Roles of Divalent Metal Ions in Oxidations Catalyzed by Recombinant Cytochrome P450 3A4 and Replacement of NADPH−Cytochrome P450 Reductase with Other Flavoproteins, Ferredoxin, and Oxygen Surrogates†

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ABSTRACT: Recombinant cytochrome P450 (P450) 3A4 was most active in nifedipine and testosterone oxidation in a system including NADPH−P450 reductase, cytochrome b_5 (b_5), a semisynthetic phospholipid mixture plus cholate, glutathione, and $MgCl_2$. The $MgCl_2$ effect could be seen with high concentrations of Ca^{2+} or Sr^{2+} but not readily when these cations were replaced with monovalent cations. The divalent cation effect was also seen in liver microsomes. Part of the basis of this effect appears to be enhanced rates of b_5 reduction, as judged from studies on deletions of reconstitution components and analysis of steady-state spectral studies. Rapid reduction of ferric P450 3A4 to ferrous was dependent upon the presence of substrate, either testosterone or ethylmorphine. When testosterone was present, reduction was also highly dependent upon the presence of b_5 and Mg^{2+}. In the case of the substrate ethylmorphine, the need to add b_5 and Mg^{2+} to obtain optimal reduction rates was less pronounced. These patterns are consistent with the dramatic dependence of testosterone 6β-hydroxylation on b_5 and the lack of dependence of ethylmorphine *N*-demethylation on b_5. Our interpretation is that divalent cations stimulate electron transfer from NADPH−P450 reductase to several acceptors and that substrates *and* b_5 can bind to P450 3A4 to influence its rate of reduction by the reductase. P450 3A4 catalyzed testosterone 6β-hydroxylation within *Escherichia coli* cells. The reactions could be supported by *E. coli* cytosol or by purified *E. coli* flavodoxin and NADPH−flavodoxin reductase. Spinach ferredoxin and NADPH−ferredoxin reductase also supported catalytic activities. The "oxygen surrogate" iodosylbenzene supported higher reaction turnover numbers than did any of the reductase-based systems, in support of the view that events related to P450 reduction and oxygen activation are generally rate-limiting for oxidations catalyzed by this enzyme.

P450[1] enzymes are the major catalysts involved in the mammalian oxidation of xenobiotic chemicals such as drugs and carcinogens, and they also oxidize many endobiotic compounds (e.g., steroids, eicosanoids, alkaloids, fat-soluble vitamins) (Porter & Coon, 1991; Guengerich, 1991; Ortiz de Montellano, 1986). In humans, P450 3A4 is usually the major enzyme expressed in liver and small intestine (Guengerich *et al.*, 1986; Shimada *et al.*, 1994; Kolars *et al.*, 1992). P450 3A4 plays a major role in the metabolism of many drugs and has been implicated as a locus for some adverse drug interactions (Yun *et al.*, 1993; Kivistö *et al.*, 1994). A general working mechanism is presented in Scheme 1 (Guengerich & Macdonald, 1990).

A historic problem with all of the P450s in the 3A subfamily has been reconstituting catalytic activity after purification (Elshourbagy & Guzelian, 1980; Schwab *et al.*, 1988; Guengerich *et al.*, 1986). b_5 has been known to be involved for many years (Guengerich *et al.*, 1986), although the exact role has not been clear. Imaoka *et al.* (1992) and others (Halvorson *et al.*, 1990) have reported the usefulness of particular phospholipid/detergent mixtures. We also found stimulation of the activity of recombinant bacterial P450 3A4 by GSH (Gillam *et al.*, 1993). Shet *et al.* (1993) reported that a recombinant bacterial P450 3A4:NADPH−P450 reductase fusion protein had catalytic activity in Tris buffers but not in phosphate; no stimulation was seen with GSH. This chimeric protein required b_5 (and Tris buffer) for oxidation of testosterone and nifedipine. However, neither phospholipid nor b_5 was necessary for *N*-demethylation of the substrates ethylmorphine, erythromycin, and imipramine. We also found similar variations in some of our own work with P450s 3A4 and 3A5 (another 3A subfamily enzyme with 85% sequence identity), in that the oxidations of nifedipine, testosterone, and aflatoxin B_1 were highly sensitive to alterations in the reaction mixture, but the oxidations of ethylmorphine, erythromycin, and *d*-benzphetamine were not (Gillam *et al.*, 1995; Ueng *et al.*, 1995).

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[1] Abbreviations: P450, cytochrome P450 [also termed "heme-thiolate protein" by the Enzyme Commission (EC 1.14.14.1)]; b_5, cytochrome b_5 (EC 4.4.2 group); Fdx, (spinach) ferredoxin (EC 5.3.2 group); Flx, (*Escherichia coli*) flavodoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; CuOOH, cumene hydroperoxide; PhIO, iodosylbenzene; GSH, (reduced) glutathione; HPLC, high-performance liquid chromatography; $E_{m,7}$, oxidation−reduction potential relative to hydrogen, at pH 7.0.

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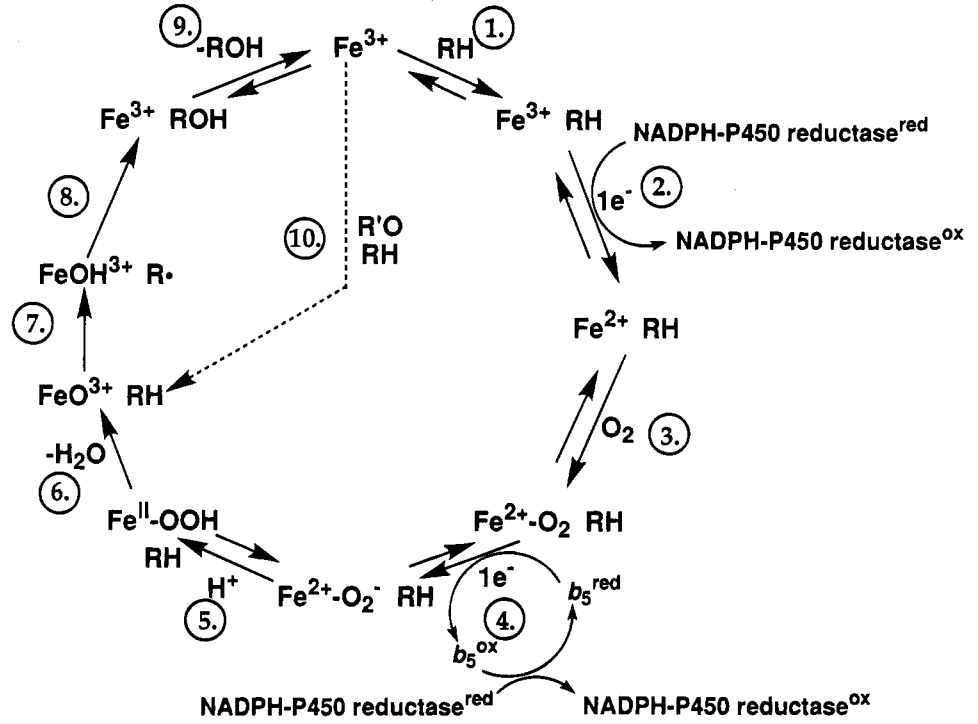
Scheme 1: General Mechanistic Cycle of P450 Catalysis[a]



[a] Fe is the iron of the P450 heme; RH = substrate; ROH = product; R'O = oxygen surrogate; and ox and red indicate oxidized and reduced states of b_5 and NADPH-P450 reductase.

