DMD Fast Forward. Published on May 3, 2005 as doi:10.1124/dmd.105.004606

DMD#4606

Role of Cytochrome b_5 in Modulating Peroxide-supported CYP3A4 Activity: Evidence for a Conformational Transition and P450 Heterogeneity

Santosh Kumar, Dmitri R. Davydov, and James R. Halpert

Department of Pharmacology and Toxicology,

University of Texas Medical Branch,

301 University Boulevard,

Galveston, Texas 77555-1031

Running Title: Modulation of CYP3A4 activity by cytochrome b_5

Corresponding Author:

Santosh Kumar, Department of Pharmacology and Toxicology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1031

Tel: (409) 772-9677, Fax: (409) 772-9642, Email: sakumar@utmb.edu

Number of text pages: 28

Number of Tables: 2

Number of Figures: 3

Number of References: 39

Number of words in Abstract: 246

Number of words in Introduction: 557

Number of words in Results and Discussion and Conclusion: 1980

Abbreviations:

CYP, Cytochrome P450; 7-BQ, 7-Benzyloxyquinoline; 7-BFC, 7-Benzyloxy-4-(trifluoromethyl)coumarin; 7-EFC, 7-Ethoxy-4-(trifluoromethyl)coumarin; α-NF, α-Naphthoflavone; SRS, Substrate recognition site; CPR, NADPH-cytochrome P450 reductase; b_5, Cytochrome b_5; H_2O_2, Hydrogen peroxide; CuOOH, Cumene hydroperoxide

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The role of cytochrome b_5 (b_5) in the α-naphthoflavone (α-NF)-mediated inhibition of H_2O_2-supported 7-benzyloxyquinoline (7-BQ) debenzylation by heterologously expressed and purified cytochrome P450 3A4 (CYP3A4) was studied. Although α-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 α-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.

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

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 α-naphthoflavone (α-NF), which may also serve as a substrate (Harlow and Halpert, 1998; He at al., 2003; Domanski et al., 2000, 2001). Interestingly, activation by α-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 α-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 α-NF of carbamazepine 10, 11-epoxidation at a single concentration of substrate, α-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

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_2O_2-supported 7-benzyloxyquinoline (7-BQ) debenzylation by CYP3A4. The results suggest an important mechanism by which b_5 modulates CYP3A4 activity and provide further evidence for P450 heterogeneity.

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

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). α-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_2O_2 (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 μM 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

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_2O_2. 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-µl 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 µM) in the absence and presence of α-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-µl 96-well microplate before initiation of the reaction with H_2O_2 (10 mM final). The total reaction volume of the assay was 100 µl. Thus, the concentration of CYP3A4 in the reaction mixture was equal to 0.25 µM, if not otherwise indicated. After 5 min of incubation, the reactions were stopped by adding 340 units (50 µl) of catalase. Subsequently, 50 µl of 100 mM Hepes buffer, pH 7.4, was added before recording the fluorescence intensity at λ_{ex} = 405 and λ_{em} = 510 nm using a fluorescence plate reader (Ascent Fluoroscan, Ramsey, MN). In all the cases, the final activity was calculated by comparison to a standard curve. The k_{cat}, S_{50}, and Hill

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_2O_2 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_2O_2. 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).

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

Results and Discussion

Hydrogen peroxide-supported 7-BQ debenzylation by CYP3A4. Previous studies showed that the activity of CYP3A4 and P450eryF in peroxide-supported substrate oxidations is inhibited by α-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 H_2O_2- and 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 H_2O_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 H_2O_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 H_2O_2-supported debenzylation 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 H_2O_2-supported oxidation of 7-BQ and inhibition by α-NF. Figure 1 illustrates the effect of α-NF on CYP3A4-catalyzed H_2O_2-dependent debenzylation of 7-BQ. In the absence of α-NF the dependence of v on [S] was given by an S-shaped curve obeying the Hill equation with k_{cat}, S_{50}, and n values of 1.4 min^{-1}, 72 μM, and 1.8, respectively. Addition of 25 μM α-

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

NF decreased the value of k_{cat} to 0.76 min^{-1}, with no effect on S_{50} (79 μM) 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 H_2O_2 system.

