Cooperativity in Oxidations Catalyzed by Cytochrome P450 3A4[†]

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ABSTRACT: Cytochrome P450 (P450) 3A4 is the most abundant human P450 and oxidizes a diversity of substrates, including various drugs, steroids, carcinogens, and macrolide natural products. In some reactions, positive cooperativity has been reported in microsomal studies. Flavonoids, e.g., 7,8-benzoflavone (α-naphthoflavone, αNF), have been shown to stimulate some reactions but not others. In systems containing purified recombinant bacterial P450 3A4, positive cooperativity was seen in oxidations of several substrates, including testosterone, 17β-estradiol, amitriptyline, and most notably aflatoxin (AF) B_1. With these and other reactions, αNF typically reduced cooperativity (i.e., the *n* value in a Hill plot) while either stimulating or inhibiting reactions. With the substrate AFB_1, αNF both stimulated 8,9-epoxidation and inhibited 3α-hydroxylation. The same patterns were seen with AFB_1 in a fused P450 3A4−NADPH-P450 reductase protein. αNF did not alter patterns of activity plotted as a function of NADPH-P450 reductase concentration in systems containing the individual proteins. The patterns of AFB_1 oxidation to the two products were modified considerably in systems in which NADPH-P450 reductase was replaced with a flavodoxin or ferredoxin system, iodosylbenzene, or cumene hydroperoxide. AFB_2, which differs from AFB_1 only in the presence of a saturated 8,9-bond, was not oxidized by P450 3A4 but could inhibit AFB_1 oxidation. These and other results are considered in the context of several possible models. The results support a model in which an allosteric site is involved, although the proximity of this putative site to the catalytic site cannot be ascertained as of yet. In order to explain the differential effects of αNF and reduction systems on the two oxidations of AFB_1, a model is presented in which binding of substrate in a particular conformation can facilitate oxygen activation to enhance catalysis.

P450s[1] are found throughout the phylogenetic spectrum and usually function as monooxygenases in the oxidation of a wide variety of natural and synthetic chemicals (Nelson et al., 1993; Porter & Coon, 1991; Guengerich, 1991, 1993; Ortiz de Montellano, 1995). The ability of these enzymes to transform drugs, pesticides, pollutants, and potential carcinogens to both inocuous and also highly electrophilic chemicals is of practical interest (Guengerich, 1991; Porter & Coon, 1991).

In humans, P450 3A4 is generally agreed to be the most abundant P450 enzyme present in both liver and small intestine, two major sites for oxidation of xenobiotic chemicals (Guengerich, 1995; Wrighton & Stevens, 1992). This protein, originally isolated on the basis of its ability to oxidize the drug nifedipine (Guengerich et al., 1986), can account for at least as much as 60% of the total P450 in a human liver (Guengerich, 1990, 1995). P450 3A4 has a number of interesting properties. (i) The enzyme has a very broad substrate specificity, with >100 substrates now identified (Guengerich, 1995, and references therein). Approximately half of the drugs currently on the market are substrates. The ability of a single protein to catalyze so many reactions has been confirmed in studies with recombinant proteins (Brian et al., 1990; Guengerich et al., 1991; Shimada et al., 1994; Guengerich, 1995, and references therein). (ii) P450 3A4, like other P450 3A subfamily enzymes, tends to lose catalytic activity during purification, and many of its reactions require special conditions for reconstitution of optimal activity. (iii) Some of the reactions are very sensitive to particular reconstitution components (e.g., b_5, Mg^{2+}, and negatively charged phospholipids), while others are not (Shet et al., 1993; Yamazaki et al., 1995; Gillam et al., 1995; Ingelman-Sundberg et al., 1996). (iv) Some reactions appear to show cooperativity, at least as judged by sigmoidal v *vs* S plots reported in studies with microsomes (Andersson et al., 1994; Schmider et al., 1995; Schwab et al., 1988; Kerr et al., 1994). (v) The effects of αNF and natural flavonoids vary, yielding either stimulation, no effect, or inhibition depending on the reaction (Shimada & Guengerich, 1989; Yun et al., 1992; Raney et al., 1992b; Ueng et al., 1995).

In this set of studies, we consider aspects of the latter two items cited above (iv and v). Mechanisms have been proposed to explain such behavior in P450s derived from experimental animals, particularly rabbit P450s. One explanation for the stimulation by flavones is enhanced affinity

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[1] Abbreviations: P450, cytochrome P450 [also termed "heme−thiolate protein" by the Enzyme Commission (EC 1.14.14.1) (Palmer & Reedijk, 1992; Nelson et al., 1993)]; b_5, cytochrome b_5 (EC 4.4.2 group); v, enzyme reaction velocity; S, substrate concentration; AF, aflatoxin; αNF, α-naphthoflavone (7,8-benzoflavone); PhIO, iodosylbenzene; CuOOH, cumene hydroperoxide; GSH, (reduced) glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Flx, (*Escherichia coli*) flavodoxin; Fdx, (spinach) ferredoxin (EC 5.3.2 group).

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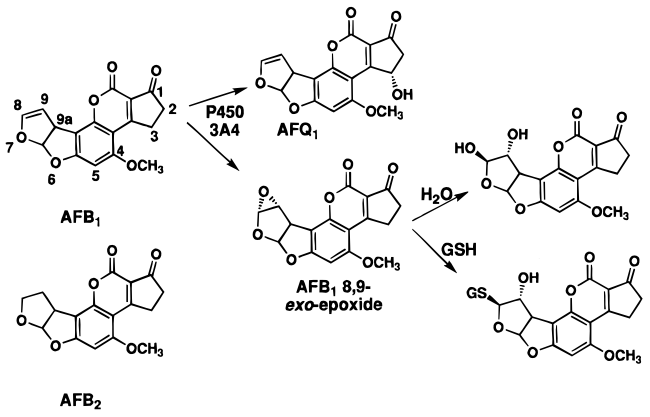
Scheme 1: Structures of AFB_1 and AFB_2 and Reactions of AFB_1



of the P450 for NADPH-P450 reductase (Huang et al., 1981b). Another mechanism proposed to explain both cooperativity and flavone stimulation is increased substrate affinity due to the presence of the effector at a distinct site (Schwab et al., 1988; Johnson et al., 1988) and involves at least some aspect of an allosteric model. More recently, Shou et al. (1994) proposed that the stimulation of P450 3A4-catalyzed benzo[*a*]pyrene oxidation by αNF is related to co-occupancy of a single, large binding site by the substrate and αNF, as opposed to an allosteric mechanism. However, no evidence for cooperativity was observed. Two other reports are of interest. Koley et al. (1995) hypothesized that multiple conformations of P450 3A4 exist, on the basis of multiphasic flash photolysis kinetics, and are modified by the presence of substrate. However, the relevance of these observations to the problems under investigation here was not investigated. Recently, Harlow and Halpert (1996) reported that mutation of residues 211 and 212 of P450 3A4 increased catalytic activity in the 6β-hydroxylations of testosterone and progesterone and blocked the stimulatory effect of αNF seen in *Escherichia coli* membranes containing recombinant P450 3A4 and fortified with NADPH-P450 reductase.

We have developed P450 3A4 reconstitution systems that appear to reflect the catalytic specificity seen in human liver microsomes (Gillam et al., 1993; Ueng et al., 1995; Yamazaki et al., 1995) and also show the patterns of cooperativity and flavone stimulation for AFB_1 (Scheme 1) (Ueng et al., 1995; Shimada & Guengerich, 1989; Guengerich et al., 1994). In this article, we use such systems to examine several substrates that have previously been suggested by others to show cooperative behavior. We also extend our earlier studies on AFB_1, a hepatocarcinogen for which we have previously reported some evidence for cooperativity and opposing αNF effects on different reaction pathways (Guengerich et al., 1994; Raney et al., 1992b; Ueng et al., 1995). The results of these studies on AFB_1 and several drugs reported to show cooperativity or αNF stimulation are considered in the context of several possible models for P450 3A4 catalysis.

EXPERIMENTAL PROCEDURES

Chemicals. AFB_1, AFB_2, AFQ_1, carbamazepine, amitriptyline, 17β-estradiol, testosterone, progesterone, diazepam, nortriptyline, 10,11-epoxycarbamazepine, temazepam, *N*-desmethyl diazepam, and miconazole were purchased from Sigma Chemical Co. (St. Louis, MO). 2β-Hydroxytestosterone, 6β-hydroxytestosterone, 6β-hydroxyprogesterone, 16α-hydroxyprogesterone, 2-hydroxy-17β-estradiol, and 4-hydroxy-17β-estradiol were purchased from Steraloids (Wilton, NH). AFB_2 was purified by reversed-phase preparative HPLC [octadecylsilane, 10 × 250 mm, Beckman Ultrasphere (Beckman, San Ramon, CA), 43% CH_3OH in 0.10 M sodium phosphate buffer at pH 3.0 (v/v)]. The GSH−AFB_1 conjugate was provided by Prof. T. M. Harris of the Center in Molecular Toxicology (Raney et al., 1992a). PhIO was prepared by hydrolysis of the diacetate (Saltzman & Sharefkin, 1973), stored at −20 °C, and sonicated in H_2O to prepare a working solution (5 mM) each day before use. CuOOH was purified by extraction with aqueous NaOH as described (Nordblom et al., 1976).