In some of our earlier studies we found an unexpected stimulation of some of the P450 3A4-catalyzed reactions by Mg^{2+} ions (Ueng *et al.*, 1995; Gillam *et al.*, 1995). We have investigated the basis of this phenomenon further and now interpret it in terms of stimulation of electron flow. The work led to other reinvestigations of the role of b_5 in P450 3A4-catalyzed reactions. We also report P450 3A4 catalytic activity within *Escherichia coli* expressing the protein, as in the case of Barnes *et al.* (1991) with P450 17A. This activity, like that of P450 17A (Jenkins & Waterman, 1994), is supported by purified Flx and also by the spinach iron–sulfur protein Fdx.

EXPERIMENTAL PROCEDURES

Chemicals. CuOOH was purchased from Aldrich Chemical Co. (Milwaukee, WI), dissolved in CH_2Cl_2, washed with dilute NaOH, dried with Na_2SO_4, and concentrated *in vacuo* before preparation of aqueous solutions (Hoch & Lang, 1944). PhIO was prepared by hydrolysis of the diacetate (Aldrich) as described elsewhere (Saltzman & Sharefkin, 1973). [1α,2α-^{3}H]Testosterone was obtained from DuPont/New England Nuclear (Boston, MA). Nifedipine was purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized from C_2H_5OH in amber glass (Guengerich, 1990); the oxidation product was synthesized by HNO_3 oxidation and recrystallized as described elsewhere (Böcker & Guengerich, 1986). The "phospholipid mixture" used in many of the P450 3A4 studies included L-α-dilauroyl-*sn*-glycero-3-phosphocholine, L-α-dioleoyl-*sn*-glycero-3-phosphocholine, and bovine brain phosphatidylserine (all purchased from Sigma) in a 1:1:1 (w/w/w) ratio (Imaoka *et al.*, 1992). This was generally used at a concentration of 20 μg (total weight) mL^{-1}, in the presence of 0.25 mM sodium cholate (Imaoka *et al.*, 1992).

Enzymes. Spinach Fdx and NADPH–Fdx reductase (EC 1.18.1.2) were purchased from Sigma. *E. coli* Flx (20 kDa) and NADPH–Flx reductase (29 kDa) were purified from *E. coli* cytosol by the methods described by Jenkins and Waterman (1994); the enzymes were electrophoretically homogeneous and showed the expected flavin spectra. b_5 was purified to electrophoretic homogeneity from human or rabbit liver microsomes as described elsewhere (Shimada *et al.*, 1986). NADPH–P450 reductase (EC 1.6.2.4) was purified to apparent homogeneity from liver microsomes of phenobarbital-treated rabbits as described (Yasukochi & Masters, 1976) with minor modification (Guengerich, 1994).

Recombinant P450s 3A4 and 3A5 were expressed in *E. coli* DH5α cells using a pCW vector system, and the proteins were purified as described elsewhere (Gillam *et al.*, 1993, 1995).

Spectroscopy. UV–visible spectra were recorded at ambient temperature using an Aminco DW2/OLIS instrument (On-Line Instrument Systems, Bogart, GA). CD spectroscopy was done using a JASCO 720 instrument (Japan Applied Spectroscopy, Tokyo).

The kinetics of P450 3A4 reduction were measured with an Otsuka RA-401 stopped-flow instrument (Otsuka Electronics, Osaka) using the general method of Taniguchi *et al.* (1979). Aliquots of two vessels were mixed; both contained glucose oxidase (Sigma, type VII, from *Aspergillus niger*, 0.25 μM), bovine liver catalase (Sigma, 0.5 μM), 30 mM glucose, and 50 mM potassium HEPES buffer (pH 7.4) under an atmosphere of purified CO. One syringe contained NADPH (0.6 mM) to start the reaction (plus 30 mM $MgCl_2$, when indicated). The other syringe contained 0.4 μM P450 3A4, 0.8 μM NADPH–P450 reductase, 1.0 mM sodium cholate, 40 μg mL^{-1} of the phospholipid mixture, 6 mM GSH, and the indicated additions of b_5 (0.40 μM), $MgCl_2$ (30 mM), and substrate (either 0.40 mM testosterone or ethylmorphine). Reactions were done at 25 °C.

Enzyme Assays. P450 3A4 or P450 3A5 was generally mixed with a 2-fold excess of NADPH–P450 reductase, b_5

(amount equimolar with P450), GSH, and sodium cholate to yield final concentrations of 3.0 and 0.25 mM, respectively, and the phospholipid mixture mentioned earlier (to give a final concentration of 20 μg mL^{-1}) (Imaoka *et al.*, 1992). The mixture, in a total volume ~1/5 that of the final assay, was allowed to stand for 10 min at ambient temperature. Buffer (generally 50 mM potassium HEPES, pH 7.7), H_2O, and substrate were then added to bring the mixture to the final volume. The general optimum reconstitution mixture included 30 mM $MgCl_2$. The mixture was incubated at 37 °C for 3 min, and the reaction was initiated by the addition of an NADPH-generating system (Guengerich, 1994). After an appropriate time the reaction was quenched, and products were analyzed using chromatography.

In some cases the components were altered, as indicated in the text. Sometimes NADPH–P450 reductase was replaced by *E. coli* cytosol, a mixture of Flx and NADPH–Flx reductase, or a mixture of Fdx and NADPH–Fdx reductase. In some cases, NADPH–P450 reductase and the NADPH-generating system were replaced with an oxygen surrogate (CuOOH or PhIO).

Testosterone 6β-hydroxylation was measured using analysis of the products by HPLC (Brian *et al.*, 1990). Nifedipine oxidation was also analyzed using HPLC (Guengerich *et al.*, 1986). Ethylmorphine *N*-demethylation was measured using the Nash assay (Nash, 1953) as modified (Macdonald *et al.*, 1989).

Assays of Catalytic Activity in Bacterial Cells. E. coli DH5α cells harboring the pCW control and P450 3A4 expression plasmids were grown in modified TB media (Gillam *et al.*, 1993) at 30 and 32 °C, respectively, for 24 h in the presence of 1.0 mM isopropyl β-D-thiogalactoside. Cells were pelleted by centrifugation at $(5 \times 10^3)g$ for 15 min, washed once in 50 mM potassium 3-(*N*-morpholino)-propanesulfonate buffer (pH 7.2) containing 100 mM KCl, 1.0 mM EDTA, and 1.0 mM dithiothreitol, and resuspended (in 1/10 the original culture volume) in the same buffer. [1α,2α-^{3}H]Testosterone (3.6 Ci mmol^{-1}, 2.5 μM) was mixed with resuspended cells containing 0.8 nmol of P450 3A4 and 10 mM glucose and incubated for 0–12 h at 32 or 37 °C. The product was analyzed by HPLC (Brian *et al.*, 1990), with the effluent passed directly into the cell of an on-line scintillation counter (β-RAM, IN/US, Tampa, FL).

RESULTS AND DISCUSSION

Effects of Divalent Cations and Other Components on Catalytic Activities of P450 3A4. Nifedipine oxidation, a rather prototypic activity of this enzyme (Guengerich *et al.*, 1986), was reconstituted under a variety of conditions, but the most active systems all included b_5 (Figure 1A). In the course of these experiments we found a rather dramatic effect of adding a high concentration of $MgCl_2$ (30 mM). Catalytic activity was observed in the presence of Tris, phosphate, or HEPES buffers. The order of activity with the buffers varied somewhat, but generally HEPES proved to yield the highest activity. The most active system contained potassium HEPES buffer, b_5, $MgCl_2$, and GSH. The stimulatory effect of GSH (Gillam *et al.*, 1993) was routinely observed and is a ~2-fold enhancement. The catalytic activity of the system, expressed on a P450 basis, appeared to be as high as in microsomes. Similar results were seen when the requirements for testosterone 6β-hydroxylation were examined (Figure 1B).