To test the hypothesis that the inhibition by α-NF in the H_2O_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 H_2O_2-supported oxidation of 7-BQ was assessed (Fig. 1). Addition of CPR, b_5, and phospholipid considerably increased the values of k_{cat}, S_{50}, and Hill coefficient to 1.9 min^{-1}, 134 μM, and 2.4, respectively. Importantly, in this case the values of k_{cat}, S_{50}, and n observed in the presence of α-NF (1.8 min^{-1}, 129 μM, 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_{cat} >2.5-fold with a significant decrease in S_{50} (Table 1). The increase in k_{cat} caused by b_5 was also observed in the presence of α-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, α-NF showed negligible effect in an NADPH-dependent reconstituted system, and removal of b_5 caused no significant change in the action of α-NF (data not shown).

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

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 μM 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)[c]. 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).

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

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 (ρ^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

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

which the maximal stimulation of testosterone 6β-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 *(ρ^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 ± 0.1 min^{-1}, k_{cat}^1 = 5.1 ± 0.2 min^{-1} and K_d = 0.042 ± 0.02 μM.

Effect of b_5 on H_2O_2-dependent heme depletion. The increased rate of H_2O_2-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_2O_2. To probe this hypothesis we studied the effect of b_5 on H_2O_2-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.

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

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

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

the presence of two conformers of the enzyme with different accessibility of the heme moiety to H_2O_2. Sixty-six percent of the wild-type enzyme in the absence of b_5 was represented by the conformer bleached in the fast phase, while the addition of b_5 decreased the fraction of the fast phase to 16% (Fig. 3). Hence, b_5 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_5. Thus, the interaction of CYP3A4 with b_5 does not increase the accessibility of P450 heme moiety for H_2O_2, 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 $\rightarrow$ 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_2O_2-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

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_2O_2-supported 7-BQ debenzylation, 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_2O_2-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 debenzylation (Table 2). While b_5 stimulated k_{cat} by ~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

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 α-NF inhibition of H_2O_2-supported 7-BQ debenzylation 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 re-distribution 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

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.

Acknowledgements

The authors thank Ms. You-Ai He and You-Qun He for proving purified CYP3A4 active site mutant proteins. We also thank Drs. Kenneth Johnson and Cheng Wang, Pharmacology and Toxicology, UTMB for allowing us to use their fluorescence plate reader (Ascent).

References

Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, and Waskell L (1998) Identification of the binding site on cytochrome P450 2B4 for cytochrome b_5 and cytochrome P450 reductase. *J Biol Chem* **273**:17036-17049.

Clarke TA, Im SC, Bidwai A, and Waskell L (2004) The role of the length and sequence of the linker domain of cytochrome b_5 in stimulating cytochrome P4502B4 catalysis. *J Biol Chem* **279**:36809-36818.

Davydov DR, Deprez E, Hui Bon Hoa G, Knyushko TV, Kuznetsova GP, Koen Y. M, and Archakov AI (1995) High-pressure-induced transitions in microsomal cytochrome P450 2B4 in solution: evidence for conformational inhomogeneity in the oligomers. *Arch Biochem Biophys* **320**:330-344.

Davydov DR, Halpert JR, Renaud JP, and Hui Bon Hoa G. (2003) Conformational heterogeneity of cytochrome P450 3A4 revealed by high pressure spectroscopy. *Biochem Biophys Res Commun* **312**:121-130.

Davydov DR and Halpert JR (2004) Allosteric mechanisms in cytochrome P450: conformational transition in CYP3A4 caused by the binding of α-naphthoflavone. *FASEB J* **18**:C-178.