Enzymes. Human liver samples were obtained through Tennessee Donor Services (Nashville, TN) and stored at −80 °C until microsomes were prepared (Guengerich, 1994). Recombinant P450s 3A4 and 3A5 were expressed in *E. coli* and purified as described previously (Gillam et al., 1993, 1995). A P450 3A4−rat NADPH-P450 reductase fusion protein, originally described by Shet et al. (1993), was expressed from a similar construction vector and purified (Chun et al., 1996). Rabbit liver NADPH-P450 reductase (EC 1.6.2.4) (Yasukochi & Masters, 1976; Guengerich, 1994) and b_5 (Strittmatter et al., 1978; Shimada et al., 1986) were purified as described elsewhere. *E. coli* Flx and NADPH-Flx reductase were expressed and purified as described elsewhere (Jenkins & Waterman, 1994). Spinach Fdx and NADPH-Fdx reductase and GSH *S*-transferases were purchased from Sigma.

Enzyme Reconstitution Conditions. The reconstituted system used for the drug oxidations consisted of 40 nM P450 3A4, 80 nM NADPH-P450 reductase, 80 nM b_5, 0.5 mM sodium cholate, 20 μg of phospholipid mixture per milliliter (L-α-dilauroyl-*sn*-glycero-3-phosphocholine/L-α-dioleyl-*sn*-glycero-3-phosphocholine/bovine brain phosphatidylserine, 1:1:1, w/w/w), 50 mM potassium HEPES buffer (pH 7.4), 30 mM $MgCl_2$, an NADPH-generating system (Guengerich, 1994), and substrate in a total volume of 0.5 mL. Most substrates were dissolved in CH_3OH and used at final concentrations in the range of 2.5 −1000 μM, the upper concentration depending upon solubility. The final concentration of CH_3OH in the reaction mixture was <1%. In assays with 17β-estradiol, 1.0 mM ascorbic acid was added to prevent oxidation of catechol products (Guengerich, 1988). In the cases of amitriptyline and carbamazepine, 5-fold greater amounts of P450, reductase, b_5, cholate, and phospholipid mixture were used, in order to detect the products formed at lower substrate concentrations. All reactions were initiated by the addition of the NADPH-generating system and stopped by the addition of 0.10 mL of 1.0 M aqueous Na_2CO_3 (pH 10.5) containing 2 M NaCl, after incubation at 37 °C for 20 min. The oxidized metabolites of each substrate were extracted with CH_2Cl_2 or ethyl acetate, and the extracts were dried under N_2 and dissolved in the mobile phase for HPLC.

In AFB_1 and αNF oxidations, a mixture containing 20 μg of phospholipid mixture per milliliter (*vide supra*), 0.5 mM sodium cholate, 0.20 μM P450, 0.20 μM b_5, and 0.40 μM NADPH-P450 reductase was preincubated at 23 °C for 10 min. After preincubation, the following components were added: 50 mM potassium HEPES buffer (pH 7.85), 30 mM $MgCl_2$, and 3 mM GSH. In AFB_1 oxidation, 4 μM mouse GSH transferase was added in order to conjugate AFB_1 *exo-*

8,9-epoxide with GSH (Raney et al., 1992a). When the P450 system was supported with NADPH-Flx reductase, a mixture of 2.0 μM P450, 2.0 μM rabbit b_5, 4.0 μM Flx, and 2.0 μM NADPH-Flx reductase was used. In NADPH-Fdx reductase-supported systems, 0.8 μM P450, 0.8 μM b_5, 1.6 μM Fdx, and 0.8 μM NADPH-Fdx reductase were added. In these two systems, the AFB_1 oxidation mixture contained 50 mM potassium HEPES buffer (pH 7.85), 30 mM $MgCl_2$, 0.4 M KCl, 3.0 mM GSH, and 12 μM rat GSH transferase. Unless indicated otherwise, 50 μM AFB_1 was used and reactions were initiated by addition of the NADPH-generating system and proceeded at 37 °C for 20 min. The CuOOH- and PhIO-supported reaction mixtures contained 1.0 μM P450, 30 mM $MgCl_2$, and 50 μM AFB_1 in 50 mM potassium HEPES buffer (pH 7.85); 0.10 mM CuOOH or 1.0 mM PhIO was added, and reactions proceeded at 37 °C for 10 min (CuOOH) or 20 s (PhIO).

In experiments with human liver microsomes, sample HL 110 [known to be high in P450 3A4 (Guengerich, 1988)] was used in the place of the purified enzymes, in the same buffer (devoid of cholate).

Assay Methods and HPLC Conditions. AFB_1 oxidations were stopped by the addition of HCO_2H (to 0.07 M). AFQ_1 and AFB_1−GSH conjugate were separated with the use of an Alltech octadecylsilane (C18) HPLC column (4.6 × 250 mm, Alltech, Deerfield, IL) using 43% CH_3OH in 0.10 M sodium phosphate buffer at pH 3.0 (v/v) at a flow rate of 0.8 mL min^{-1} and were detected by A_{360} measurements. AFQ_1 was quantitated with the use of external standards, and AFB_1−GSH was quantitated by using the extinction coefficient of AFB_1 (ϵ_{362} = 21.8 mM^{-1} cm^{-1}). In αNF oxidations, the reaction mixture was stopped and extracted with an ethyl acetate/$(CH_3)_2CO$ mixture (2:1, v/v). The organic layer was dried under N_2, dissolved in CH_3OH, and then analyzed using an ISCO octadecylsilane (C18) HPLC column (4.6 × 250 mm, ISCO, Lincoln, NE) using an isocratic mixture of 80% CH_3OH in H_2O (v/v) at a flow rate of 0.8 mL min^{-1}. Product was detected by A_{290} measurements, and αNF 5,6-oxide formation was estimated using the extinction coefficient of αNF (ϵ_{290} = 23.7 mM^{-1} cm^{-1}).

Concentrations of drug oxidation products were determined by HPLC. The columns, flow rates, mobile phases, and detection wavelengths for the assays were as follows: testosterone, Partisil 5 ODS-3, 4.6 × 250 mm (Phenomenex, Torrance, CA), 1.0 mL min^{-1}, 65:35 CH_3OH/H_2O, v/v, and 254 nm; progesterone, YMC-Pack ODS AM302, 4.6 × 150 mm (YMC Co., Kyoto, Japan), 1.2 mL min^{-1}, 75:25 CH_3OH/H_2O, v/v, and 239 nm; 17β-estradiol, YMC-Pack ODS AM302, 4.6 × 150 mm, 1.2 mL min^{-1}, 60:40:0.5 $CH_3OH/H_2O/CH_3CO_2H$, v/v/v, and 280 nm; amitriptyline, Develosil ODS-HG-5, 4.6 × 150 mm (Nomura Chemicals Co. Ltd., Aichi, Japan), 1.5 mL min^{-1}, 40:60 10 mM potassium phosphate buffer (pH 4.0)/CH_3CN, v/v, containing 0.02% $(C_2H_5)_3N$, v/v, and 240 nm; carbamazepine, Develosil ODS-HG-5, 4.6 × 150 mm, 1.2 mL min^{-1}, 65:35 50 mM phosphate buffer (pH 6.0)/CH_3CN, v/v, containing 0.05% $(C_2H_5)_3N$, v/v, and 236 nm; diazepam, Develosil ODS-HG-5 4.6 × 150 mm, 1.2 mL min^{-1}, 45:20:35 75 mM potassium phosphate buffer (pH 6.0)/CH_3CN/CH_3OH, v/v/v, containing 0.05% $(C_2H_5)_3N$, v/v, and 236 nm.

Spectroscopy. P450 and NADPH-P450 reductase spectra were recorded using an OLIS/Aminco DW2 instrument (On-Line Instrument Systems, Bogart, GA). CD spectra were recorded with a JASCO J-720 instrument (Japan Applied Spectronics Co., Tokyo), and α-helicity was estimated as described elsewhere (Greenfield & Fasman, 1969; Chen & Yang, 1971).

Data Analysis. Kinetic analysis of P450 3A4 oxidations was done using a sigmoidal V_{max} model. Values of v at various substrate concentrations were fit using nonlinear least-squares regression with a weight of $1/v$ for the drug substrates and without weight for AFB_1 oxidations, due to the varying S ranges, to the equation

$$v = (V_{max}S^n)/(S_{50}{}^n + S^n)$$

where S_{50} is the substrate concentration showing a half-maximal velocity, n is a measure of cooperativity (Kuby, 1991), and V_{max} is the maximal velocity (Sigma Plot, Jandel Scientific, San Rafael, CA). The n value was obtained from plots of $\log[v/(V_{max} - v)]$ vs $\log S$ (Hill plot) through linear regression without weighting using Sigma Plot. Estimates of variance (denoted by ±) are presented from analysis of individual sets of data (i.e., internal estimates of error). The sets of results presented in this paper are representative of other studies [in the case of AFB_1, going back several years (Guengerich et al., 1994)].