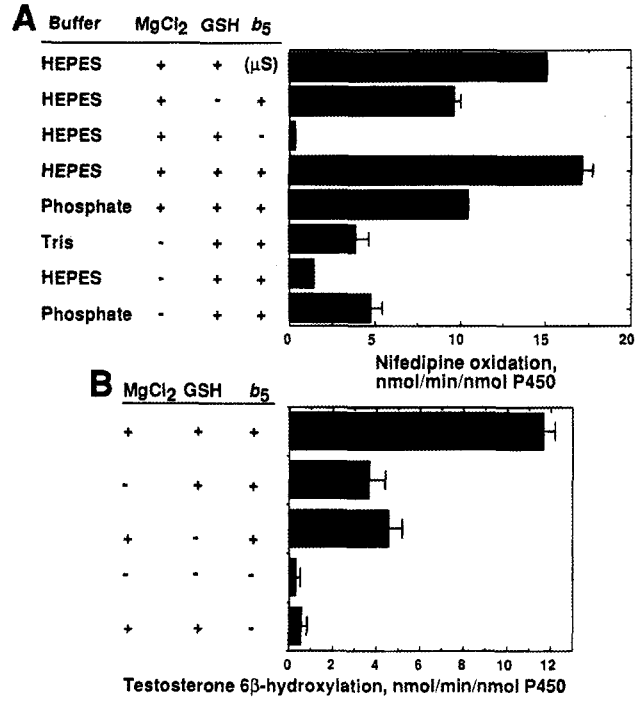


FIGURE 1: Effects of reconstitution system components on P450 3A4-catalyzed oxidations. Systems were set up as outlined in the general procedure described under Experimental Procedures for (A) nifedipine oxidation and (B) testosterone 6β-hydroxylation. The top bar in part A indicates a system containing human liver microsomes (μS) [liver sample HL110, a sample known to be high in P450 3A4 (Guengerich, 1988)]. All other values were for systems reconstituted with purified recombinant P450 3A4 and are means of triplicate experiments ± SD. In part B all studies were done using 50 mM potassium HEPES (pH 7.7) buffer.

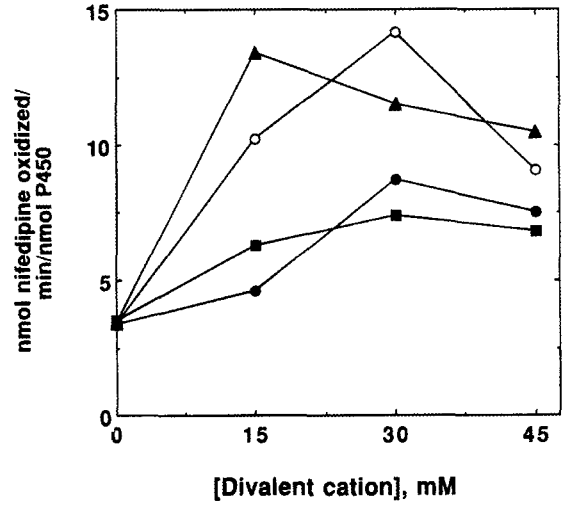


FIGURE 2: Dependence of rates of nifedipine oxidation on divalent cation concentrations. The standard reconstituted P450 3A4 system using potassium HEPES buffer was used as described under Experimental Procedures except in the case of microsomes, where only the buffer, substrate, and NADPH-generating system were added: P450 3A4 and $MgCl_2$ (●); P450 3A4 and $CaCl_2$ (○); P450 3A5 and $MgCl_2$ (▲); human liver microsomes (sample HL110) and $MgCl_2$ (■).

In a preliminary series of experiments, the maximum stimulation of catalytic activity was seen with ~30 mM $MgCl_2$ (Figure 2). $CaCl_2$ was also effective, and the effect was also seen with the close relative P450 3A5 [85% sequence identity (Gillam *et al.*, 1995)] and human liver microsomes. The cation effect was examined further, since

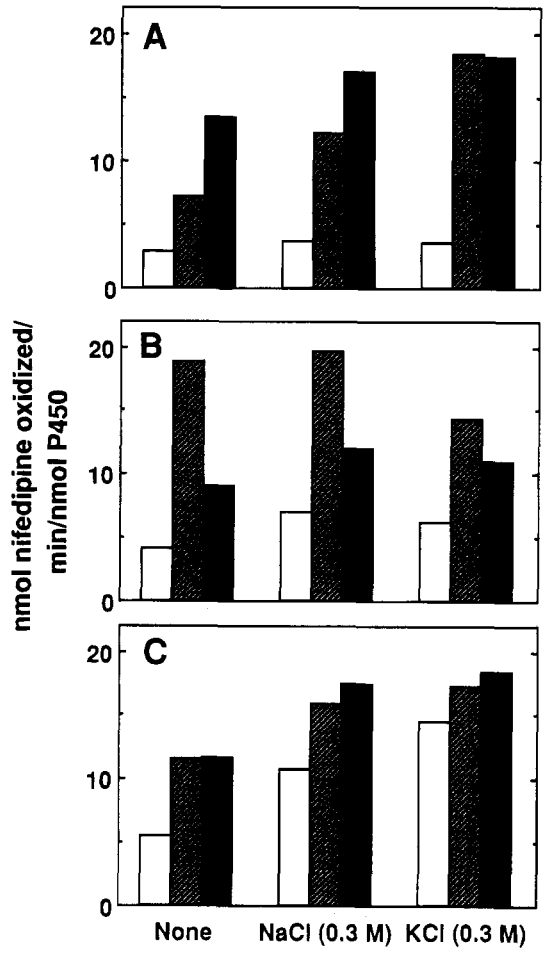


FIGURE 3: Nifedipine oxidation as a function of addition of monovalent and divalent cations. Rates are shown (means of duplicate experiments) for the standard complete P450 3A4 system (see Experimental Procedures) set up in the absence or presence of the indicated monovalent cation. Either no divalent cation (open bars), 30 mM $MgCl_2$ (hatched bars), or 30 mM $CaCl_2$ (solid bars) was present: (A) P450 3A4; (B) P450 3A5; (C) human liver microsomes [sample HL110, known to be high in P450 3A4 (Guengerich, 1988)].