Domanski TL, He YA, Harlow GR, and Halpert JR. (2000) Dual role of human cytochrome P450 3A4 residue Phe-304 in substrate specificity and cooperativity. *J Pharmacol Exp Ther* **293**:585-591.

Domanski TL, He YA, Khan KK, Roussel F, Wang Q, and Halpert JR (2001) Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and cooperativity. *Biochemistry* **40**:10150-10160.

Guengerich FP (1999) Cytochrome P450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39:**1-17.

Guryev OL, Gilep AA, Usanov SA, and Estabrook RW (2001) Interaction of apo-cytochromes P450 3A4 and P450 17A: relevance of heme transfer reactions. *Biochemistry* **40**:5018-5031.

He YA, Roussel F, and Halpert JR (2003) Analysis of homotropic and heterotropic cooperativity of diazepam oxidation by CYP3A4 using site-directed mutagenesis and kinetic modeling. *Arch Biochem Biophys* **409**:92-101.

Harlow GR and Halpert JR (1997) Alanine-scanning mutagenesis of putative substrate recognition sites in human cytochrome P4503A4. *J Biol Chem* **272**:5396-5402.

Harlow GR and Halpert JR (1998) Analysis of human cytochrome P4503A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proc Natl Acad Sci USA* ***95***:6636-6641.

Hutzler JM and Tracy TS (2002) Atypical kinetic problems in drug metabolism reactions. *Drug Metab Dispos* **30**:355-362.

Jansson I, Tamburini PP, Favreau LV, and Schenkman JB (1985) The interaction of cytochrome b_5 with four cytochrome P450 enzymes from the untreated rat. *Drug Metab Dispos* **13**:453-458.

Khan KK and Halpert JR (2002) 7-Benzyloxyquinoline oxidation by P450eryF A245T: finding of a new fluorescent substrate probe. *Chem Res Toxicol* **15**:806-814.

Khan KK, Liu H, and Halpert JR (2003) Homotropic versus heterotropic cooperativity of cytochrome P450eryF: a substrate oxidation and spectral titration study. *Drug Metab Dispos* **31**:356-359.

Koley AP, Buters JT, Robinson RC, Markowitz A, and Friedman FK (1995) CO binding kinetics of human cytochrome P450 3A4: Specific interaction of substrates with kinetically distinguishable conformers. *J. Biol. Chem.* **270**:5014-5018.

Kumar S, Chen CS, Waxman DJ, and Halpert JR (2005) Directed evolution of mammalian cytochrome P450 2B1: mutations outside of the active site enhance the metabolism of several substrates including the anticancer prodrugs cyclophosphamide and ifosfamide. *J. Biol. Chem.* [E. publication, March 17]

Nebert DW and Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* **360**:155-1162.

Perret A and Pompon D (1998) Electron shuttle between membrane-bound cytochrome P450 3A4 and b_5 rules uncoupling mechanisms. *Biochemistry* **37**:11412-11424.

Pikuleva IR, Lapko AG, and Chashchin VL (1992) Functional reconstitution of cytochrome $P450_{scc}$ with hemin activated with woodward's reagent K. *J Biol Chem* **267**:1438-1442.

Reed JR and Hollenberg PF (2003) Examining the mechanism of stimulation of cytochrome P450 by cytochrome b_5: the effect of cytochrome b_5 on the interaction between cytochrome P450 2B4 and P450 reductase. *J Inorg Biochem* **97**:265-273.

Reed JR and Hollenberg PF (2003) Comparison of substrate metabolism by cytochromes P450 2B1, 2B4, and 2B6: relationship of the heme spin state, catalysis, and the effects of cytochrome b_5. *J Inorg Biochem* **93**:152-160.

Renaud JP, Davydov DR, Heirwegh KPM, Mansuy D, and Hui Bon Hoa G. (1996). Thermodynamic studies of substrate binding and spin transition in human cytochrome P450 3A4 expressed in yeast microsomes. *Biochem J* **319**:675-681.