RESULTS

Requirements for System Components in P450 3A4-Catalyzed Reactions. The requirements for individual system components in the oxidations of testosterone and AFB_1 by P450s 3A4 (Gillam et al., 1993; Yamazaki et al., 1995; Ueng et al., 1995) and 3A5 (Gillam et al., 1995) have been reported elsewhere. The oxidations examined here also showed strong dependence on both b_5 and Mg^{2+} (Figure 1A). The only exceptions were 17β-estradiol 2-hydroxylation and diazepam 3-hydroxylation, which showed less dependence on b_5. In contrast to earlier studies with P450 3A4 (Gillam et al., 1993), we found only slight stimulation with GSH. This has been a general experience with the P450 3A4 and 3A5 systems containing HEPES and Mg^{2+} (Yamazaki et al., 1995) instead of phosphate buffers (Gillam et al., 1993). The rate of testosterone 6β-hydroxylation was in the range of the higher rates reported from this (Yamazaki et al., 1995; Ingelman-Sundberg et al., 1996) and other laboratories (Shet et al., 1993) for this and similar systems. The rate of progesterone 6β-hydroxylation was twice as fast as that measured for testosterone.

αNF stimulated the rates of several of the activities (*vide infra*). A concentration of 30 μM was used in most of the studies because prior work with AFB_1 had shown this to be optimal for altering AFB_1 oxidation rates (Ueng et al., 1995). The component requirements for αNF-stimulated carbamazepine 10,11-epoxidation were similar to those for the reaction in the absence of αNF; i.e., b_5 and Mg^{2+} were both required for optimal activity (Figure 1B). It is also of interest to note that the activity of the enzyme system was also stimulated by αNF in the absence of b_5 or Mg^{2+}; thus, the presence of these components is not a requirement *per se* for αNF stimulation.

Steady-State Kinetics of P450 3A4-Catalyzed Reactions and Effects of αNF. Rates of oxidation of six drugs, in the presence and absence of 30 μM αNF, were fit to both Michaelis−Menten (v vs S) and Eadie−Hofstee plots (v vs v/S), using nonlinear regression (see Experimental Procedures) (Figure 2). The resulting calculated parameters are presented in Table 1.

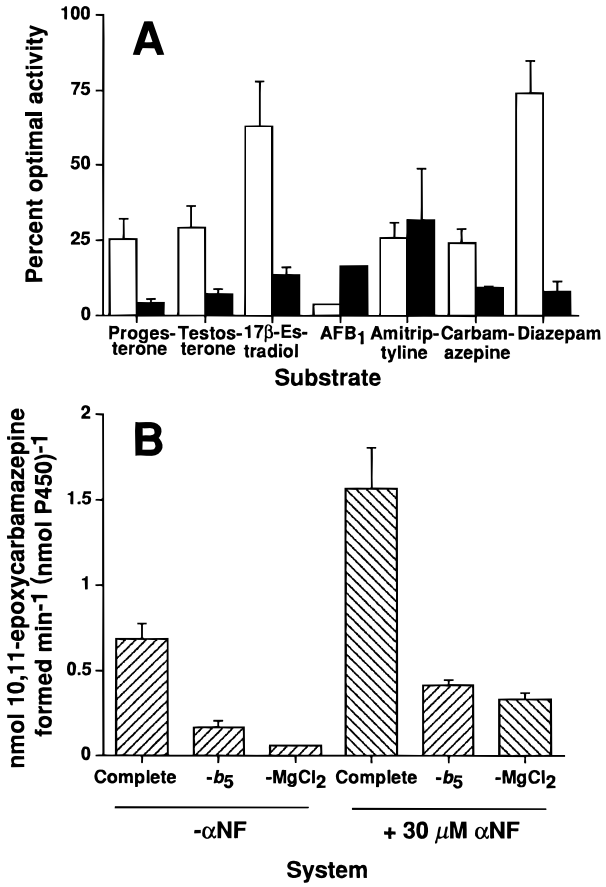


FIGURE 1: Effects of b_5 and Mg^{2+} on oxidations catalyzed by P450 3A4 in reconstituted systems. (A) Results for seven different reactions (in the absence of αNF), using a substrate concentration of 100 μM in each case: minus b_5 (□) and minus $MgCl_2$ (■). Rates (nanomoles of product formed per minute per nanomole of P450) were as follows: progesterone 6β-hydroxylation, 36 ± 3; testosterone 6β-hydroxylation, 16 ± 3; 17β-estradiol 2-hydroxylation, 3.2 ± 0.2; AFB_1 3α-hydroxylation, 7.5 ± 0.2; amitriptyline N-demethylation, 1.4 ± 0.3; carbamazepine 10,11-epoxidation, 0.6 ± 0.1; and diazepam 3-hydroxylation, 6.8 ± 1.0. (B) Carbamazepine 10,11-epoxidation in the absence and presence of αNF (30 μM). All results are presented as $\bar{X}$ ± SD of three separate experiments.

Progesterone 6β-hydroxylation (Figure 2B) showed no significant departure from linearity in the v *vs* v/S plot (n = 1.1 ± 0.1) (a third significant digit is not presented in this work). Testosterone 6β-hydroxylation showed a slight deviation from linearity (n = 1.3 ± 0.1) (Figure 2B, Table 1). The n values were near unity in the presence of αNF. The experiments with these two substrates were repeated with human liver microsomes, and very similar results were obtained (results not shown), except that S_{50} (i.e., K_m) was decreased ~1/2 in the presence of 30 μM αNF (change of 36 ± 6 to 14 ± 2 μM).

17β-Estradiol 2-hydroxylation yielded an n value of 1.3 ± 0.2 (Figure 2, Table 1). The reaction was stimulated several-fold (V_{max}) by αNF, and the n value was decreased (0.9 ± 0.1). Amitriptyline N-demethylation had an n value of 1.4 ± 0.1 (Figure 2, Table 1), which dropped to 1.1 ± 0.1 in the presence of αNF.

Carbamazepine 10,11-oxidation showed a response indicative of a linear v *vs* S plot, i.e., a vertical line in the v *vs* v/S plot (Figure 2B); thus, an n value could not be calculated. αNF increased the rate of epoxidation at low but not at high substrate concentrations. Diazepam 3-hydroxylation also showed linear plots of v *vs* S and v *vs* v/S (Figure 2B) in the absence of αNF. In the presence of αNF, the rates were decreased at the higher substrate concentrations, yielding a hyperbolic v *vs* S plot (Figure 2), with an n of 1.0 (±0.1) (Table 1).

Several of the drug substrates were also oxidized to minor products, i.e., 2β-hydroxytestosterone, 16α-hydroxyprogesterone, 4-hydroxy-17β-estradiol, *N*-desmethyl diazepam, and an unidentified product of carbamazepine (rates were 15−25% of formation of the major products). v *vs* S and derived patterns (obtained in the absence and presence of αNF) were not analyzed in detail, and the sensitivity is a limitation. However, in most cases, the cooperativity and the response to αNF appeared to be very similar to those seen with the major product. The only apparent exception was testosterone, where αNF stimulated 2β-hydroxylation ~2-fold and had a somewhat inhibitory effect on 6β-hydroxylation (Figure 2A).

Oxidations of AFB_1 by P450 3A4 and P450 3A4−NADPH-P450 Reductase Fusion Protein. The sigmoidal nature of v *vs* S plots for AFB_1 oxidation to AFQ_1 (3α-hydroxylation) and the 8,9-epoxide (with product trapped as a GSH conjugate) was noticed previously (Guengerich et al., 1994; Ueng et al., 1995). The work has been repeated several times with human liver microsomal preparations (Guengerich et al., 1994) and with recombinant P450 3A4. The shape of the v *vs* S plots is shifted from sigmoidal to hyperbolic in the presence of αNF, and oxidation is also shifted from 3α-hydroxylation to 8,9-epoxidation (Ueng et al., 1995). A set of plots obtained with a human liver microsomal preparation known to be high in P450 3A4 (Guengerich, 1988) is shown (Figure 3). The general trends are seen in the calculated parameters presented in Table 2, although the Hill plot did not yield much difference in the n value for 3α-hydroxylation in the presence of αNF.

One set of experiments with the reconstituted P450 3A4 system is shown in Figures 4 and 5. The results were also converted to linear transformations [$\log[v/(V_{max} - v)]$ *vs* $\log S$], and linear regression analysis was done (Table 2). For 3α-hydroxylation, the Hill coefficient n decreased from 2.1 to 1.3 and the Eadie−Hofstee n from 2.4 to 1.4 in the presence of αNF. For 8,9-epoxidation, the Hill plot value of n decreased from 2.3 to 1.5 and the Eadie−Hofstee plot n from 4.1 to 1.6.

The patterns observed with the P450 3A4−NADPH-P450 reductase fusion protein were qualitatively similar to those of reconstituted P450 3A4 (Figures 6 and 7 and Table 2), although the rates were lower. High n values were measured for both oxidations in the absence of αNF, and αNF had the effects of (i) decreasing the V_{max} for 3α-hydroxylation, (ii) increasing the V_{max} for 8,9-epoxidation, and (iii) decreasing n values for both reactions. No major effect on S_{50}, an index of positive cooperativity and an approximation of K_m, was observed (Table 2).

Dependence of Oxidations by P450 3A4 on the Concentration of NADPH-P450 Reductase. The ratio of NADPH-P450 reductase to P450 3A4 was varied from 0.2 to 10, in the presence and absence of αNF. Maximum catalytic activity was observed with a NADPH-P450 reductase to P450 3A4 ratio of ~2, and αNF did not appear to shift the pattern for oxidations of AFB_1, amitriptyline, or 17β-estradiol (results not shown).