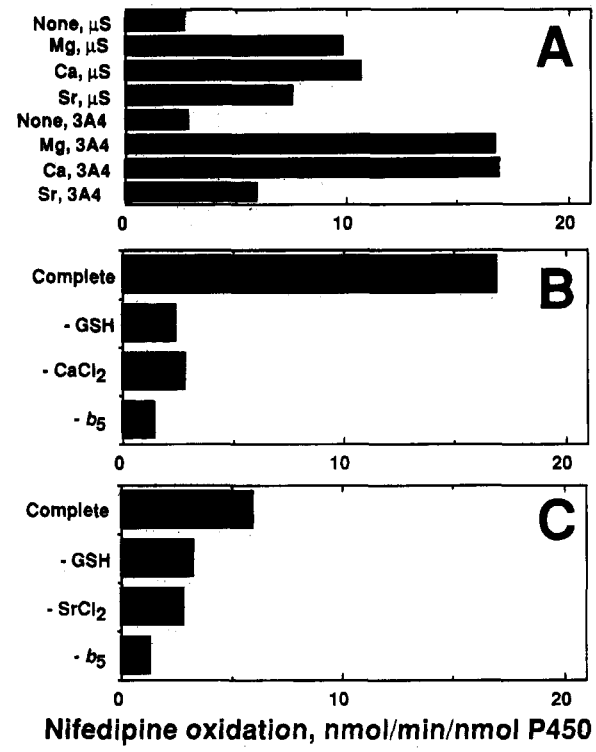


FIGURE 4: Effects of divalent cations and reconstitution system components upon nifedipine oxidation. (A) Either human liver microsomes (μS) or the standard P450 3A4 reconstitution system was used in the absence or presence of 30 mM concentrations of the indicated divalent cations. (B) P450 3A4 system including 30 mM $CaCl_2$, with various components deleted. (C) P450 3A4 system including 30 mM $SrCl_2$ with various components deleted. All values are means of duplicate experiments.

some P450-catalyzed reactions are stimulated by high concentrations of monovalent cations (Schenkman *et al.*, 1994). High concentrations of NaCl or KCl (0.3 M) showed little stimulation of nifedipine oxidation by P450s 3A4 and 3A5, and the stimulatory effect of $MgCl_2$ or $CaCl_2$ was seen in the presence of high levels of monovalent cations (Figure 3). In liver microsomes the stimulation of activity by NaCl and KCl is more pronounced, but the divalent cations also show a definite effect (Figure 3C).

Further experiments with P450 3A4 and human liver microsomes showed that the divalent cation effect extended from Mg^{2+} not only to Ca^{2+} but even to Sr^{2+}, although these latter two ions were less effective (Figure 4). In all systems, GSH and b_5 needed to be present for optimal activity (Figure 4B,C). In the optimal reconstitution system, the need for b_5 was substoichiometric (Figure 5A) (i.e., a b_5:P450 ratio of 0.5 was already optimal) and an equimolar concentration of NADPH-P450 reductase (to P450) was saturating (Figure 5B). Mg^{2+} did not appear to alter the saturability in either case.

Mechanism of Enhancement of P450 3A4-Catalyzed Oxidation by Divalent Cations: Steady-State Experiments. The marked divalent cation stimulation of a P450 reaction has little firm precedent in the literature (Cinti, 1978), and the effect appears to be highly repeatable. We made the general observation that the Mg^{2+} effect was only apparent when b_5 was present in a system [*vide supra*; see also Tamura *et al.* (1990)]. We also found, in agreement with the report of Shet *et al.* (1993) with the P450 3A4:rat NADPH–P450 reductase fusion protein, that the *N*-demethylations of *d*-benzphetamine, ethylmorphine, and erythromycin were relatively fast (rates ~ microsomes) and not stimulated by b_5. Further, these reactions were not stimulated by $MgCl_2$ or GSH (Gillam *et al.*, 1995).

We considered the possibility that high Mg^{2+} concentrations may be stimulating the transfer of electrons to or from b_5. Tamura *et al.* (1990) have reported stimulation of reduction of exogenous, trypsin-cleaved b_5 in porcine liver microsomes by Mg^{2+} or Ca^{2+}. The effect may not only be on transfer to b_5, since the divalent cations also stimulated microsomal NADPH–ferricyanide reduction rates. Thus, either the rate of P450 reduction (step 2 of Scheme 1), the rate of b_5 reduction (part of step 4), or perhaps electron transfer from b_5 to P450 (step 4) might be involved.

The work of Tamura *et al.* (1990) showed a 20-fold enhancement of the rate of microsomal NADPH-dependent reduction of b_5 by $MgCl_2$. However, that work involved protease-cleaved b_5, which does not bind well to the reductases containing membrane anchor regions. In other experiments, we found that $MgCl_2$ (30 mM) increased the rate of NADPH–P450 reductase-catalyzed reduction of b_5 (measured in the absence of P450) from 0.67 to 2.7 nmol (b_5) reduced min^{-1} (nmol of NADPH–P450 reductase)$^{-1}$.

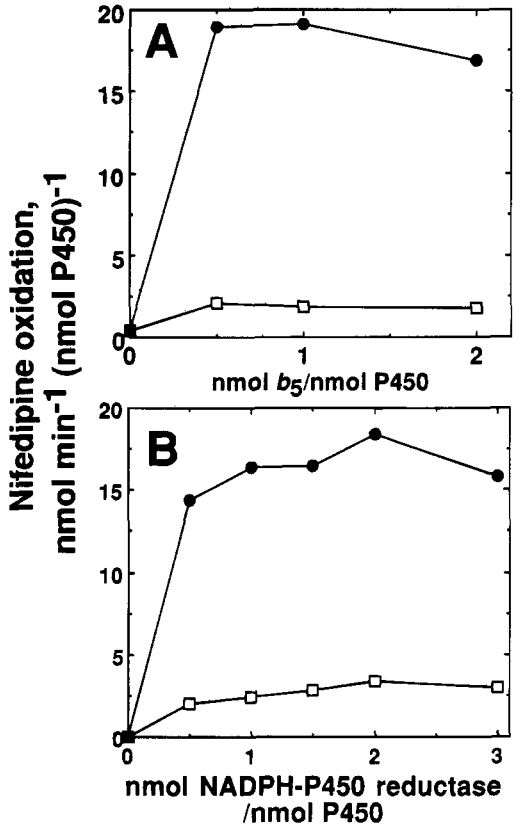


FIGURE 5: Dependence of P450 3A4-catalyzed nifedipine oxidation activity on b_5 (A) and NADPH−P450 reductase (B) in the presence (●) and the absence (□) of $MgCl_2$. The standard P450 3A4 reconstitution system was used, with a P450 concentration of 0.20 μM.

The other possible reactions, P450 reduction at either the first or the second electron stage (steps 2 and 4 of Scheme 1), are more difficult to measure. We examined the steady-state spectra of reaction systems to estimate effects of various components.

The first experiment involved observation of the various redox states of P450 3A4 (Figure 6). The system was set up without b_5 to simplify spectral assignments, and the reference cuvette had all components except NADPH. Thus, the ferric P450 system is the baseline. Addition of NADPH to the semianaerobic sample cuvette reduced the P450 to the ferrous state (λ_{max}, 424 nm). When the cuvette was opened and O_2 was added, a new spectrum appeared and persisted (λ_{max}, 417 nm). This spectrum is assigned to be that of the ferrous • O_2 complex, which is qualitatively similar to that reported previously for P450 2B1 in the presence of cyclohexane (Guengerich, 1983). Further evidence that this spectrum is that of a high-valent P450−oxygen complex was obtained by adding $Na_2S_2O_4$, which yielded the ferrous spectrum again (λ_{max} 424 nm).

Steady-state spectra were examined under approximate conditions where rates of testosterone 6β-hydroxylation had been measured, with P450 3A4 and NADPH−P450 reductase present at concentrations of 2.0 μM each (and b_5 at 2.0 μM when present). [Nifedipine interfered with the spectra; i.e., see Guengerich *et al.* (1986).] Trace catalase (0.15 μM) was added to block heme destruction (Guengerich, 1978), which confounded the Soret spectra. The reference cuvette contained all of the components except the NADPH-generating system.

