamaguchi et al., 2003). Residues at these sites were replaced by smaller and larger side chains, and kinetic analysis of H<sub>2</sub>O<sub>2</sub>-supported 7-BQ oxidation was performed as presented in Table 2. S119A, I301A, I301F, and A370F exhibited a  $\geq 2$ -fold decrease in the  $k_{cat}$ . T309F showed very low activity and kinetics could not be measured (data not shown). S119W demonstrated a complete loss of enzyme cooperativity (Table 2), suggesting an important site for allosteric modulation.

The most interesting observations, however, were the effect of the active site mutations on  $b_5$ -stimulated 7-BQ debenzoylation (Table 2). While  $b_5$  stimulated  $k_{cat}$  by  $\sim 2.5$ -fold in wild-type CYP3A4, the stimulation by  $b_5$  was more or less abolished in S119W and I301F, and A305F was inhibited by  $b_5$ . (The lack of correlation between the specific content of the mutants and their stimulation by  $b_5$ , suggests that any interactions of  $b_5$  with apo-protein may be neglected). Another important observation was that I369F and A370F demonstrated a loss of cooperativity upon  $b_5$  addition. The changes in the profiles of regioisomer formation from phenyldiazene suggest that in S119W (B'-C loop) active site topology is altered upon  $b_5$ -interaction (Yamaguchi et al., 2003). In this

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context it is important to note that some basic amino acid residues in the C and C' helix are thought to be involved in binding between P450 2B4 and  $b_5$  (Bridges et al., 1998; Clarke et al., 2004). Likewise, the substitutions at Ile-301 and Ala-305 in the SRS-4 region, which is closest to the heme (Yano et al., 2004; Williams et al., 2004), with larger side chains may cause a structural transition leading to impaired  $b_5$ -interaction. Recently resolved X-ray crystal structures of CYP3A4 have indicated a Phe-cluster that includes SRS 1 residue Phe-108 and SRS 4 residue Phe-304 (Yano et al., 2004; Williams et al., 2004). It has been suggested that conformational movements in the Phe-cluster could be triggered by interaction with redox partners, which is consistent with our results with active site mutants. Significantly reduced activation by  $b_5$  along with abolished enzyme cooperativity upon  $b_5$ -interaction in A370F suggests an important role of SRS-5, as predicted earlier based on the altered regioisomers formation upon interaction with redox partners (Yamaguchi et al., 2003).

## Conclusions

Stimulation of CYP3A4 activity by  $b_5$  and its ability to reverse  $\alpha$ -NF inhibition of  $H_2O_2$ -supported 7-BQ debenzoylation suggests an important role of  $b_5$  as an allosteric modulator. The results of titration with  $b_5$  on CYP3A4 activity and of heme-depletion measurements in the absence and presence of  $b_5$  suggest that the interaction with  $b_5$  results in redistribution of the CYP3A4 pool between two distinct populations with different susceptibility to  $H_2O_2$ -dependent bleaching. Furthermore, the active site mutants (S119W, I301F, and A305F), which show abolished  $b_5$ -activation, suggest an impaired P450- $b_5$  interaction. To our knowledge this is the first report on the effect of  $b_5$  in the

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peroxide-supported system, and illustrates one possible mechanism by which  $b_5$  modulates CYP3A4 heterotropic cooperativity. We are now actively engaged in studying the oligomerization-related heterogeneity of P450 as a putative mechanistic basis of CYP3A4 allosteric modulation.

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### Footnotes

- a) This work was supported by National Institutes of Health grants GM54995 and Center Grant ES06676.
- b) Addition of apo-CYP3A4 at different P450: $b_5$  molar ratios did not decrease the stimulation by  $b_5$ , suggesting that any apo-protein present in the original preparation does not interfere with the results (data not shown).



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### Figure Legends:

**Fig. 1:** Steady-state kinetic analysis of H<sub>2</sub>O<sub>2</sub>-supported 7-BQ debenzoylation by CYP3A4 in the absence and presence of a standard reconstituted system (RS). The plots of the fit to the Hill equation and the respective kinetic parameters are as follows: Without  $\alpha$ -NF and without RS (open circles;  $k_{\text{cat}} - 1.4 \text{ min}^{-1}$ ,  $S_{50} - 72 \text{ }\mu\text{M}$ ,  $n - 1.8$ ); 25  $\mu\text{M}$   $\alpha$ -NF and without RS (closed circles;  $k_{\text{cat}} - 0.76 \text{ min}^{-1}$ ,  $S_{50} - 79 \text{ }\mu\text{M}$ ,  $n - 1.9$ ); without  $\alpha$ -NF and with RS (open squares;  $k_{\text{cat}} - 1.9 \text{ min}^{-1}$ ,  $S_{50} - 134 \text{ }\mu\text{M}$ ,  $n - 2.4$ ); 25  $\mu\text{M}$   $\alpha$ -NF and with RS (closed squares;  $k_{\text{cat}} - 1.8 \text{ min}^{-1}$ ,  $S_{50} - 129 \text{ }\mu\text{M}$ ,  $n - 2.4$ ). The standard reconstituted system included P450:CPR: $b_5$ : 1:4:2 molar ratio, CHAPS - 0.04%, and DOPC - 0.1  $\mu\text{g}/\mu\text{l}$ .

**Fig. 2:** Effect of increasing concentrations of  $b_5$  on the parameters of the Hill equation for H<sub>2</sub>O<sub>2</sub>-supported 7-BQ debenzoylation by 1  $\mu\text{M}$  CYP3A4. The main plot shows the effect of  $b_5$  on the  $k_{\text{cat}}$ . These plots correspond to the results of fitting of the experimental data to equation 1 assuming the concentration of P450 to be 1  $\mu\text{M}$  (solid lines) and 0.65  $\mu\text{M}$  (dashed lines). Inserts shows the effect of  $b_5$  on the values of  $n$  (circles) and  $S_{50}$  (squares).

**Fig. 3:** CYP3A4-heme depletion assays at 60 mM H<sub>2</sub>O<sub>2</sub> and 1  $\mu\text{M}$  P450. The inset represents a semi log re-plot of the heme-depletion kinetics. Open and closed circles represent the absence of  $b_5$  and the presence of 1  $\mu\text{M}$   $b_5$ . The plots of the fit to equation 2

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yielded kinetic parameters as follows: Without  $b_5$  (open circles;  $k_f = 0.34 \text{ min}^{-1}$ ,  $k_s = 0.06 \text{ min}^{-1}$ ,  $F_f = 66\%$ );  $b_5$  (closed circles;  $k_f = 1.0 \text{ min}^{-1}$ ,  $k_s = 0.12 \text{ min}^{-1}$ ,  $F_f = 16\%$ ).

Table 1. Steady-state kinetics: Effect of redox-partners on H<sub>2</sub>O<sub>2</sub>-supported CYP3A4 activity.

Sample	- $\alpha$ -NF			25 $\mu$ M $\alpha$ -NF		
	$k_{\text{cat}}$ (min <sup>-1</sup> )	$S_{50}$ ( $\mu$ M)	$n$	$k_{\text{cat}}$ (min <sup>-1</sup> )	$S_{50}$ ( $\mu$ M)	$n$
NIL	1.36 $\pm$ 0.23	72.2 $\pm$ 7.5	1.88 $\pm$ 0.13	0.696 $\pm$ 0.07	85.0 $\pm$ 12	1.87 $\pm$ 0.16
CHAPS	1.46 $\pm$ 0.17	87.0 $\pm$ 7.0	1.81 $\pm$ 0.06	1.17 $\pm$ 0.15*	142 $\pm$ 22*	1.48 $\pm$ 0.10*
CPR	1.29 $\pm$ 0.17	97.3 $\pm$ 9.4	2.30 $\pm$ 0.14*	0.836 $\pm$ 0.18	143 $\pm$ 16*	2.27 $\pm$ 0.07*
$b_5$	3.79 $\pm$ 0.36*	52.6 $\pm$ 1.5*	1.86 $\pm$ 0.26	4.73 $\pm$ 0.21*	126 $\pm$ 5.3*	1.30 $\pm$ 0.09*
CPR + $b_5$	3.34 $\pm$ 0.13*	93.3 $\pm$ 16	2.38 $\pm$ 0.12*	3.56 $\pm$ 0.38*	115 $\pm$ 20	1.82 $\pm$ 0.16
DOPC	1.66 $\pm$ 0.14	175 $\pm$ 18*	2.78 $\pm$ 0.43*	1.06 $\pm$ 0.20*	144 $\pm$ 5.3*	2.75 $\pm$ 0.06*
$b_5$ + DOPC	1.99 $\pm$ 0.03*	53.6 $\pm$ 3.1*	2.05 $\pm$ 0.23	2.57 $\pm$ 0.32*	83.6 $\pm$ 16	1.39 $\pm$ 0.14*
CPR + $b_5$ + DOPC	1.91 $\pm$ 0.31*	128 $\pm$ 23*	2.14 $\pm$ 0.28	2.20 $\pm$ 0.39*	110 $\pm$ 10	2.09 $\pm$ 0.17