Alterations of Patterns of AFB_1 Oxidation of P450s 3A4 and 3A5 by Modification of the Reductase System or Oxygen Surrogates. The typical pattern of effects of αNF on P450

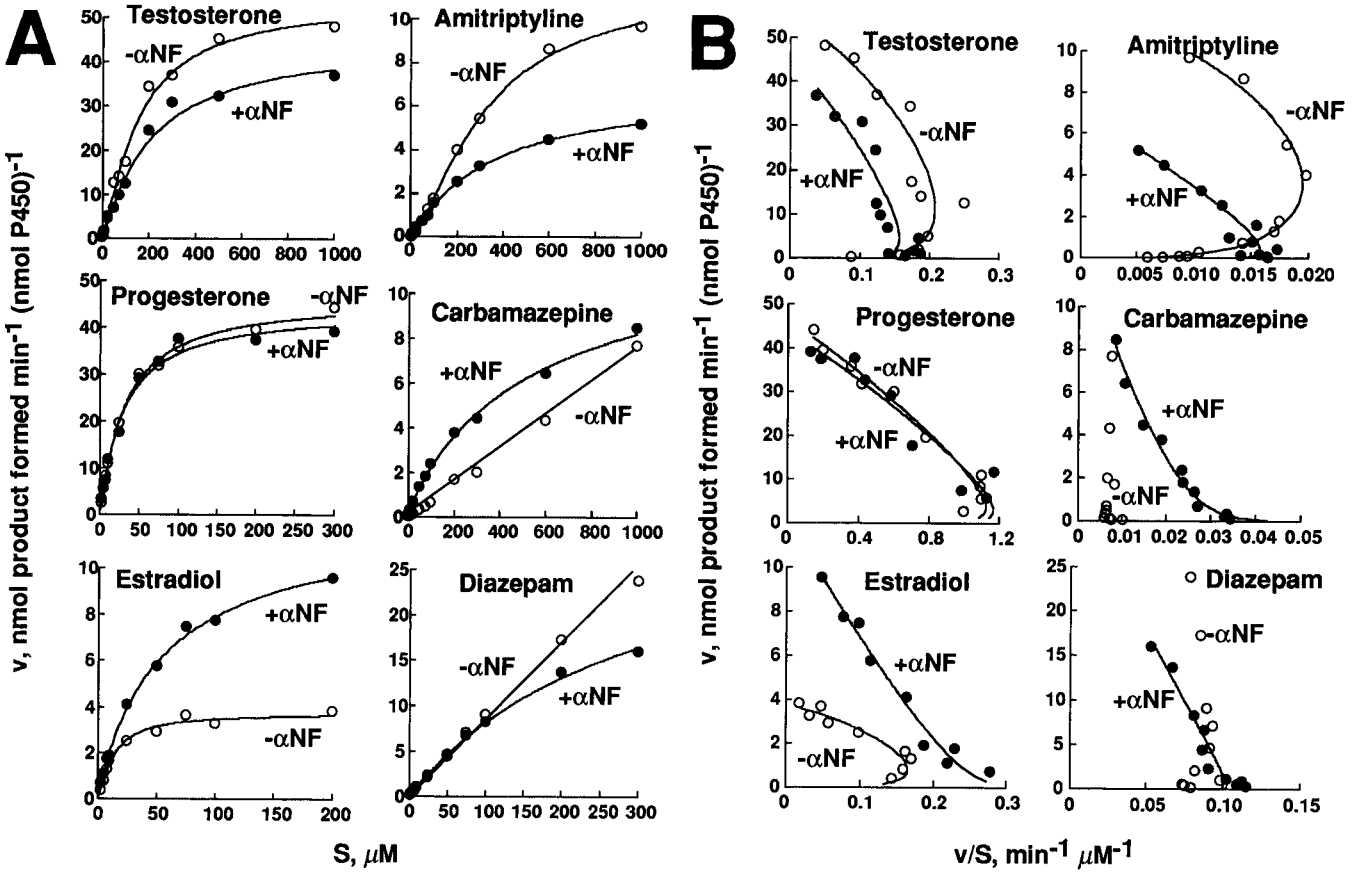


FIGURE 2: Steady-state kinetics of six P450 3A4 reactions and the effects of αNF. Plots of v, expressed in nanomoles of product formed per minute per nanomole of P450, *vs* S, substrate concentration (A), and v *vs* v/S (B) are presented, with lines drawn using the Sigma-Plot program and the equation $v = (V_{max}S^n)/(S_{50}^n + S^n)$ for testosterone 6β-hydroxylation, progesterone 6β-hydroxylation, 17β-estradiol 2-hydroxylation, amitriptyline N-demethylation, carbamazepine 10,11-epoxidation, and diazepam 3-hydroxylation: minus αNF (○) and plus 30 μM αNF (●).

Table 1: Kinetic Parameters for P450 3A4-Catalyzed Drug Oxidations[a]

	minus αNF			plus αNF (30 μM)		
reaction	S_{50} (μM)	V_{max} [nmol of product min^{-1} (nmol of P450)$^{-1}$]	n	S_{50} (μM)	V_{max} [nmol of product min^{-1} (nmol of P450)$^{-1}$]	n
progesterone 6β-hydroxylation	32 ± 3	46 ± 2	1.1 ± 0.1	30 ± 5	43 ± 3	1.1 ± 0.1
testosterone 6β-hydroxylation	160 ± 25	54 ± 3	1.3 ± 0.1	210 ± 45	45 ± 4	1.1 ± 0.1
17β-estradiol 2-hydroxylation	13 ± 2	3.7 ± 0.2	1.3 ± 0.2	56 ± 11	13 ± 1	0.9 ± 0.1
amitriptyline N-demethylation	330 ± 2	12 ± 3.6	1.4 ± 0.1	340 ± 40	6.9 ± 0.4	1.1 ± 0.1
carbamazepine 10,11-epoxidation	—[b]	—[b]	—[b]	610 ± 20	18 ± 4	0.9 ± 0.1
diazepam 3-hydroxylation	—[b]	—[b]	—[b]	300 ± 70	33 ± 9	1.0 ± 0.1

[a] Each value represents the parameter determined by the nonlinear least-squares method. [b] See Figure 2B.

3A4-catalyzed AFB_1 oxidation was seen for a P450 3A4−NADPH-P450 reductase fusion protein, although the ratios of products were noticeably shifted in both the presence and absence of αNF (Table 3). When the NADPH-P450 reductase component, unbound or fused, was replaced by either a Flx/Flx reductase or Fdx/Fdx reductase system [which have been demonstrated to be capable of (slowly) transferring electrons to P450 3A4 (Yamazaki et al., 1995)], no 3α-hydroxylation was observed (Table 3). The low rates of 8,9-epoxidation were enhanced by 1 order of magnitude in the presence of αNF. The oxygen surrogates PhIO and CuOOH yielded AFB_1 8,9-dihydrodiol [the hydrolysis product of the 8,9-epoxide (Johnson et al., 1996)] but no detectable AFQ_1 (the epoxide was not trapped in these reactions because of the interfering oxidation of GSH by these oxidants). αNF inhibited the epoxidation of AFB_1 (Table 3); the concentration of αNF needed for 50% inhibition was ~10 μM (results not shown).

Somewhat similar patterns were seen with P450 3A5, another human P450 in the P450 3A subfamily [85% sequence identity (Aoyama et al., 1989; Gillam et al., 1995)]. As reported earlier (Gillam et al., 1995), the ratio of 3α-hydroxylation to 8,9-epoxidation is reversed relative to P450 3A4. αNF stimulated 8,9-epoxidation but did not appreciably affect the low rate of 3α-hydroxylation (Table 3). As with P450 3A4, the oxygen surrogates PhIO and CuOOH yielded only 8,9-epoxide.

Attempted Oxidation of AFB_2 by P450 3A4. AFB_2 differs from AFB_1 only in the presence of a saturated 8,9-bond (Scheme 1). Since the major product of the P450 3A4 oxidation of AFB_1 results from 3α-hydroxylation, on the other end of the molecule, we were interested in the oxidation of AFB_2 and the effects of αNF. However, we were unable to detect any oxidation products under the conditions used with the substrate AFB_1. Previously, Roebuck et al. (1978) had reported trace oxidation of AFB_2 to a putative AFQ_2