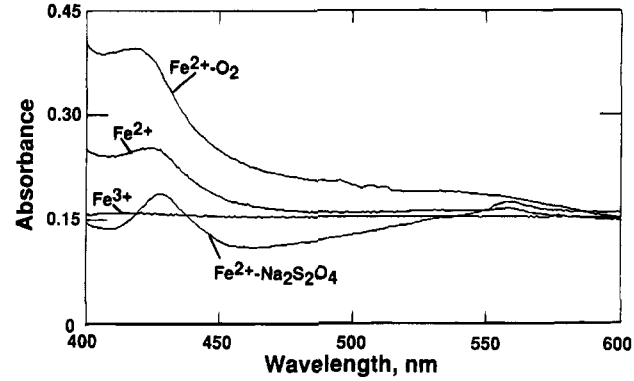


FIGURE 6: Difference spectra of various oxidation states of P450 3A4 in the presence of testosterone. The sample cuvette contained 50 mM potassium HEPES buffer (pH 7.4), the standard cholate−phospholipid mixture, GSH (3.0 mM), $MgCl_2$ (30 mM), testosterone (2.0 mM), P450 3A4 (1.3 μM), NADPH−P450 reductase (0.4 μM), and an NADPH-generating system devoid of $NADP^+$. Ar was bubbled through the cuvette, and the cuvette was sealed with a septum cap. The reference cuvette contained all of the same components except P450. A baseline spectrum was recorded and is labeled Fe^{3+}, indicating oxidized P450. $NADP^+$ (50 μM) was added to both cuvettes; in the case of the sample, $NADP^+$ was added through the septum with the use of a microsyringe. Several spectra were recorded, until the system came to apparent equilibrium (traced labeled Fe^{2+} indicating ferrous P450). The sample cuvette was then opened, and air was bubbled through for ~10 s. Spectra were then recorded at 2-min intervals. An intermediate trace is labeled $Fe^{2+} \cdot O_2$ and these continued but the peak in the Soret region shifted to the red with time. Finally, a few grains of $Na_2S_2O_4$ were added to both cuvettes to record the Fe^{2+}-$Na_2S_2O_4$ spectrum.

In the absence of b_5 (Figure 7A), the first spectral scans indicated flavin reduction (broad trough at 450 nm in difference spectrum). This gradually slowly shifted to show some of the putative $Fe^{2+} \cdot O_2$ complex at 417 nm and then the Fe^{2+} spectrum (λ_{max}, 424 nm) as O_2 was consumed. Addition of $Na_2S_2O_4$ yielded ferrous P450, with a λ_{max} at 424 nm. When the experiment was repeated with b_5 and $MgCl_2$ present (Figure 7B), under conditions where testosterone 6β-hydroxylation is optimal, the spectra were dominated by reduced b_5 throughout the course of the assay. Because of the similarity of the P450 ferrous and ferrous • O_2 spectra to the ferrous b_5 spectrum (Figure 6), contributions of the P450 spectra may be obscured.

The experiment of Figure 7B was repeated in the absence of $MgCl_2$ (Figure 7C). Again the spectrum is dominated by that of ferrous b_5, but the level and rate of reduction are considerably lower than when Mg^{2+} is present (Figure 7B). Since reduced b_5 did not accumulate in the absence of Mg^{2+} (Figure 7C), transfer of electrons from b_5 to $P450^{2+} \cdot O_2$ (Scheme 1, step 4) does not appear to be rate-limiting. However, transfer of electrons from NADPH−P450 reductase to b_5 might be, as judged by the time course of b_5 reduction (Figure 7B,C).

Similar spectral studies were done with ethylmorphine as the substrate instead of testosterone, and rather qualitatively similar results were obtained. However, rates of ethylmorphine oxidation (*N*-demethylation) are not dependent upon $MgCl_2$ or b_5 (Gillam *et al.*, 1995). Thus, we cannot attribute the varying dependence of the testosterone and ethylmorphine reactions on components only to differences in step 4 of Scheme 1.

Measurement of Rates of Reduction of P450 3A4 by NADPH−P450 Reductase in the Presence of Various System

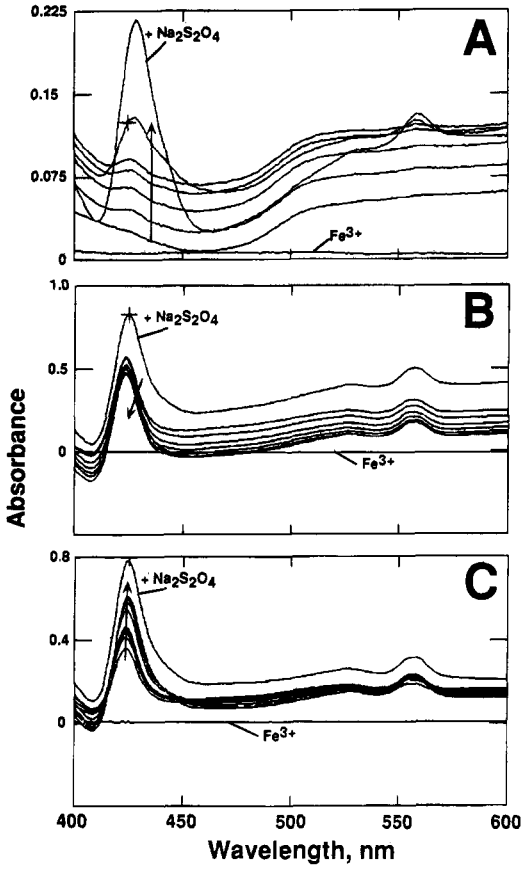


FIGURE 7: Changes in steady-state optical spectra of reconstituted P450 3A4 systems during testosterone hydroxylation. The basic P450 3A4 reconstitution system was used, with deletion of components as indicated and with 0.20 mM testosterone present. Both the sample and reference cuvettes contained the same components, and the NADPH-generating system was added only to the sample cuvette. Spectra were recorded at 2.0-min intervals (at 23 °C), in the directions indicated with the arrows (offset for clarity). The final spectrum labeled $+Na_2S_2O_4$ was recorded after the addition of a few grains of solid $Na_2S_2O_4$. (A) System minus b_5 (cross at 417 nm). (B) Complete system. (C) System minus $MgCl_2$.

Components. The earlier studies mentioned above indicated that $MgCl_2$ enhanced rates of b_5 reduction but did not yield a clear answer as to why a dramatic difference was observed in the oxidations of testosterone and ethylmorphine, in terms of dependence upon b_5 and $MgCl_2$. Part of the problem in interpretation of the results of the steady-state spectral measurements (Figure 7) was that the b_5 spectra interfere with those of the P450 Fe^{3+}, Fe^{2+}, and $Fe^{2+} \cdot O_2$ forms.

We examined the rates of the first reduction step (step 2 of Scheme 1) under anaerobic conditions, trapping the Fe^{2+} product as the $Fe^{2+} \cdot CO$ complex to avoid problems with b_5 (Figure 8). P450 3A4 reduction was rapid if testosterone, b_5, and $MgCl_2$ (Figure 8A) were all present (estimated first-order rate constant of ~58 min^{-1} for the initial phase of reduction at 25 °C).[2] Reduction was very slow in the absence of any substrate (even with $MgCl_2$, GSH, and b_5 present; Figure 8B). When the substrate ethylmorphine was present, the optimal rate of P450 reduction was also seen in the presence of all components. The rate was decreased (to ~13 min^{-1}) when either b_5 or $MgCl_2$ was deleted, but not to the extent with testosterone as the substrate (Figure 8A).