Results are the mean  $\pm$  standard deviation of at least three independent experiments. \* $p \leq 0.05$  based on comparison with the corresponding NIL value using a two-sample equal variance two-tailed Student's t test. CHAPS (0.04%) is included in all the experiments, except the NIL. CPR and  $b_5$  were used at 4:1 and 2:1 molar ratios, respectively. P450 concentration was 0.25  $\mu$ M. DOPC concentration was 0.1  $\mu$ g/ $\mu$ l.

Table 2. Steady-state kinetics: Effect of  $b_5$  on H<sub>2</sub>O<sub>2</sub>-supported activity of CYP3A4 wild-type and active site mutants.

P450s	$k_{\text{cat}}$ (min <sup>-1</sup> )		$S_{50}$ (μM)		$n$	
	NIL	$b_5$	NIL	$b_5$	NIL	$b_5$
WT (15) <sup>a</sup>	1.44 (0.02) <sup>b</sup>	3.58 (0.01)	71.7 (2.2)	50.6 (0.5)	1.86 (0.11)	1.56 (0.03)
S119A (12)	0.77 (0.02)	3.09 (0.14)	88.4 (4.0)	116 (7.2)	2.04 (0.41)	1.71 (0.22)
S119W (10)	1.31 (0.08)	1.19 (0.06)	195 (21)	169 (14)	1.01 (0.03)	1.04 (0.02)
I301A (11)	0.48 (0.01)	2.56 (0.05)	93.3 (3.0)	160 (4.4)	1.56 (0.04)	1.43 (0.04)
I301F (6)	0.61 (0.00)	0.72 (0.02)	100 (1.4)	82.0 (4.7)	2.09 (0.07)	1.45 (0.14)
A305F (10)	1.41 (0.05)	0.68 (0.03)	173 (7.1)	135 (8.5)	1.64 (0.12)	1.53 (0.13)
I369F (12)	0.98 (0.03)	3.04 (0.03)	122 (4.1)	159 (2.7)	1.85 (0.13)	1.01 (0.02)
A370F (12)	0.56 (0.03)	0.82 (0.05)	146 (7.6)	146 (15)	2.25 (0.29)	1.24 (0.20)
L373F (3.0)	3.02 (0.22)	17 (1.0)	212 (14)	190 (11)	1.78 (0.22)	1.55 (0.15)

Data are representative of at least two independent experiments. The variation between the experiments is  $\leq 15\%$ .

<sup>a</sup> Values are specific content in nmol of P450 per mg protein. <sup>b</sup> Values are the standard error for fit to the Hill equation. P450 and  $b_5$  were at 0.25 μM concentrations in the presence of 0.04% CHAPS.