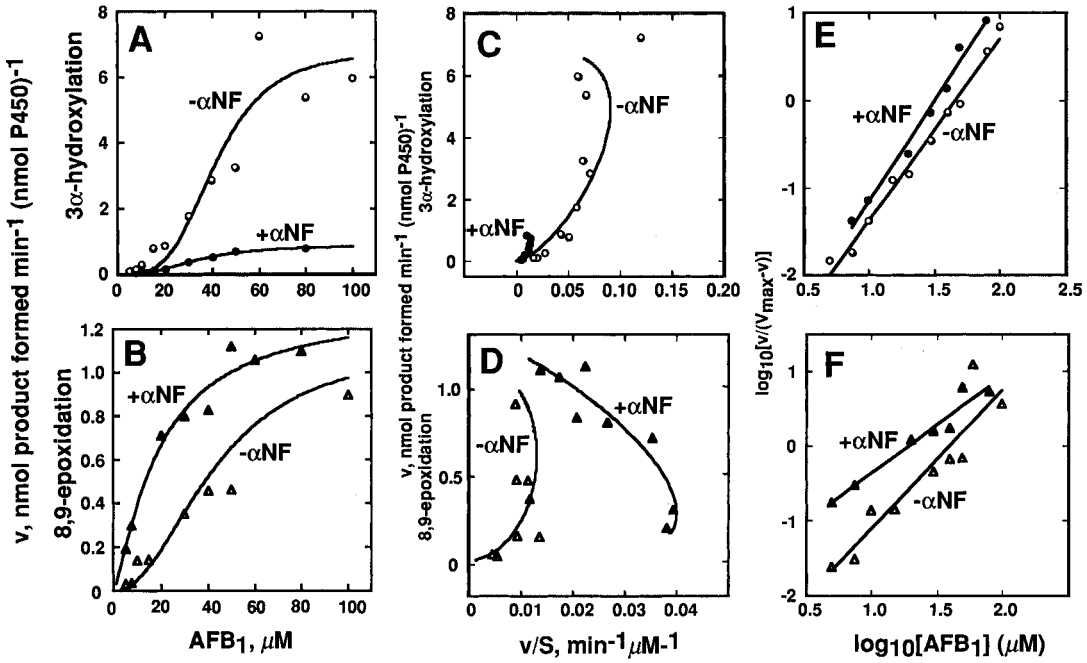


FIGURE 3: Cooperativity in the oxidations of AFB_1 in human liver microsomes. Parts A and B show 3α-hydroxylation and 8,9-epoxidation in the absence and presence of 30 μM αNF, respectively, in plots of v *vs* S. Parts C and D show the results of parts A and B, with lines fitted from v *vs* S plots, in plots of v *vs* v/S. Parts E and F show the same results fitted to plots of $\log[v/(V_{max} - v)]$ *vs* log S. See Table 2 for calculated parameters: open symbols, incubations without αNF; and filled symbols, incubations done in the presence of 30 μM αNF.

Table 2: Parameters for AFB_1 Oxidations Catalyzed by P450 3A4 and P450 3A4−NADPH-P450 Reductase Fusion Protein

	3α-hydroxylation		8,9-epoxidation	
enzyme	minus αNF	plus αNF[a]	minus αNF	plus αNF[a]
Human Liver Microsomes				
plot of v vs v/S[b]				
S_{50} (μM)	42 ± 5	34 ± 3	44 ± 17	19 ± 6
V_{max} (min^{-1})	6.8 ± 1.2	0.89 ± 0.08	1.1 ± 0.4	1.3 ± 0.2
n	3.6 ± 1.5	2.7 ± 0.4	2.1 ± 1.1	1.3 ± 0.3
plot of $\log[(V_{max} - v)/v]$ *vs* log S				
n	2.1 ± 0.1	2.3 ± 0.1	1.8 ± 0.3	1.3 ± 0.1
P450 3A4				
plot of v vs v/S[b]				
S_{50} (μM)	43 ± 7	48 ± 26	27 ± 2	15 ± 2
V_{max} (min^{-1})	8.1 ± 1.2	2.6 ± 0.8	0.9 ± 0.1	1.8 ± 0.1
n	2.4 ± 0.6	1.4 ± 0.4	4.1 ± 0.8	1.6 ± 0.2
plot of $\log[(V_{max} - v)/v]$ *vs* log S				
n	2.1 ± 0.1	1.3 ± 0.1	2.3 ± 0.3	1.5 ± 0.1
P450 3A4−NADPH P450 Reductase Fusion Protein				
plot of v vs v/S[b]				
S_{50} (μM)	27 ± 1	36 ± 6	33 ± 2	30 ± 8
V_{max} (min^{-1})	0.96 ± 0.04	0.52 ± 0.05	0.14 ± 0.01	1.1 ± 0.2
n	3.6 ± 0.5	1.6 ± 0.2	3.8 ± 0.7	1.2 ± 0.2

[a] αNF at 50 μM in the case of microsomes and 30 μM in the case of purified enzymes. [b] V_{max} (actually k_{cat}) from a plot of v *vs* S.

product with one of the two human liver microsomal preparations [rate of 0.02 nmol of product min^{-1} (mg of protein)$^{-1}$, i.e., ∼0.05 nmol of product min^{-1} (nmol of P450)$^{-1}$].

However, 30 μM AFB_2 inhibited the oxidation of AFB_1 (used at 50 μM) to both AFQ_1 and AFB_1 *exo*-8,9-epoxide. This latter result is in contrast with an earlier report from our group, in which liver microsomes were used and AFB_2 preferentially inhibited 8,9-epoxidation to a greater extent than 3α-hydroxylation (Raney et al., 1992b).

Oxidation of αNF and Effect of AFB_1. αNF was transformed to a single major product in the P450 3A4-reconstituted system. The HPLC retention time of the product matched that of the product observed in the oxidation of αNF by P450 1A1 (Bauer et al., 1995), and the UV spectra of the product measured at different pH values matched those reported for the 5,6-epoxide (Nesnow & Bergman, 1981; Vyas et al., 1983). The mass spectrum (MH^+ at *m/z* 289, fast atom bombardment) is that of a monooxygenated product.

The rate of oxidation of αNF to the 5,6-oxide is subject to uncertainty because no authentic product was available to determine the extinction coefficient (Nesnow & Bergman, 1981; Vyas et al., 1983). However, the rate was not changed by the presence of AFB_1 in either of two experimental approaches designed to examine competition (Figure 8).

Stoichiometry of NADPH Oxidation, H_2O_2 Production, and AFB_1 and αNF Product Formation by P450 3A4. The P450 3A4 system is relatively leaky in its coupling efficiency. In the absence of substrate, electrons are used to reduce O_2 to

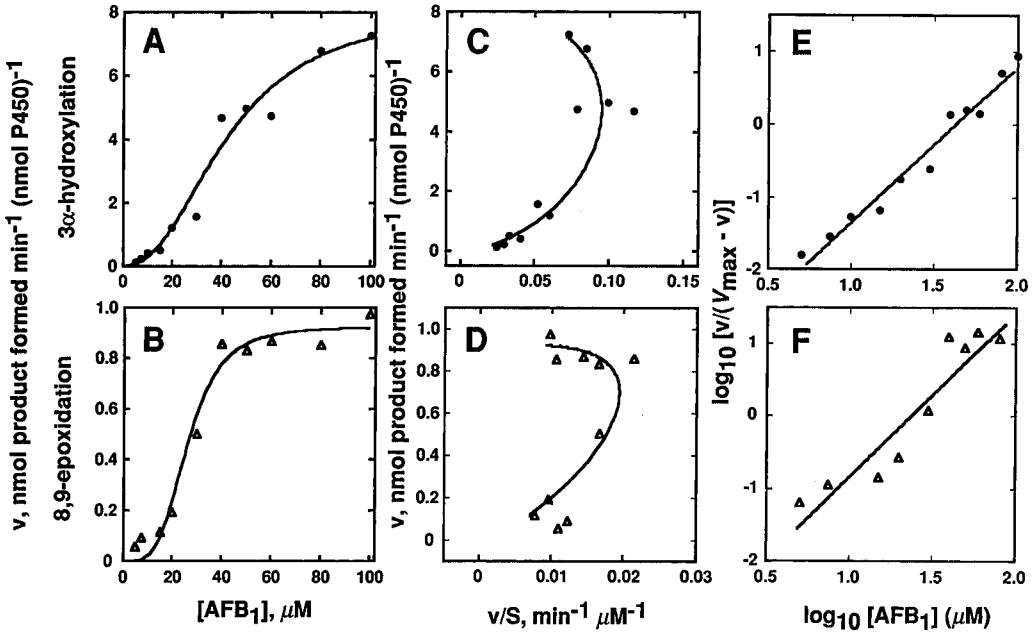


FIGURE 4: Cooperativity in the oxidations of AFB_1 by P450 3A4 in the absence of αNF. The standard reconstitution system was used with P450 3A4 and varying concentrations of AFB_1, in the presence of mouse GSH transferase to trap all *exo*-8,9-epoxide as GSH−AFB_1 conjugate: (A and B) 8,9-epoxidation and 3α-hydroxylation; (C and D) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted from plots of v *vs* S, in plots of v *vs* v/S; and (E and F) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted to plots of the Hill equation, $\log[v/(V_{max} - v)]$ *vs* log S. For lists of calculated parameters, see Table 2.