These results are consistent with experiments in which product formation was measured (Shet *et al.*, 1993; Gillam *et al.*, 1995). That is, testosterone 6β-hydroxylation is dependent upon b_5 and Mg^{2+} (Figure 1B), but ethylmorphine *N*-demethylation is not (Gillam *et al.*, 1995). The reduction of ferric P450 occurs very slowly in the former case unless b_5 and a divalent cation are present. This difference in reduction can account for at least part of the divergent behavior of the two reactions catalyzed by the same enzyme.

The question arises as to why b_5 is required for reduction of ferric P450 3A4 to ferrous. Such an effect has apparently not been reported before. Previously Ca^{2+} had been shown to stimulate rates of electron transfer from NADPH–P450 reductase to b_5, cytochrome *c*, and ferricyanide (Tamura *et al.*, 1990). We postulate that the divalent cations also stimulate electron transfer from NADPH–P450 reductase directly to P450 3A4, particularly when some substrates and b_5 are bound (Figure 8A; Scheme 2). Apparently b_5 binds to P450 3A4, or to a P450 3A4:NADPH–P450 reductase complex, in such a way as to facilitate electron transfer. An alternative view is that electron transfer is from NADPH–P450 reductase to b_5 to P450 3A4. This possibility cannot be dismissed, although the order of $E_{m,7}$ values for the oxidation–reduction couples would make this possibility unlikely, since the $E_{m,7}$ of b_5 is ~0 mV, and since most P450 $E_{m,7}$ values examined to date are <-250 mV, even in the presence of their substrates (Guengerich, 1983).

Spectral Differences in the Interaction of P450 3A4 with Testosterone and Ethylmorphine. We examined the binding of testosterone and ethylmorphine to P450 3A4 b_5 (Figure 10). Testosterone yielded the typical type I difference spectrum (Schenkman & Sato, 1968), corresponding to a shift from low- to high-spin iron, regardless of whether b_5 and $MgCl_2$ were present. The apparent K_s was 78($\pm$1) μM in the absence of these components and 164($\pm$18) μM in the presence of both; the extrapolated $\Delta A_{390-420,max}$ values were 0.075 and 0.065 (μM P450)$^{-1}$, respectively. When ethylmorphine was added to P450 3A4 in the absence of b_5 and $MgCl_2$, only a decrease in the magnitude of the Soret peak at 417 nm was observed [K_s, 46 $\pm$ 9 μM; $\Delta A_{417,max}$, 0.01 (μM P450)$^{-1}$]. When b_5 and $MgCl_2$ were present, a "reverse type I" difference spectrum was observed, with a K_s of 14($\pm$1) μM and a $\Delta A_{420-389,max}$ of 0.01 (μM P450)$^{-1}$. These changes cannot be directly linked to differences in rates of reduction (Figure 9) and argue that the shift of low- to high-spin iron seen with testosterone is not sufficient to enhance the rate of reduction. However, the results do suggest that the two substrates do interact differently with P450 3A4.

A number of CD studies were also done with P450 3A4 and the substrates testerone and ethylmorphine under various conditions. However, we have not been able to associate any consistent changes in the far-UV region (200–220 nm) with the various catalytic phenomena observed here.

P450 3A4 Oxidation Reactions Supported by Flx and Fdx. We were interested in the selectivity of electron-transfer components interacting with P450s, particularly in light of the requirement of P450 3A4 for b_5 in many reactions. Barnes *et al.* (1991) reported that recombinant bovine P450 17A catalyzes steroid hydroxylation within *E. coli* cells, and Jenkins and Waterman (1994) showed that the *E. coli*

[2] These rates were estimated using the initial portions of the reduction reactions (3–8 s total). When the entire reaction is considered, there is a multiphasicity of reduction, as reported for other P450s (Peterson *et al.*, 1976; Oprian *et al.*, 1979; Backes *et al.*, 1980; Eyer & Backes, 1992). Further characterization of this phenomenon is in progress.

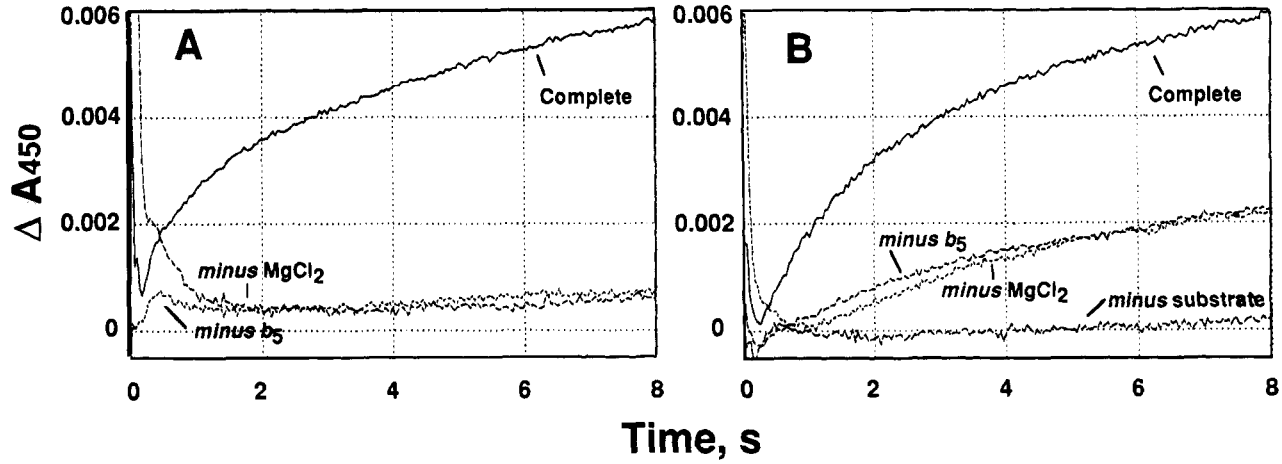


FIGURE 8: Effects of various components on rates of reduction of ferric P450 3A4. Rates were measured under anaerobic conditions (CO) with the various components added as described under Experimental Procedures. The complete system contained P450 3A4, NADPH–P450 reductase, b_5, GSH, $MgCl_2$, NADPH, and the indicated substrate: (A) testosterone; (B) ethylmorphine.

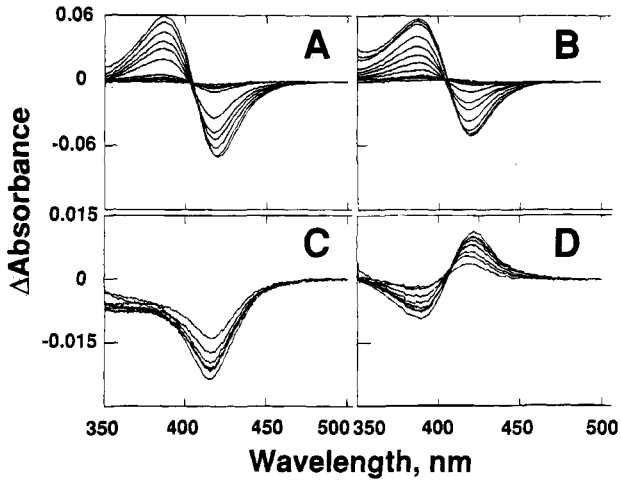


FIGURE 9: Changes in P450 3A4 spectra due to interactions with testosterone and ethylmorphine. Increasing amounts of testosterone (added in 1.0-μL aliquots from a CH_3OH solution) or ethylmorphine (1.0-μL aliquots of aqueous solutions) were added over the range of 5–500 μM to a 2.0 μM solution of P450 3A4 in 50 mM potassium HEPES buffer (pH 7.4) containing the standard phospholipid mixture, and spectra were recorded (all at 23 °C). In parts A and B testesterone was added, and in parts C and D ethylmorphine was added. In parts B and D the system also contained 2.0 μM b_5 and 30 mM $MgCl_2$.