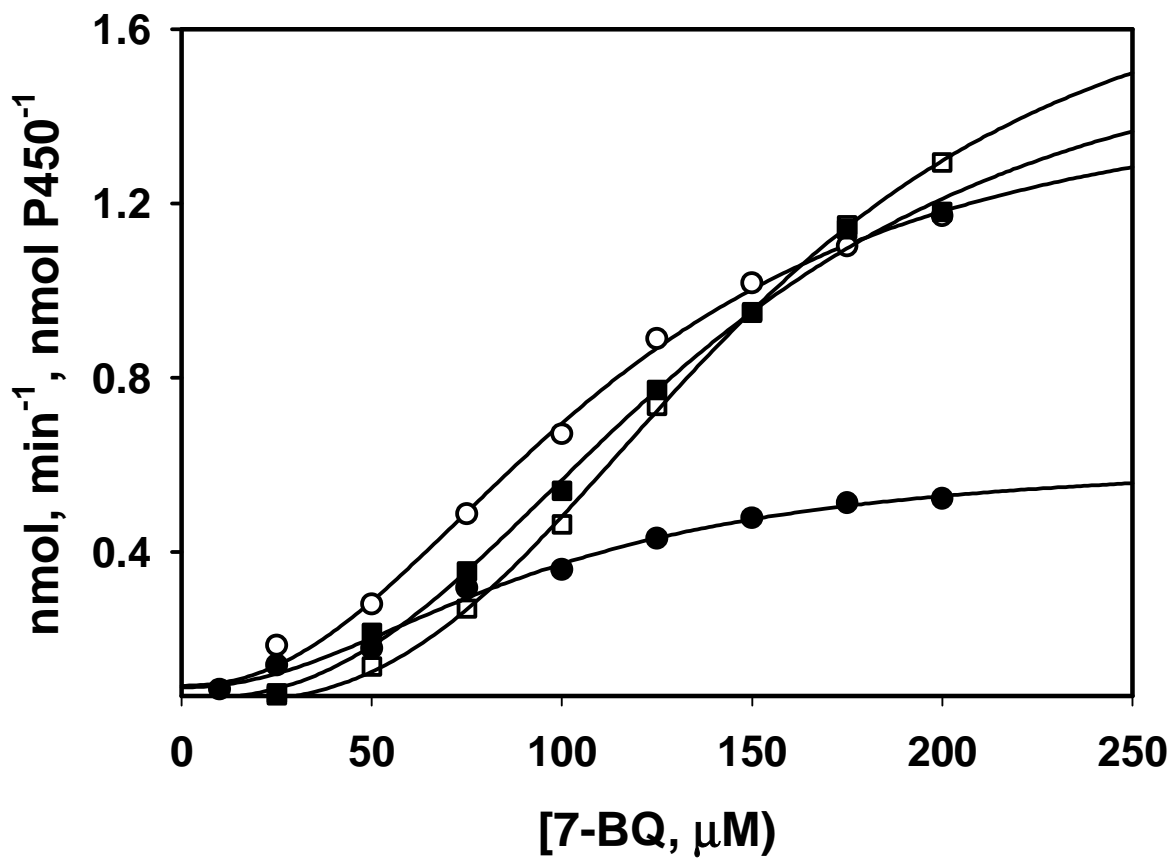


Figure 1

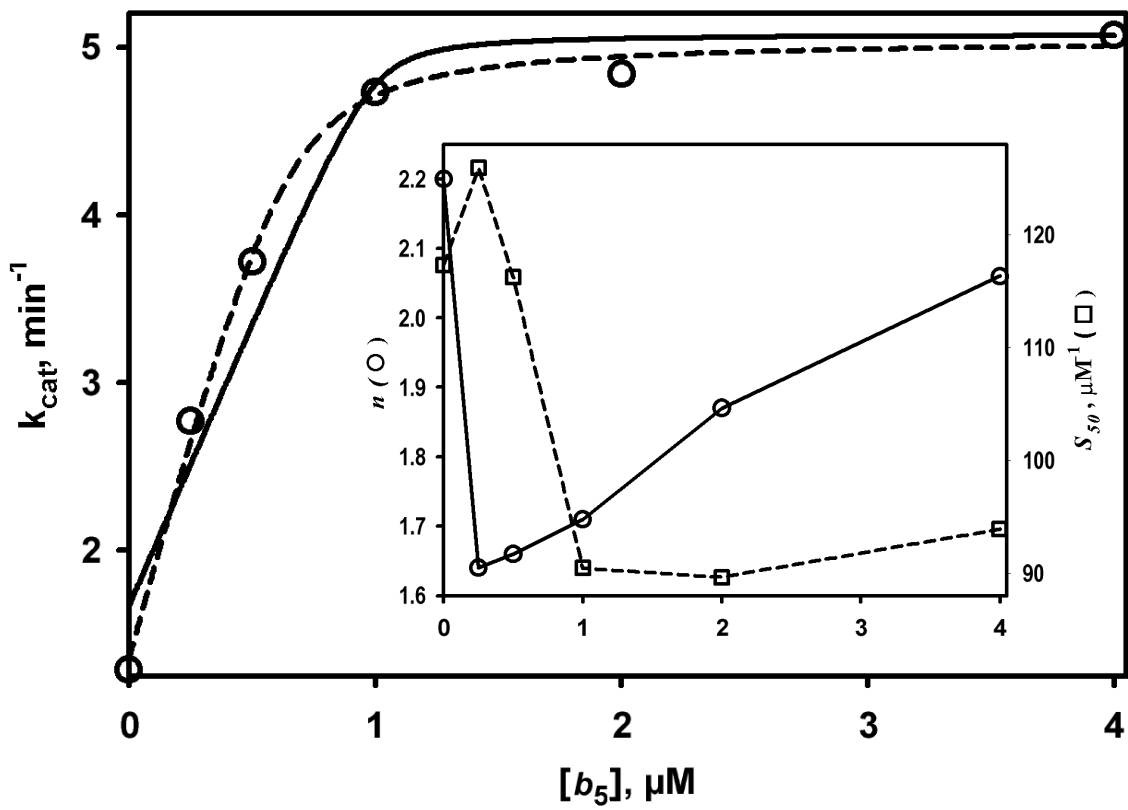