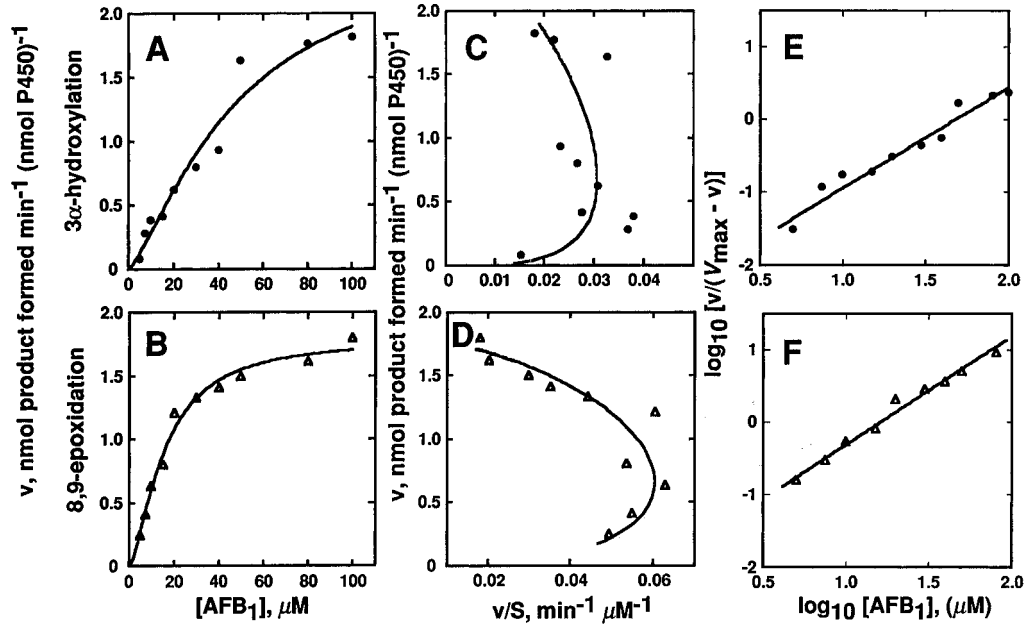


FIGURE 5: Cooperativity in the oxidations of AFB_1 by P450 3A4 in the presence of 30 μM αNF. The standard reconstitution system was used with P450 3A4 and varying concentrations of AFB_1, in the presence of mouse GSH *S*-transferase to trap all *exo*-8,9-epoxide as GSH−AFB_1 conjugate: (A and B) 8,9-epoxidation and 3α-hydroxylation; (C and D) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted from plots of v *vs* S, in plots of v *vs* v/S; and (E and F) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted to plots of the Hill equation, $\log[v/(V_{max} - v)]$ *vs* log S. For lists of calculated parameters, see Table 2.

H_2O_2 and apparently to H_2O (Table 4). In the presence of AFB_1 or αNF, most of the electrons from NADPH are still used nonproductively, i.e., not for oxidation of the substrate. We considered the possibility that the presence of αNF might render the oxidation of AFB_1 more efficient. However, when both molecules were present, the rate of NADPH oxidation was higher, with only low productive utilization of NADPH for product formation.

DISCUSSION

P450 3A4 is the major P450 enzyme present in human liver and small intestine, and the levels of the protein can vary 40-fold among individuals (Guengerich, 1988, 1995). The enzyme has a very broad catalytic specificity and appears to play a major role in the metabolism of about half of the drugs on the market. Interactions among substrates and inhibitors can have serious consequences [e.g., terfenadine (Yun et al., 1993; Guengerich, 1995)]. The interactions of P450 3A4 and other P450 3A subfamily enzymes with ligands and accessory proteins appear to be more complex than those of other P450s.

We found that a number of P450 3A4 substrates showed cooperative interactions, and some catalytic activities were stimulated by the flavonoid αNF (Table 1). These two types

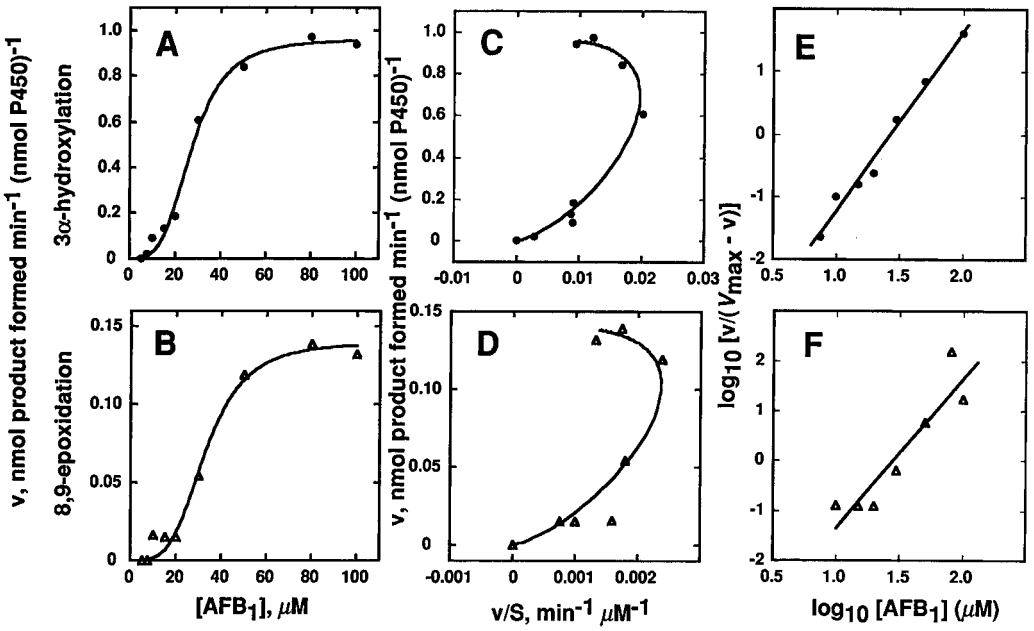


FIGURE 6: Cooperativity in the oxidations of AFB_1 by P450 3A4−NADPH-P450 reductase fusion protein in the absence of αNF. The standard reconstitution system was used with P450 3A4 and varying concentrations of AFB_1, in the presence of mouse GSH transferase to trap all *exo*-8,9-epoxide as GSH−AFB_1 conjugate: (A and B) 8,9-epoxidation and 3α-hydroxylation; (C and D) 8,9-epoxidation and 3a-hydroxylation, respectively, fitted to plots of v *vs* S, in plots of v *vs* v/S; and (E and F) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted to plots of the Hill equation, $\log[v/(V_{max} - v)]$ *vs* $\log S$. For lists of calculated parameters, see Table 2.

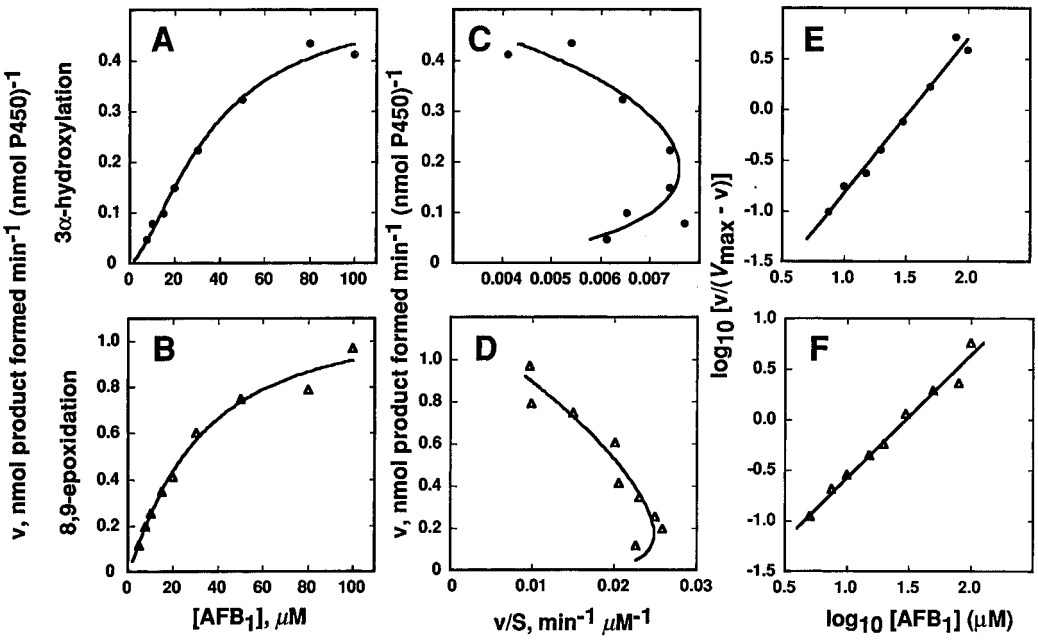


FIGURE 7: Cooperativity in the oxidations of AFB_1 by P450 3A4−NADPH-P450 reductase fusion protein in the presence of 30 μM αNF. The standard reconstitution system was used with P450 3A4 and varying concentrations of AFB_1, in the presence of mouse GSH transferase to trap all *exo*-8,9-epoxide as GSH−AFB_1 conjugate: (A and B) 8,9-epoxidation and 3α-hydroxylation; (C and D) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted to plots of v *vs* S, in plots of v *vs* v/S; and (E and F) 8,9-epoxidation and 3α-hydroxylation, respectively, fitted to plots of the Hill equation, $\log[v/(V_{max} - v)]$ *vs* $\log S$. For lists of calculated parameters, see Table 2.

of interactions are termed "homotropic" and "heterotropic", respectively (Creighton, 1993). However, not all of the reactions previously reported to show such behavior did so in the recombinant P450 3A4 systems used here. Some of the discrepancies may be due to the small degree of cooperativity reported in microsomal preparations and the need to add inhibitors to mask the contributions of other P450s (Schmider et al., 1995). Progesterone and testosterone 6β-hydroxylations have been reported to show αNF stimulation in bacterial membranes containing recombinant P450 3A4 and added NADPH-P450 reductase (Harlow & Halpert, 1996); however, neither αNF stimulation nor cooperativity was detected in our studies (Figure 2). The basis of the discrepancy is unknown, although the rates measured in the completely reconstituted system are much higher (Table 1). Some of the heterotropic interactions may have not been observed because a single concentration of αNF (30 μM), found to be useful with AFB_1, may not be optimal for other substrates. The drug reactions that showed cooperativity were testosterone 6β-hydroxylation, 17β-estradiol 2-hydroxylation, and amitriptyline N-demethylation (Figure 2, Table 1). Carbamazepine 10,11-epoxidation and diazepam 3-hydroxylation showed rather vertical v *vs* v/S plots (Figure 2B). The general effect of αNF was to render the v *vs* S