NADPH–Flx reductase/Flx system is the electron-transfer system involved.

In our initial studies, we found that *E. coli* expressing P450 3A4 would slowly convert testosterone to the 6β-hydroxy product. Further studies showed time dependence, with a rather linear production over time (up to 12 h at 32 or 37 °C; results not presented). After 12 h, about 12 pmol of 6β-hydroxytestosterone had been formed per nanomole of P450. The abilities of the *E. coli* cytosolic fraction and the Flx system to support nifedipine oxidation by purified recombinant P450 3A4 were examined, with all systems containing the typical P450 3A4 phospholipid system. The effects of certain components on the NADPH–P450 reductase-supported reaction were examined in direct comparisons, and neither $MgCl_2$, b_5, KCl (0.4 M), nor the substitution of phosphate for HEPES buffer had a consistent effect on the activity in these systems.

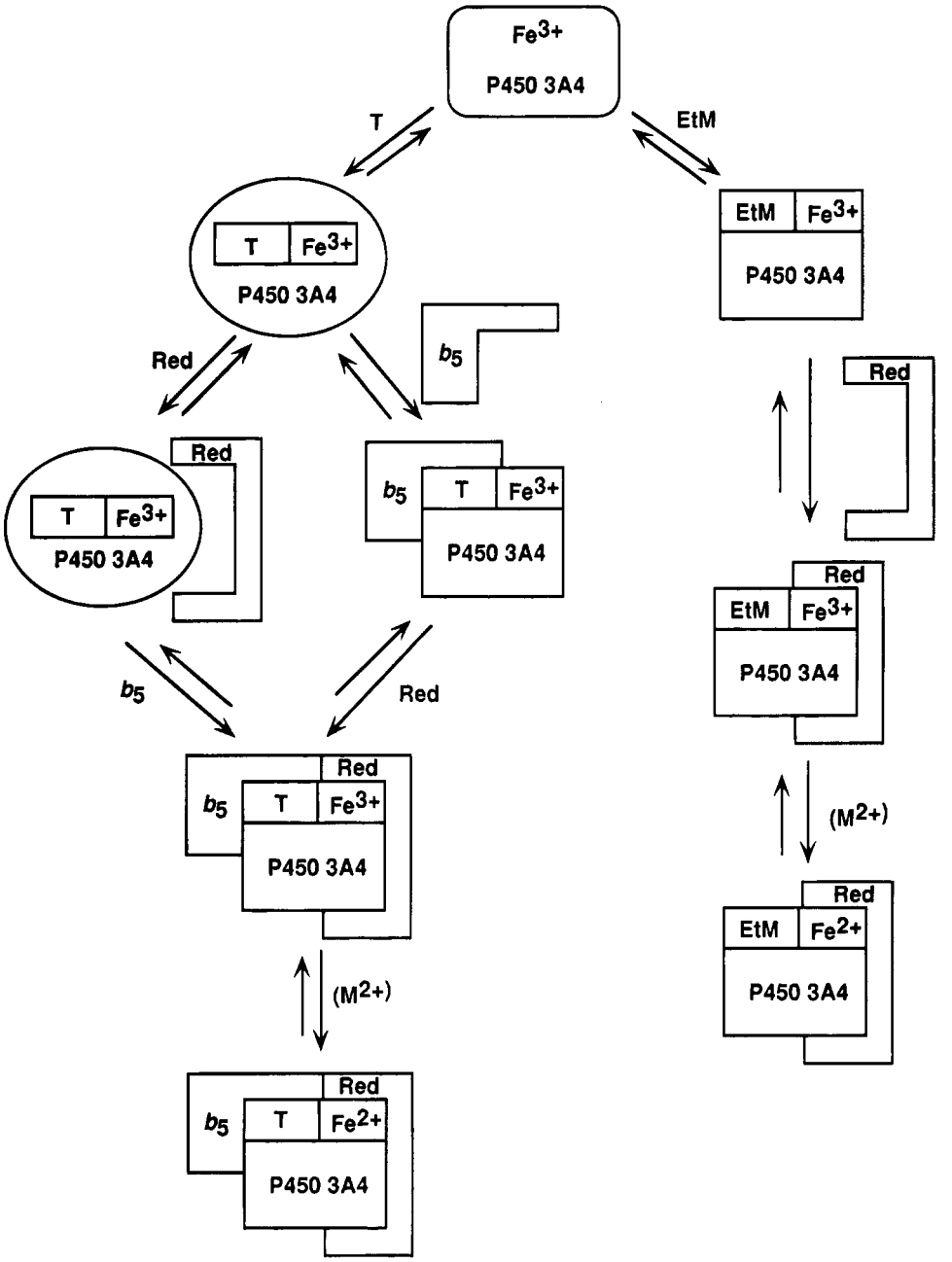
We also found that the reaction could be supported by a spinach NADPH–Fdx reductase/Fdx system. As with the Fdx system, the presence of monovalent or divalent cations or b_5 or changes in buffers did not have a dramatic effect on catalytic activity. The effects of varying concentrations of Fdx and Fdx components on P450 3A4-catalyzed nifedipine oxidation are presented in Figure 10. Although the rates measured here with NADPH–P450 reductase are far less than optimal (because of the lack of high $MgCl_2$, GSH, and b_5; cf. Figures 1–5), it should be emphasized that the rates seen with the artificial electron donors are real and not small when compared to NADPH–P450 reductase in the absence of b_5 and $MgCl_2$ (Figures 1A and 14A).

Catalytic Activities of P450s Supported by Oxygen Surrogates. Several aspects of this work support the view that reduction is a rate-limiting process in overall catalysis of oxidation reactions by P450 3A4. "Oxygen surrogates" are hypervalent oxygenated compounds that can transfer oxygen to P450 to raise the oxidation state to $(FeO)^{3+}$ directly, without the need for reduction and O_2 binding (Lichtenberger *et al.*, 1976; Kadlubar *et al.*, 1973). In some cases the rates are considerably higher than those measured in the typical systems involving NADPH–P450 reductase (Lichtenberger *et al.*, 1976; Macdonald *et al.*, 1989).

Experiments were done with P450 3A4 with two oxygen surrogates, CuOOH and PhIO (Table 1). CuOOH did not yield particularly high rates, even when care was taken to ensure that product formation was linear with time. However, the rates measured in the PhIO-supported reactions were considerably higher than seen in the NADPH–P450 reductase-supported reactions.

