Lack of Electron Transfer from Cytochrome \(b_5\) in Stimulation of Catalytic Activities of Cytochrome P450 3A4

CHARACTERIZATION OF A RECONSTITUTED CYTOCHROME P450 3A4/NADPH-CYTOCHROME P450 REDUCTASE SYSTEM AND STUDIES WITH APO-CYTOCHROME \(b_5\)

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Many catalytic activities of cytochrome P450 (P450) 3A4, the major human liver P450 enzyme, require cytochrome \(b_5\) (\(b_5\)) for optimal rates. The stimulatory effect of \(b_5\) on P450 reactions has generally been thought to be due to transfer of electrons from ferrous \(b_5\) to the ferrous P450-O\(_2\)-substrate complex. We found that apo-\(b_5\), devoid of heme, could substitute for \(b_5\) in stimulating two prototypic activities, testosterone 6\(\beta\) hydroxylation and nifedipine oxidation. The stimulatory effect was not seen with albumin, hemoglobin, catalase, or cytochrome \(c\). Apo-\(b_5\) could not substitute for \(b_5\) in a testosterone 6\(\beta\) hydroxylation system composed of NADH-\(b_5\) reductase and P450 3A4. Rates of electron transfer from NADPH-P450 reductase to ferric P450 3A4 were too slow (<2 min\(^{-1}\)) to support testosterone 6\(\beta\) hydroxylation (<14 min\(^{-1}\)) unless \(b_5\) or apo-\(b_5\) was present, when rates of ~700 min\(^{-1}\) were measured. The oxidation-reduction potential \(E_{\text{m,7}}\) of the ferric/ferrous couple of P450 3A4 was unchanged (~−310 mV) under different conditions in which the kinetics of reduction were altered by the addition of substrate and/or apo-\(b_5\). Rapid reduction of P450 3A4 required substrate and a preformed complex of P450 3A4, NADPH-P450 reductase, and \(b_5\); the rates of binding of the proteins to each other were 2–3 orders of magnitude less than reduction rates. We conclude that \(b_5\) can facilitate some P450 3A4-catalyzed oxidations by complexing with P450 3A4 and enhancing its reduction by NADPH-P450 reductase, without directly transferring electrons to P450.

More than 40 P450\(^1\) enzymes are found in a single mammalian species (2). The proteins constitute a superfamily and collectively contribute extensively to the oxidation of xenobiotic chemicals (e.g. drugs, carcinogens, pesticides, alkaloids, and other natural products) and also endobiotics (e.g. steroids, eicosanoids, fat-soluble vitamins, fatty acids) (3–6). The contributions of these P450 enzymes to metabolism in humans are well recognized, particularly regarding issues of drug clearance (7–9). There is general agreement that, in most humans, P450 3A4 is the most abundant of the P450s in both liver and small intestine (8, 9); it can constitute up to 60% of the total P450 in the liver (10). The intestinal enzyme has been implicated in variation in the bioavailability of many orally administered drugs (11).

P450 3A4 has a very broad range of substrates, with more than 60 drugs having been already identified (9). These vary widely in structure, and one of the questions about this enzyme has been the molecular basis of its broad catalytic specificity (12, 13). Other mechanistic questions involve the basis of the sigmoidal plots of enzyme velocity versus substrate seen with some compounds (14–16) and the stimulation of activity by chemicals other than the substrate (14, 17, 18). The purified enzyme, along with other P450 3A subfamily enzymes, is much more sensitive to its reconstitution environment than are most other P450s (19, 20). A variety of components have been reported to stimulate catalytic activity, including long chain unsaturated phosphatidylcholines (21), phosphatidylserine (20, 22), ionic detergents (21, 22), GSH (23), divalent cations (24, 25), and \(b_5\) (19, 21). Not all of these components are directly relevant to the membrane-bound enzyme, but Mg\(^2+\) has been shown to stimulate activity of the enzyme in microsomes (24, 25) and antibodies raised against \(b_5\) can inhibit some catalytic activities of P450 3A4 in microsomes (19, 25). Somewhat surprisingly, certain catalytic activities of P450 3A4 are quite refractory to alterations in lipids and \(b_5\) (24, 26).