Table 3: AFB_1 Oxidation by P450s 3A4 and 3A5 in Various Systems

system	rate of product formation [pmol min^{-1} (nmol of P450)$^{-1}$]			
	8,9-epoxide (GSH−AFB) or AFB 8,9-diol[a]		3α-hydroxylation (AFQ_1)	
	minus αNF	plus αNF[b]	minus αNF	plus αNF[b]
P450 3A4 and NADPH-P450 reductase	850 ± 20	1800	4800 ± 150	1600
fused P450 3A4−NADPH-P450 reductase	140 ± 50	780 ± 50	1900 ± 130	400 ± 10
P450 3A4, Flx, and Flx reductase	1.4 ± 1.2	12 ± 2	<1	<1
P450 3A4, Fdx, and Fdx reductase	0.4	5.3	<1	<1
P450 3A4 and PhIO	22000 ± 3000	8300 ± 1300	<10	<10
P450 3A4 and CuOOH	150 ± 10	63 ± 10	<10	<10
P450 3A5 and NADPH-P450 reductase	370 ± 50	660 ± 20	70 ± 20	80
P450 3A5 and PhIO	2900	2300	<10	<10
P450 3A5 and CuOOH	60	40	<10	<10

[a] Detected as GSH−AFB_1 in the reductase-containing systems and as AFB 8,9-diol in the systems containing PhIO and CuOOH. [b] 30 μM.

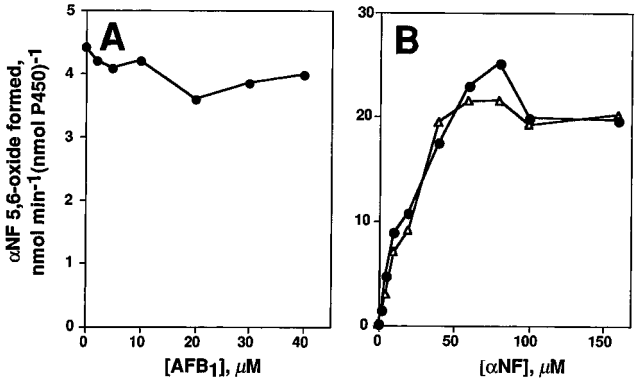


FIGURE 8: Lack of effect of AFB_1 on oxidation of αNF. The usual reconstituted P450 3A4 system was used. In part A, 10 μM αNF and the indicated concentrations of AFB_1 were present. In part B, the AFB_1 concentration was held at 40 μM (△) or else no AFB_1 was present (●), and the αNF concentration was varied.

plots more hyperbolic, i.e., decrease *n* (Table 1) and give a linear *v vs v*/*S* plot normally seen in Michaelis−Menten kinetics. αNF stimulated some reactions but not others (Table 1). In some cases, V_{max} was modified; in other cases, curve shapes were altered, with enhancement and inhibition both seen, depending upon the substrate concentration (e.g., Figure 2).

The results obtained with AFB_1 were quite striking in many respects. Plots of *v vs S* were the most sigmoidal of any of the reactions examined (Figures 3−7). The presence of αNF in the reactions produced a consistent shift toward hyperbolic patterns (i.e., normal Michaelis−Menten plots) and low *n* values as judged by any of the methods used, in both the 3α-hydroxylation and 8,9-epoxidation reactions. As reported earlier (Raney et al., 1992b; Ueng et al., 1995), αNF inhibited 3α-hydroxylation and enhanced 8,9-epoxidation (Figures 3−7). This pattern was also seen with the fused P450 3A4−NADPH-P450 reductase protein (Figure 6, Table 2) and also the related subfamily protein P450 3A5 (Table 3). The pattern of products was highly dependent upon the reduction or oxygen surrogate system used to support the reaction (Table 3). Different product ratios were seen with the fusion protein in which NADPH-P450 reductase was covalently attached, and no 3α-hydroxylation product (AFQ_1) was found in the systems containing Flx, Fdx, PhIO, or CuOOH. AFB_2 differs from AFB_1 only in the presence of a saturated 8,9-bond (Scheme 1), and we hypothesized that 8- or 9-hydroxylation might occur. We should have been able to find 3-hydroxy-AFB_2 (AFQ_2) with an HPLC retention time near that of AFQ_1 (Roebuck et al., 1978), but none was seen.

Our results are considered in the context of three major models. Work on the stimulation of benzo[*a*]pyrene 3-hydroxylation activity of several rabbit P450s was interpreted in the context of a model in which the binding of the effector to the P450 increases the apparent affinity of the P450 for NADPH-P450 reductase (Huang et al., 1981a; Miller et al., 1983). However, this model does not seem to be applicable here, at least not for the oxidations of the substrates examined. αNF stimulates AFB_1 8,9-epoxidation but also inhibits AFB_1 3α-hydroxylation (Figures 3−7), so the stimulation cannot be accounted for only by enhanced reductase binding. Further, the same pattern of stimulation of AFB_1 8,9-epoxidation and inhibition of 3α-hydroxylation was seen in the P450 3A4−NADPH-P450 fusion protein (Figures 6 and 7). Intermolecular electron transfer in that protein cannot be ruled out,[2] but no clear differences in the patterns of reductase dependence of activities upon NADPH-P450 concentration were seen with either AFB_1, amitriptyline, or 17β-estradiol as the substrate.

A second possible model is presented in Scheme 2. This model was developed on the basis of enhancement of the P450 3A4-catalyzed oxidation of benzo[*a*]pyrene and other polycyclic hydrocarbons by αNF (Shou et al., 1994). These workers discounted allosteric models (*vide infra*) and favored a paradigm in which there is a single, large substrate binding site that is capable of holding two molecules of substrate, or one each of substrate and effector. The primary rationale for this appears to be the ability of P450 3A4 to oxidize αNF and the increase in V_{max} without a change in K_m (for polycyclic hydrocarbon oxidation) (Shou et al., 1994). A size for the site was calculated, although there is no experimental evidence that both the substrate and αNF are together in the site. An advantage of this model is that it provides the possibility for one molecule (effector) to enter the site and stimulate reduction, since it appears from stopped-flow kinetic experiments that the presence of substrate (or αNF) is critical for rapid reduction of ferric P450 3A4 (Yamazaki et al., 1996). However, there are some deficiencies in this model, at least with regard to the reactions studied here. There is no rationale for homotropic cooperativity, in the form of the sigmoidal plots of *v vs S* [apparently, these were not observed with the polycyclic

[2] Reduction rates of the oxidized enzyme have been measured using stopped-flow techniques. The rate of heme reduction (ferrous enzyme trapped with CO) appears to be invariant over a 12-fold concentration range.[3] However, intermolecular electron transfer could be occurring within an aggregate of the protein.

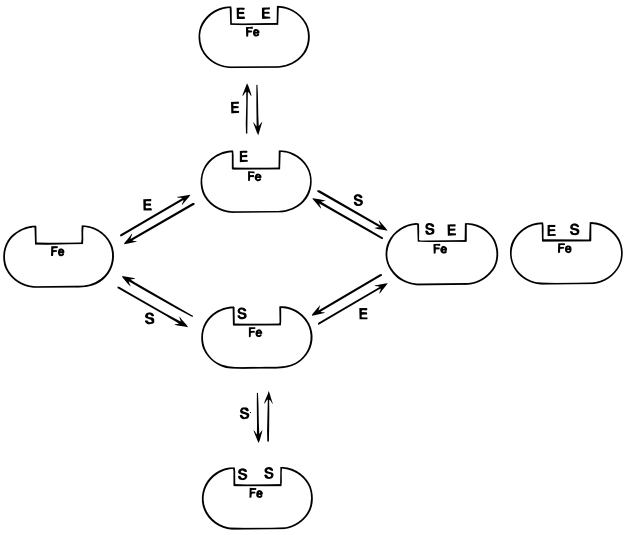
[3] F. P. Guengerich, W. W. Johnson, and Y.-J. Chun, unpublished results.

Table 4: Stoichiometry of Oxidations by P450 3A4

substrate	NADPH oxidation [nmol min^{-1} (nmol of P450)$^{-1}$]	H_2O_2 formation [nmol min^{-1} (nmol of P450)$^{-1}$]	H_2O formation [nmol min^{-1} (nmol of P450)$^{-1}$][c]	nanomoles of product formed per minute per nanomole of P450		
				AFQ_1	AFB_1 oxide	αNF 5,6-oxide
none	86	13	36	–	–	–
AFB_1[a]	120	24	45	4.5 ± 0.5	0.7 ± 0.1	–
αNF[b]	130	27	45	0	0	11.2
αNF[b] + AFB_1[a]	210	31	84	1.1	1.3	11.2

[a] 50 μM. [b] 30 μM. [c] Calculated by difference (Gorsky et al., 1984).