Schenkman JB and Jansson I (1999) Interactions between cytochrome P450 and cytochrome b_5. *Drug Metab Rev* **31**:351-364.

Schenkman JB and Jansson I (2003) The many roles of cytochrome b_5. *Pharmacol Ther* **97**:139-152.

Segel IH (1993) Kinetic behavior at high enzyme concentrations. In *Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-State enzyme systems.* (Segel, IH, Ed.) pp 72-75, Wiley classic library, New York.

Shou M, Grogan J, Mancewicz JA, Krausz KW, Gonzalez FJ, Gelboin, HV, and Korzekwa KR (1994) Activation of CYP3A4: evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. *Biochemistry* **33**:6450-6455.

Tamburini PP and Gibson GG (1983) Thermodynamic studies of the protein-protein interactions between cytochrome P450 and cytochrome b_5. Evidence for a central role of the cytochrome P450 spin state in the coupling of substrate and cytochrome b_5 binding to the terminal hemo-protein. *J Biol Chem* **258**:13444-13452.

Tracy TS (2003) Atypical enzyme kinetics: their effect on in vitro-in vivo pharmacokinetic predictions and drug interactions. *Curr Drug Metab* **4:**341-346.

Ueng YF, Kuwabara T, Chun YJ, and Guengerich PF (1997) Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* **36**:370-380.

Uvarov VY, Tretiakov VE, Archakov AI (1990) Heme maintains catalytically active structure of cytochrome P450. *FEBS lett.* **260:** 309-312.

Williams PA Cosme J, Vinkovic DM, Ward A, Angove HC, Day PJ, Vonrhein C, Tickle IJ, and Jhoti H. (2004) Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* **305**: 683-686.

Yamada M, Ohta Y, Bachmanova GI, Nishimoto Y, Archakov AI, Kawato S (1995) Dynamic interactions of rabbit liver cytochromes P450IA2 and P450IIB4 with cytochrome b_5 and NADPH-cytochrome P450 reductase in proteoliposomes. *Biochemistry* **34**: 10113-10119.

Yamaguchi Y, Khan KK, He YA, He YQ, and Halpert JR (2003) Topological changes in the CYP3A4 active site probed with phenyldiazene: effect of interaction with NADPH-cytochrome P450 reductase and cytochrome b_5 and of site-directed mutagenesis. *Drug Metab Dispos* **32**:155-161.

Yamaori S, Yamazaki H, Suzuki A, Yamada A, Tani H, Kamidate T, Fujita KI, and Kamataki T (2003) Effects of cytochrome b_5 on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7. *Biochem Pharmacol* **66**:2333-2340.

Yamazaki H, Johnson WW, Ueng YF, Shimada T, and Guengerich FP (1996) Lack of electron transfer from cytochrome b_5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome b_5. *J Biol Chem.* **271**: 27438-27444.

Yamazaki H, Shimada T, Martin MV, and Guengerich FP (2001) Stimulation of cytochrome P450 reactions by apo-cytochrome b_5. Evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b_5 or heme oxygenase. *J Biol Chem* **276**:30885-30891.

Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, and Johnson EF (2004) The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-angstrom resolution. *J Biol Chem* **279**:38091-38094.

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).

Figure Legends:

Fig. 1: Steady-state kinetic analysis of H_2O_2-supported 7-BQ debenzylation 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 α-NF and without RS (open circles; k_{cat} - 1.4 min^{-1}, S_{50} – 72 μM, n – 1.8); 25 μM α-NF and without RS (closed circles; k_{cat} - 0.76 min^{-1}, S_{50} – 79 μM, n – 1.9); without α-NF and with RS (open squares; k_{cat} - 1.9 min^{-1}, S_{50} – 134 μM, n - 2.4); 25 μM α-NF and with RS (closed squares; k_{cat} - 1.8 min^{-1} , S_{50} – 129 μM, n - 2.4). The standard reconstituted system included P450:CPR:b_5: 1:4:2 molar ratio, CHAPS - 0.04%, and DOPC - 0.1 μg/μl.