In order to better understand this complex but important system, we initiated a systematic investigation of some of the system components on individual steps in the catalytic cycle of purified recombinant P450 3A4 (14, 19, 20, 23–25). A general conclusion about the role of \(b_5\) in modulating P450 reactions has been that electron transfer from \(b_5\) to P450 occurs in step 4 of Scheme 1 (27). Recently we found qualitative evidence that \(b_5\) could also stimulate the reduction of P450 3A4 by the flavoprotein NADPH-P450 reductase (24). We now report that apo-\(b_5\) (devoid of heme) can replace \(b_5\) in the efficient oxidation of the prototypic P450 substrates testosterone and nifedipine and that apo-\(b_5\) can also replace \(b_5\) in the facilitating electron flow from NADPH-P450 reductase to P450 3A4, in the absence of electron transfer from \(b_5\) or modulation of the \(E_{\text{m,7}}\) of P450 3A4.

\(^1\) The abbreviations used are: P450, cytochrome P450 (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1 (1)); \(E_{\text{m,7}}\), oxidation-reduction potential at pH 7.0, relative to hydrogen, with \(n\) being the experimentally determined number of electrons transferred; \(b_5\), cytochrome \(b_5\) (EC 4.4.2.5 group) (also referred to as holo-\(b_5\), to distinguish it from apo-\(b_5\), from which the heme has been removed); \(E_{\text{oxy}}\), oxidation potential relative to hydrogen; HPLC, high performance liquid chromatography; DLPC, L-\(\alpha\)-dioleoyl-sn-glycero-3-phosphocholine.
Interactions of Cytochromes P450 3A4 and b₅

EXPERIMENTAL PROCEDURES

Chemicals—Nifedipine was purchased from Sigma and recrystallized from aqueous CH₂OH in amber glass (28). Proflavin sulfite and benzyl viologen were purchased from ICN Pharmaceuticals (Plainview, NY), and safranine T was from Fluka (Ronkonkoma, NY).

Enzymes—Recombinant P450 3A4 was expressed in Escherichia coli and purified as described elsewhere (23). Bovine liver L-glutamate dehydrogenase (type IV), bovine serum albumin, human serum albumin, horse heart cytochrome c, human hemoglobin, Aspergillus niger glucose oxidase (type V), and bovine liver catalase were purchased from Sigma; the latter two enzymes were dialyzed twice overnight at 4 °C versus 500 volumes of 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA before use. Protocatechuate dioxygenase was a gift of Prof. D. P. Ballou, University of Michigan, Ann Arbor. Rabbit liver NADPH-P450 reductase (15 units/ml), 15 min, 4°C) was dried under an N₂ stream and homogenized in 4.0 ml of a second solution (34). b₅ (735 nmol, in 1.2 ml of H₂O) was added dropwise to 25 ml of cold acetone containing 0.2% HCl (–20 °C), and the mixture was stirred for 15 min at 0 °C. The white precipitate recovered by centrifugation (10,000 g, 15 min, 4 °C) was washed several times with P450 3A4 and protocatechuate dioxygenase and 80 μM protocatechuate, with similar results.

An anaerobic solution of photoreduced safranine T, in Tris-EDTA buffer, was used to deplete O₂ from the syringes and lines of the instrument prior to introduction of anaerobic protein samples, utilizing a lamp with the dye stored in the drive syringes.

Measurement of Eₐ₅₇₁—The general procedure involved equilibration with dyes of known potential and is described elsewhere (35, 37). In general, P450 3A4 (5–5 μM) was photoreduced (anaerobically, under argon) in the presence of safranine T (0.15 to 20 μM, Eₐ₅₇₁ = −289 mV (37), with proflavin sulfite (0.3–1 μM) and benzyl viologen (1–20 μM, Eₐ₅₇₁ = −352 mV) (37) present in some cases (all in the presence of 0.10 μM Tris as an electron source). At intermediate points in the photoreduction, the spectrum of P450 3A4 was recorded (Cary 14/OLIS spectrophotometer) and the Eₐ₅₇₁ was determined from the fraction of safranine T reduced (as determined by measurements of Aₐ₅₇₁ or Fₐ₅₇₁) and the Nernst equation Eₐ₅₇₁ = −289 + (2.3 RT/n)Flog₁₀[Safranine T]/[reduced safranine T], where (2.3 RT/nF) = 29.5 mV at 23 °C (37). The equation Eₐ₅₇₁ = Eₐ₅₇₁ = 2.3 RT/nFlog₁₀[P450 3A4]₀/[P450 3A4]ₜ was used to determine n and Eₐ₅₇₁. In a trial experiment with recombinant human P450 1A2 (38), values of n = 0.82 and Eₐ₅₇₁ = −313 mV were obtained (cf. −299 and −357 mV for rat and rabbit P450 1A2, respectively) (35, 39).