Figure 2

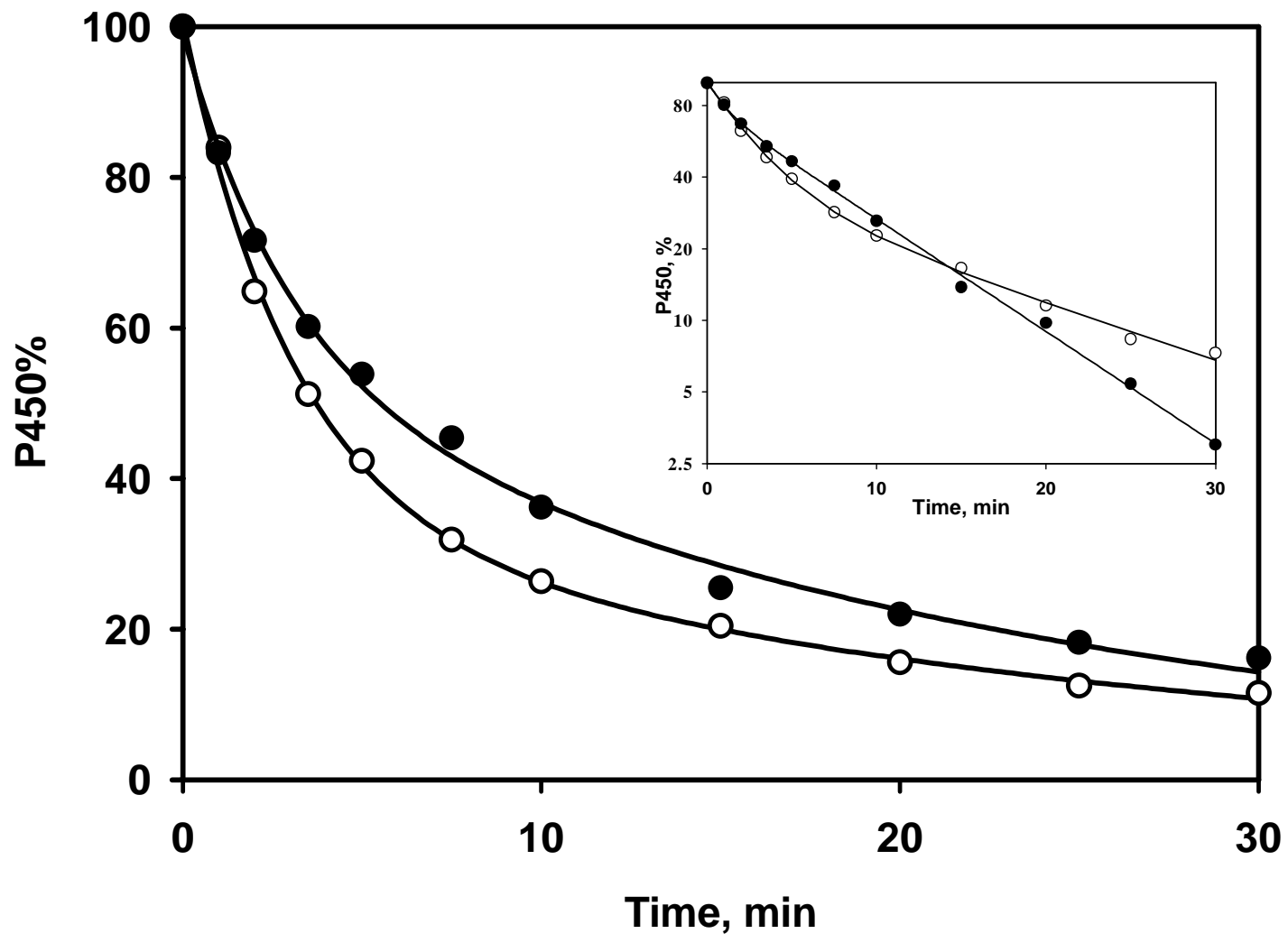


Figure 3