Fig. 2: Effect of increasing concentrations of b_5 on the parameters of the Hill equation for H_2O_2-suppported 7-BQ debenzylation by 1 μM CYP3A4. The main plot shows the effect of b_5 on the k_{cat}. These plots correspond to the results of fitting of the experimental data to equation 1 assuming the concentration of P450 to be 1 μM (solid lines) and 0.65 μM (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_2O_2 and 1 μM 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 μM b_5. The plots of the fit to equation 2

DMD Fast Forward. Published on May 3, 2005 as DOI: 10.1124/dmd.105.004606
This article has not been copyedited and formatted. The final version may differ from this version.

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

Table 1. Steady-state kinetics: Effect of redox-partners on H_2O_2-supported CYP3A4 activity.

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

Results are the mean ± 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 μM. DOPC concentration was 0.1 μg/μl.

Table 2. Steady-state kinetics: Effect of b_5 on H_2O_2-supported activity of CYP3A4 wild-type and active site mutants.

P450s	k_{cat} (min^{-1})		S_{50} (μM)		*n*	
	NIL	b_5	NIL	b_5	NIL	b_5
WT (15)[a]	1.44 (0.02)[b]	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 ≤ 15%.

[a] Values are specific content in nmol of P450 per mg protein. [b] 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.

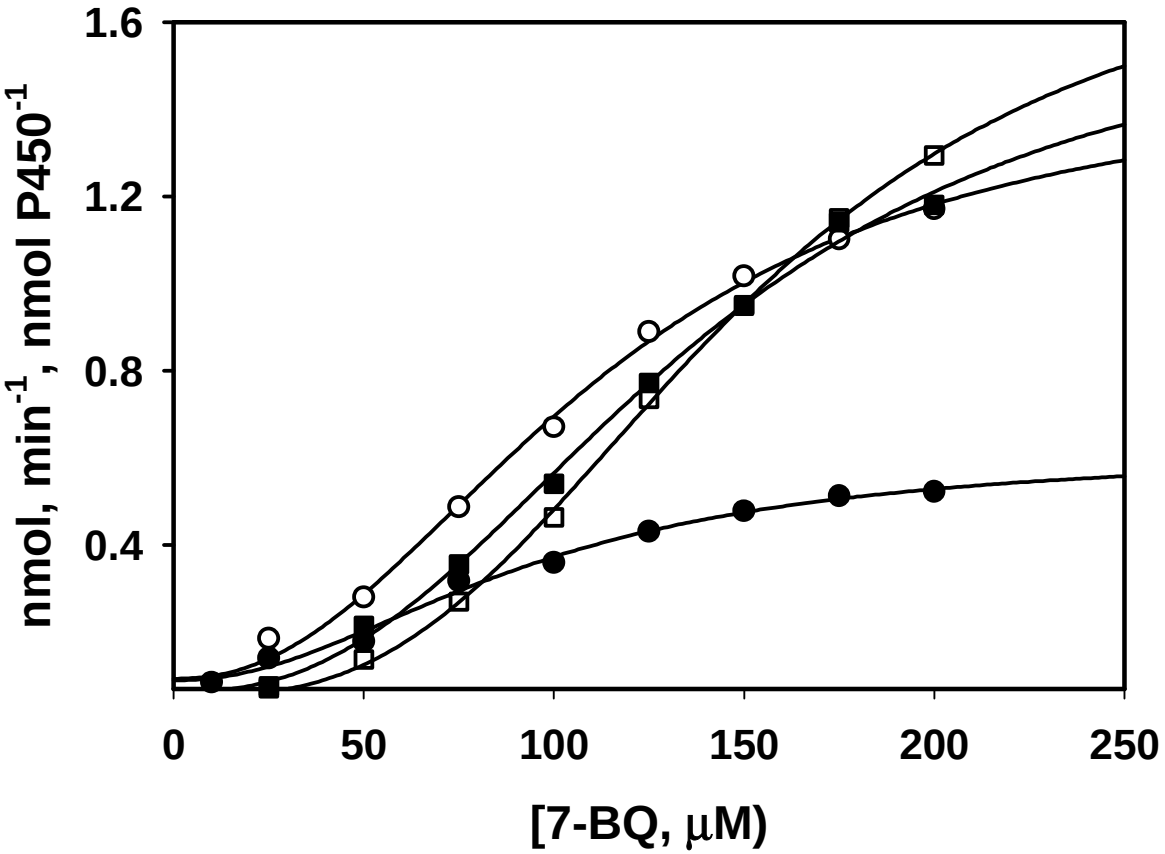

Figure 1

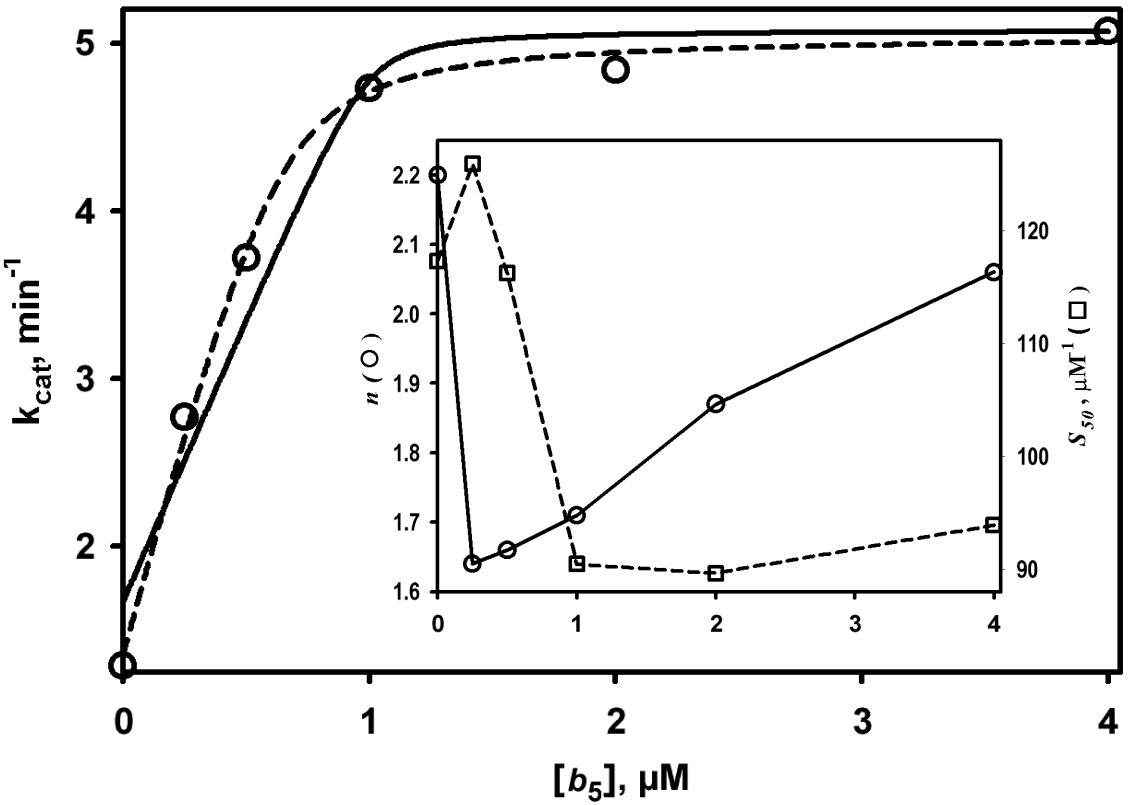

Figure 2

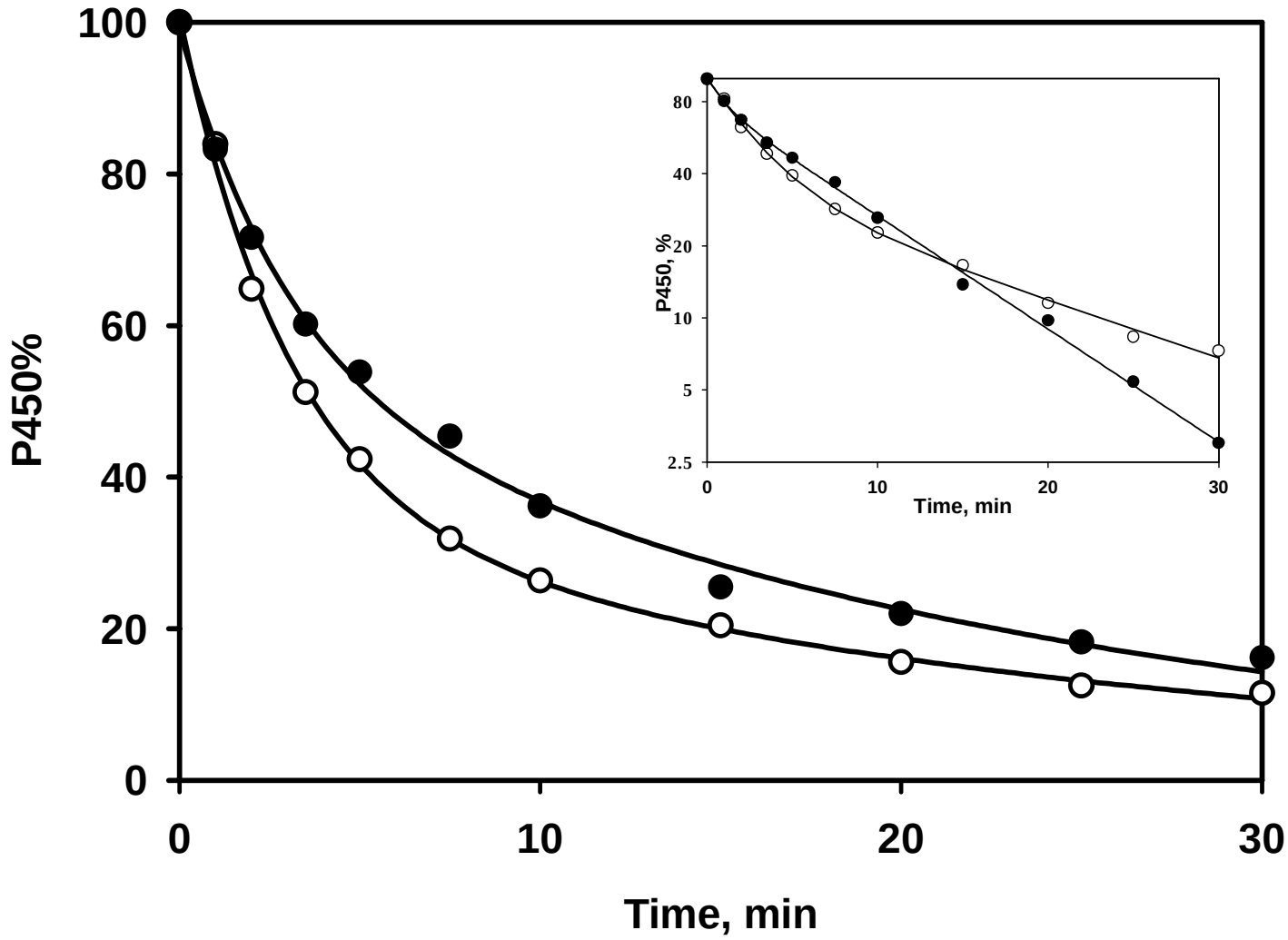

Figure 3