Steady-state Kinetic Experiments—With many of the reactions catalyzed by P450 3A4, the system is sensitive to the concentrations of individual components and even the order of mixing. The following order of mixing was found to be optimal and was used here (all concentrations are for the final incubation mix): P450 3A4 (40 nM), NADPH-P450 reductase (120 nM), b₅ (80 nmol), sodium cholate (to 0.5 mM, diluted from a 25 mM stock), and a phospholipid mixture (to 20 mg/ml), diluted from a stock of 0.5 mg/ml and consisting of a 1:1:1 mixture (by mass) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DPPC) and bovine brain phosphatidylserine (sonicated under argon); these components were mixed and allowed to stand 10 min at room temperature, followed by the addition of potassium HEPES (50 mM, pH 7.7), substrate (200 μM), and aromatic peptide and testosterone or nifedipine, GSH (3.0 mM), and MgCl₂ (30 mM). These components were mixed and incubated for 3 min at 37 °C; reactions were initiated by the addition of an NADPH-generating system consisting of (final concentrations) 10 mM glucose 6-phosphate, 0.5 mM NADP⁺, and 1 IU yeast glucose 6-phosphate dehydrogenase ml⁻¹.

Incubations with the substrates nifedipine and testosterone were generally done for 10 min at 37 °C. Reactions were terminated and the products were analyzed by HPLC using methods described elsewhere (12, 19).

Reduction Kinetics—In general, the components were mixed in the same order as described for steady-state reactions, except that the concentrations of P450 3A4, NADPH-P450 reductase, and b₅ were 0.35, 0.80, and 0.35 μM, respectively, unless indicated otherwise. All experiments were done in the stopped-flow apparatus at 37 °C under a CO atmosphere (positive pressure), with data collected at 447 nm (Fe(II)–CO complex). In some cases the disappearance of the oxidized P450 (Fe(III)) was monitored at 390 nm. Reduction of b₅ was monitored by the increase in Aₐ₅₇₁ or decrease in Aₐ₅₇₁.
The product 6β-hydroxytestosterone was then tipped into the main compartment (final concentration of 34 μM) and the residual testosterone eluted at tR 7 min. The traces in panel C correspond to a rate of 0.27 nmol of product formed min⁻¹ (nmol P450)⁻¹ (cf. Tables I and II).

**Table I**

| Components in system | Testosterone 6β hydroxylation | nmol product formed min⁻¹ (nmol P450)⁻¹ |
|----------------------|-------------------------------|---------------------------------------|
| b5                   | Lipid MgCl₂ Recombinant P450 3A4 Liver P450 3A4 |
| None                 | Mix – 0.6 ± 0.3 0.2 ± 0.2 |
| None                 | Mix + 4.2 ± 1.1 2.1 ± 0.2 |
| Holo                 | Mix – 1.0 ± 0.3 0.3 ± 0.2 |
| Holo                 | Mix + 14.4 ± 0.9 6.1 ± 0.3 |
| Apo                  | Mix – 1.0 ± 0.1 0.5 ± 0.2 |
| Apo                  | Mix + 12.9 ± 0.2 5.3 ± 0.2 |

An alternate system involved enzymatic reduction of NADPH by reduced P450. The cuvette was made up with 0.25 μM NADPH-P450 reductase, plus all of the other usual components, 0.15 mM NH₄Cl, and 6.7 mM α-ketoglutarate. After the system was rendered anaerobic, NADPH (33 μM) was tipped from a side arm into the main compartment and the course of reduction of the P450 3A4 was monitored (ΔA₃₄₀, ΔA₄₁₈) in the Cary 14 spectrophotometer (~30 min at 23 °C). l-Glutamate dehydrogenase was then tipped into the main compartment (final concentration, 0.02 IU ml⁻¹) from a second side arm to remove residual NADPH. This process was monitored by the increase in A₃₄₀. The rate of oxidation of ferrous P450, which occurred subsequently, was also monitored (increase in A₃₄₀ and decrease in A₃₄₈). Under these conditions, the rate of oxidation of photochemically reduced P450 3A4 was negligible.