Conclusions. P450 3A4, the major human liver P450, is notorious for difficulty in reconstitution of catalytic activity after purification from membranes. We developed a component mixture that reproducibly yielded high catalytic activity toward the prototypic substrates nifedipine and testosterone. This system is somewhat influenced by the particular buffer used; the most important components appear to be a previously reported phospholipid mixture containing phosphatidylserine plus sodium cholate, GSH, b_5, and Mg^{2+}. The role of the GSH remains unclear. $MgCl_2$ increases electron transfer from NADPH–P450 reductase to b_5 and P450. The Mg^{2+} effect is seen in microsomes, as well as in reconstituted systems, and Mg^{2+} can be substituted with Ca^{2+} or Sr^{2+}. Steady-state spectra and direct NADPH–P450 reductase:b_5 transfer measurements indicated that b_5 reduction

Scheme 2: Possible Model for Regulation of P450 3A4 Reduction by b_5, Divalent Cations, and Substrates[a]



[a] T = testosterone; EtM = ethylmorphine; Red = NADPH-P450 reductase; and M^{2+} = divalent cation.

is stimulated by Mg^{2+}. When we did anaerobic measurements of the reduction of ferric P450 3A4, we were surprised to find that reduction was strongly dependent upon the presence of substrate, b_5, and Mg^{2+} (Figure 8). The rate of reduction was considerably higher in the presence of ethylmorphine than in the presence of testosterone when b_5 and Mg^{2+} were not present. This pattern is consistent with the dependence of the two different substrate oxidations on b_5 in catalytic assays (Shet *et al.*, 1993; Gillam *et al.*, 1995). Our explanation for the results is that some substrates bind to P450 3A4 in such a way as to facilitate input of electrons from NADPH−P450 reductase (Scheme 2). We do not feel that these differences are alterations of $E_{m,7}$, since the various components (b_5, Mg^{2+}, etc.) did not influence overall catalytic rates in the systems supported by Flx and Fdx. In this scheme, different substrates bind to change the conformation of P450 3A4 in different ways. The binding of ethylmorphine is viewed as converting P450 3A4 to a form that readily binds the reductase and enables electron transfer to occur (stimulated further by divalent metal ions). Binding of testosterone, on the other hand, does not convert P450 3A4 to a form that is so readily reducible; binding of b_5 seems to be needed. This paradigm may be too simplistic, since b_5 did stimulate P450 3A4 reduction in the presence of ethylmorphine (Figure 8), and the possibility of electron transfer to P450 3A4 via b_5, although unlikely, cannot be completely ruled out.

It appears that the rates of electron transfer into P450 and oxygen activation are rate-limiting in the reactions catalyzed by P450 3A4, since the rates measured in the reactions supported by the oxygen surrogate PhIO were considerably higher than those measured in the normal NADPH−P450 reductase-supported system. CuOOH was not as effective in this regard. On the basis of reports that bacterial Flx can

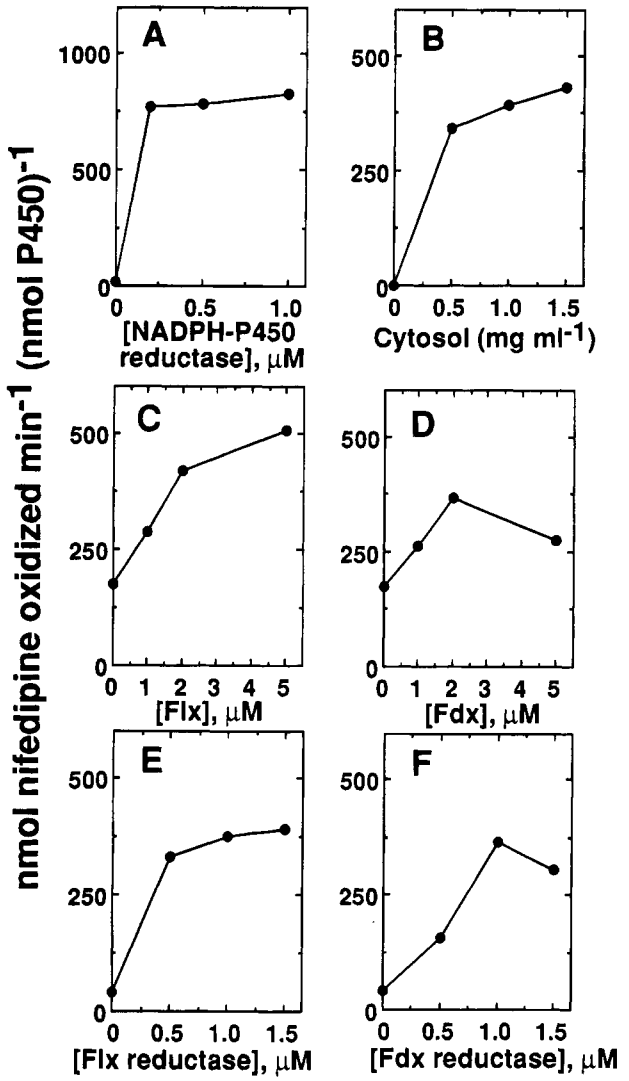


FIGURE 10: P450-catalyzed nifedipine oxidation: effects of various reduction components. All systems contained 0.10 μM P450 3A4, the standard phospholipid–cholate mixture, 0.20 mM nifedipine, 50 mM potassium phosphate buffer (pH 7.4), 400 mM KCl, 5 mM $MgCl_2$, an NADPH-generating system, and the indicated enzyme. (A) NADPH–P450 reductase; (B) *E. coli* cytosol; (C) *E. coli* Flx (in the presence of 1.0 μM Flx reductase); (D) spinach Fdx (in the presence of 1.0 μM spinach Fdx reductase); (E) *E. coli* Flx reductase (in the presence of 2.0 μM Flx reductase); (F) spinach Fdx reductase (in the presence of 2.0 μM spinach Fdx reductase).

Table 1: Rates of P450 3A4-Catalyzed Oxidations Measured in the Presence of Oxygen Surrogates

reaction	rate, nmol of product formed min^{-1} (nmol P450)$^{-1}$ [a]	
	CuOOH	PhIO
nifedipine oxidation	3.6	120
testosterone 6β-hydroxylation	1.2	30
ethylmorphine *N*-demethylation	1.2	240

[a] Means of duplicate experiments, done at incubation times at which product formation was linear with time (5 min for CuOOH, 10 s for PhIO).

support the reduction of a microsomal P450 (Jenkins & Waterman, 1994) we examined the possibility of Flx electron transfer to P450 3A4. Rates of hydroxylation were ~25–50% of those supported by NADPH–P450 reductase in the absence of additional factors such as b_5, Mg^{2+}, GSH, etc. We also found that spinach Fdx was effective in this regard. Spinach Fdx is a well-studied iron–sulfur protein with a low $E_{m,7}$ [−420 mV (Tagawa & Arnon, 1968)]. Thus, in this system, electrons flow from NADPH ($E_{m,7}$, −320 mV) to NADPH–Fdx reductase [$E_{m,7}$, −360 mV (Keirns & Wang, 1972)] to Fdx ($E_{m,7}$, −420 mV) (Tagawa & Arnon, 1968) and then to the P450 [$E_{m,7} \sim -300$ mV (Guengerich, 1983)]. Fdx contains an iron–sulfur cluster but no flavin, but there is some precedent for the interchangeability of these flavoproteins and iron–sulfur proteins in electron-transfer chains (Vetter & Knappe, 1971). Although Avadhani and his group have reported that P450s with similarity to the microsomal ones are found in mitochondria and accept electrons from adrenodoxin (Shayiq & Avadhani, 1989), we are unaware of reports of efficient (or "semi-efficient") transfer of electrons from ferredoxins to microsomal P450s. Electron transfer from Flx to P450 17A has been rationalized in terms of similarity of the FMN-binding domains of Flx and NADPH–P450 reductase (Jenkins & Waterman, 1994). With P450 3A4, Fdx was as efficient as Flx in supporting reactions, so the iron–sulfur system seems to be capable of substituting. The exact mode of this interaction is yet unknown, and we have not investigated binding of Fdx and P450 3A4.

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