Scheme 2: Model for P450 3A4 Stimulation Based on Binding of Two Ligands in a Single Site[a]

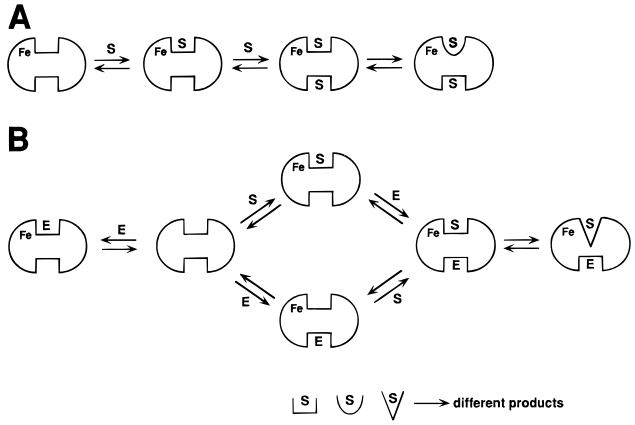


[a] S = substrate, E = effector (αNF), and Fe = iron of heme moiety.

hydrocarbons (Shou et al., 1994)]. If the substrate binding constants are unaffected by the presence of effector (bound to the protein), then there should be no cooperativity. Perhaps more problematic is the ability of αNF to modulate oxidations of AFB_1 in both negative and positive directions. However, AFB_1 did not influence the rate of αNF oxidation (Figure 8). One possibility to be considered is that αNF can only fit into one defined portion of the binding site that is occupied by AFB_1 in the position required for 3α-hydroxylation. PhIO and CuOOH could occupy this site, and the exclusive 8,9-epoxidation seen in the presence of these oxygen surrogates (Table 3) and inhibition of CuOOH-supported 8,9-epoxidation by αNF might be consistent with such a hypothesis. However, αNF 5,6-epoxidation was supported by PhIO and CuOOH (results not shown). We also considered the possibility that, if such a model (Scheme 2) is viable, some inhibitors might differentially affect the rates of the two AFB_1 oxidations. However, miconazole, gestodene (Guengerich, 1990), AFB_2 (*vide supra*), erythromycin, and L-754,394 [*N*-[2-(*R*)-hydroxy-1-(*S*)-indanyl]-5-[2-(*S*)-[[(1,1-dimethylethyl)amino]carbonyl]-4-[(furo[2,3-*b*]-pyrin-5-yl)methyl]piperazin-1-yl]-4-(*S*)-hydroxy-2-(*R*)-(phenylmethyl)pentamide] (Chiba et al., 1995) all inhibited both reactions to the same extent in the usual P450 3A4-reconstituted system (results not presented).

The model presented in Scheme 3 is an allosteric one, with two distinct sites. The mechanism in part A is presented to explain the cooperativity (i.e., sigmoidal plot of v *vs* S) seen in several of the reactions of interest. The substrate binds at the catalytic site (top) of the protein in Scheme 3. Filling the effector site (at the bottom) with the substrate could change the character of the substrate binding site, depicted

Scheme 3: Allosteric Model for P450 3A4 Cooperativity and Stimulation[a]



[a] S = substrate, E = effector, and Fe = iron of heme moiety.

as a change from a square to an oval shape here. This could increase the affinity of the substrate binding site for the substrate. As indicated, such changes in the shape of the binding site might also alter the juxtaposition of the substrate and influence the product distribution. In part B, the effector is shown occupying the effector site. Such occupation may increase substrate affinity and also perturb the shape of the substrate binding site in such a way as to influence product distribution. The effect may be the same as in part A or, as shown in part B with a different shape, different than when the substrate occupies the E site. Indeed, the reduction in apparent S_{50} values for AFB_1 when αNF is present is consistent with this theory (Figures 3−7) (see also the case of carbamazepine; Figure 2, Table 1). It is also possible for the effector (αNF) to move into the substrate site for oxidation to occur.

This latter model has several advantages. A distinct, classical allosteric binding site can be used to explain the sigmoidal kinetics. Many of the αNF interactions can be rationalized. There is some precedent with rabbit P450 3A6 for the enhanced binding of a substrate analog when an effector is present (Johnson et al., 1988). A deficiency of the model is that AFB_1 did not inhibit the oxidation of αNF (Figure 8), which might have been expected from Scheme 3B. However, the same results can be used as an argument for the independence of the two putative sites.

A further modification of Scheme 3 is considered in a model in which ferric P450 3A4 can bind AFB_1 in either of two configurations. In the absence of αNF, a juxtaposition is favored that has the 3α position close to the iron. The conformation in the presence of αNF has the 8,9-double bond close to the iron. This 8,9 conformation is postulated to facilitate the reduction of the iron by NADPH-P450 reductase (one or both steps) and possibly the proton transfers needed for oxygen activation. Thus, αNF shifts the equilibrium to a form where the 8,9-bond is targeted and a transition state

for oxygen activation is lowered. With the oxygen surrogates PhIO and CuOOH, there is an even lower barrier to oxygen activation, and the 8,9-epoxidation pathway is favored. This explanation is also useful for rationalizing the enhancement of 8,9-epoxidation by αNF in the systems that use Flx and Fdx for reduction (Table 3), although oxygen activation and substrate oxidation in these systems seem to be generally inefficient. αNF does inhibit AFB_1 8,9-epoxidation in the systems supported by the oxygen surrogates by a competitive mechanism (Table 3). This model is difficult to provide clear evidence for, since distinguishing between the putative binding conformations of AFB_1 is not possible, particularly in the absence of any structures from diffraction work. A conformational change is postulated. CD spectroscopy (of P450 3A4) showed an apparent shift in the calculated α-helicity from 43 ± 3% ($\bar{X}$ ± SD, $n = 3$) to 35 ± 5% in the presence of 30 μM αNF, but the meaning of such a difference, even if real, is unclear. P450 3A5 showed no change (31 *vs* 30% α-helicity). We also considered the possibility that studies with AFB_2 oxidation (Scheme 1) might be useful. However, this derivative did not appear to be oxidized at all. Thus, the structural features of AFB_1 related to its oxidation must be quite delicate.

Can a single model or any one of the three major ones presented here (Schemes 2 and 3) explain all of the results? Our own view is that Scheme 3 is preferred, although aspects of Scheme 2 cannot be ruled out. A problem with both is the lack of competition for AFB_1 with αNF 5,6-epoxidation (Figure 8). However, the situation may be confounded by a situation in which AFB_1 is an effector while αNF is in the substrate binding site. Schemes 2 and 3 are not mutually exclusive, with the major distinction being the proximity of the two sites. The best model may be more complex than any of these presented here. One possibility is a combination of those in Schemes 2 and 3, where there are two substrate binding sites and a distinct effector site. The two substrate sites may be nonequivalent and differentially perturbed by the presence of an effector, bound at a third site. A major question is whether two (or more) binding sites really exist. Stoichiometry results have never been presented, and our own preliminary efforts with AFB_1 have been thwarted by the insolubility of unbound AFB_1 in solution. Nevertheless, there are physical approaches (e.g., fluorescence) that, in principle, can be used to approximate distances between ligands in proteins, and ultimately, either measurements of this type or crystallographic determinations will be needed.

With the results that are available, we can eliminate some of the possible explanations for the appearance of sigmoidal v *vs* S plots (Kuby, 1991). First, the observed results are clearly not due to substrate depletion in the assays done at low substrate concentrations (e.g., Figures 3−7). Because the work was done with a recombinant enzyme, the sigmoidal patterns are not due to the presence of two distinct enzymes. Also, the patterns are not due to the presence of an inhibitor (in either the substrate or enzyme) that blocks catalysis only at low substrate concentrations, since the addition of αNF to AFB_1 assays yields hyperbolic plots of v *vs* S. Our working hypothesis, then, is that the mechanism involves a conformational change induced by the substrate or effector. Whether this postulated allosterism is best described as sequential (Koshland et al., 1966) or concerted (Monod et al., 1965) is unknown. Further evidence for an allosteric mechanism would be added if the effector site could be eliminated under conditions where there is no loss of catalytic activity (i.e., the enzyme is rendered insensitive to the modifier by mutation or covalent modification) (Kuby, 1991; Harlow & Halpert, 1996).

An unanswered question is how b_5 fits into the mechanism of substrate cooperativity. All of the reactions studied here, with the exception of 17β-estradiol 2-hydroxylation and diazepam 3-hydroxylation, were highly dependent upon b_5 (and also Mg^{2+}) (Figure 1A). The low rates of reaction seen in the absence of these components did not allow us to do careful studies to determine if cooperativity occurred in their absence. However, it is of interest to note that αNF stimulated carbamazepine 10,11-epoxidation even in the absence of b_5 or Mg^{2+} (Figure 1B). Thus, we can postulate that the putative αNF site is distinct from that used for binding b_5. It should be noted that P450 3A4 is reduced much faster when b_5 is present in the presence of some substrates (including testosterone and AFB_1) but not others (Yamazaki et al., 1995, 1996). (We have also noted that αNF stimulates ferric P450 3A4 reduction in the presence of b_5.[3])

We feel that the model presented as a variant of Scheme 3, in which the substrate can bind in either of two modes and one of these can influence events related to oxygen activation, has some merit in offering an explanation for several of the observations reported here. This scheme is of interest, in that the addition of substrate is influencing catalysis, in a manner not unlike classical induced fit. Recently, the interactions of the substrate with bacterial $P450_{eryF}$ (P450 108) have been found to behave in this manner (Cupp-Vickery et al., 1996). The interaction of the polyhydroxy substrate with the enzyme leads to the reorganization of bound H_2O molecules hydrogen bonded in the active site, where one appears to be able to participate in the mechanism of O−O bond cleavage. Such a mechanism may have relevance in explaining these reactions of P450 3A4 as well.

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