**RESULTS**

Steady-state Assays: Stimulation of Catalytic Activities by Apo-b5—Apo-b5 failed to substitute for b5 in a testosterone 6β-hydroxylation system consisting of NADH-b5 reductase, b5, and recombinant P450 3A4 (Fig. 2), because NADH-b5 reductase cannot directly transfer electrons to P450 3A4 (25). However, apo-b5 was as effective as b5 in stimulating the testosterone 6β hydroxylation activity of recombinant P450 3A4 in a system containing NADPH-P450 reductase (Table I). The need for Mg²⁺ in this system was seen, as reported earlier (24). When P450 3A4 purified from human liver was substituted for recombinant P450 3A4, the same patterns of dependence upon assay components were seen. Apo-b5 was nearly as effective as b5, on a concentration basis, in stimulating testosterone 6β hydroxylation in a system containing NADPH-P450 reductase and P450 3A4 (Fig. 3A). Similar enhancement was seen with another P450 3A4 reaction, nifedipine oxidation (Fig. 3B). It is also of interest to note that maximal stimulation was observed at a ratio of 0.25–0.5 b₅ or apo-b₅ per P₄₅₀.

Neither bovine nor human serum albumin nor several hemoproteins (cytochrome c, hemoglobin, catalase) could substitute for the stimulatory effect of b₅ or apo-b₅ (Table II). The effect of both b₅ and apo-b₅ was to decrease the Kₘ (for testosterone) and to increase Vₘₐₓ (Table III).

**Kinetics of P450 3A4 Reduction: Reconstituted System—Previous work had qualitatively demonstrated the ability of b₅ to enhance rates of P450 3A4 reduction by NADPH-P450 reductase (24, 25). These results were confirmed and extended here. P450 3A4 reduction was rapid and appeared to be first-order under what seem to be optimal conditions, identical to those used in steady-state assays, with a rate of ~750 min⁻¹ at 37 °C.**
For comparison, the estimated $Em_{7b}$ for asimilar system devoid of apo-$b_5$ and testosterone was $-316 \pm 7$ mV ($n = 1.2$) and $-320$ mV ($n = 0.9$) for a system devoid of apo-$b_5$ but with ethylmorphine substituted for testosterone.

**Kinetics of Interaction of P450 3A4 with NADPH-P450 Reductase and $b_5$**—The reduction experiments mentioned previously had all been done with preformed complexes of P450 3A4 with other enzymes, mixing with a solution of NADPH from the second reaction syringe. When NADPH-P450 reductase was moved to the NADPH syringe (and P450 3A4 and $b_5$ were in the other syringe), reduction was very slow (Fig. 6A, $k = 1.2$ min$^{-1}$). The experiment was repeated with P450 3A4 and NADPH-P450 reductase in one syringe and with $b_5$ and NADPH in the other (all other components in both). Again, reduction was very slow (Fig. 6B, $k = 0.2$ min$^{-1}$).

The results indicate that the three protein components associate rather slowly. If this is true, then one might expect to observe “burst” kinetics for the reduction of P450 3A4 and $b_5$ in a system in which the reductase is the limiting component. This is indeed the case, as shown by the results presented in Fig. 7. The reduction of P450 3A4 showed a rapid burst (Fig. 7A), followed by a lag and then a single-exponential reduction of the rest of the P450. The rates of the two phases were 920 and 0.7 min$^{-1}$ with $b_5$, part is reduced at 830 min$^{-1}$.

**Estimation of $E_{m,7}$ of P450 3A4**—Several different experimental designs were utilized in measuring the $E_{m,7}$ of P450 3A4, including the addition of dyes of varying potential at low concentrations (<1 µM), with safranine T reduction monitored by fluorescence with excitation at 520 nm and emission at 580 nm or high concentrations (with safranine T and benzyl violagen reduction both measured by visible spectroscopy). The results shown in Fig. 5 are representative and presented to demonstrate the proximity of the $E_{m,7}$ of P450 3A4 to that of safranine T. In this particular experiment, done in the presence of testosterone and apo-$b_5$, the $E_{m,7}$ was $-312$ mV ($n = 0.8$) (mean of three experiments). For comparison, the estimated $E_{m,3}$ for a similar system devoid of apo-$b_5$ and testosterone was $-316 \pm 7$ mV ($n = 1.2$) and $-320$ mV ($n = 0.9$) for a system devoid of apo-$b_5$ but with ethylmorphine substituted for testosterone.

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**DISCUSSION**

Evidence for the involvement of $b_5$ in a P450 catalytic system was first published in 1971 (44) and the literature now contains

### Table III

| Addition     | $k_m$ (for testosterone) | $V_{max}$
|--------------|---------------------------|--------|
| None         | 0.140 ± 0.042             | 12.9 ± 1.8 |
| $b_5$        | 0.065 ± 0.014             | 21.3 ± 1.8 |
| Apo-$b_5$    | 0.066 ± 0.015             | 21.1 ± 1.9 |

**Fig. 4. Kinetics of reduction of P450 3A4 and effect of $b_5$.** Traces ($\Delta A_{340}$) are shown for a system containing 0.35 µM P450 3A4, 0.80 µM NADPH-P450 reductase, 200 µM testosterone, and other reaction components (in one mixing syringe) cited under “Experimental Procedures,” “Reduction Kinetics,” reduction with 0.35 µM $b_5$ (A), 0.35 µM apo-$b_5$ (B), or no added $b_5$ or apo-$b_5$ (C). The second mixing syringe contained an equal volume of 0.4 mM NADPH. The solid traces in panels A and B correspond to single-exponential fits of 730 ± 25 min$^{-1}$ and 950 ± 30 min$^{-1}$, respectively. The residual analysis for a first-order plot is shown at the bottom of panels A and B. For further analysis related to panel C, see Fig. 6B.

**Table IV**

| Components in system | $k$          |
|---------------------|--------------|
|                     | P450 3A4     | $b_5$          |
| T                   | Testosterone | 730            | 660 |
| A                   | Testosterone | 960            |     |
| A                   | Testosterone | <1$^*$         |     |
| A                   | Aflatoxin B1 | 630            | 1100|
| A                   | Aflatoxin B1 | 690            |     |
| A                   | None         | 7              |     |
| A                   | Ethylmorphine| 660            |     |

$^*$ See Figs. 4C and 7B.
numerous reports of P450 reactions in which there is either (i)
enhancement, (ii) no effect, or (iii) inhibition by b5 (45–52).
Evidence for the role of b5 in the transfer of an electron to the
P450 ferrous-O2 complex has been presented (44, 53), and this
is almost universally accepted to be the basis for enhancement
of catalysis by b5 (Scheme 1). In several studies, derivatives of
b5 that were devoid of electron transfer capability were not
effective in stimulating reactions catalyzed by other P450 s (21,
47). We have previously presented qualitative evidence for the
stimulation of the reduction of ferric P450 3A4 by b5 (24). The
possibility exists that this phenomenon could occur via transfer
of electrons from b5 to P450 3A4, although the thermodynamics
are unfavorable (i.e. the Em,7 of b5 is ~ +20 mV and that of most
P450 s is ~ -300 mV) (35, 54). Rates of steady-state reactions
and ferric P450 3A4 reduction in systems composed of
NADH-b5 reductase and b5 are too slow to be compatible with
the rates of NADPH-P450 reductase-dependent reactions (25).
We now provide evidence that apo-b5, devoid of electron trans-
fer capability, can stimulate steady-state catalytic activity and
the reduction of P450 3A4 (Tables I-III, Figs. 3 and 4). This
effect on reduction rate may contribute to the enhancement of
P450 3A4-catalyzed testosterone 6β-hydroxylation. These ef-
fects were also seen with the substrates nifedipine and afla-
toxin B1 (Fig. 3, Table IV), reactions that require b5 (14, 19).
With ethylmorphine, a substrate whose oxidation does not
require b5 (24, 26), the substrate enhanced P450 3A4 redu-
tion to the rate seen with a combination of testosterone plus b5
(Table IV).

The enhancement of the rate of P450 3A4 reduction is ap-
parently not linked to a change in Em,7 of the ferrous/ferrous
couple (Fig. 5). Although the spin-state, kinetics, and thermo-
dynamics of P450 reduction are often suggested to be linked,
based upon work with bacterial P450 101 (55), this is not
generally the case with microsomal P450 s (35), and it is not the
case here. Neither testosterone, ethylmorphine, nor apo-b5,
appeared to change the Em,7, even though about one-half of
the P450 3A4 was converted from the low spin to high spin iron
configuration by the addition of testosterone, and it appears
possible to have two orders of magnitude difference in reduc-
tion rates in the absence of a change in Em,7. Other literature
argues for lack of linkage of substrate binding, spin state, Em,7,
and rate of reduction (35, 39, 41), and it would appear this
dependence is not very common among P450 s.

Previously evidence has been presented for slow association
and dissociation of P450s and NADPH-P450 reductase (40, 41).
We also found that these were relatively slow processes with
P450 3A4 for both NADPH-P450 reductase and b5 (Fig. 6).
However, the kinetics vary among P450 s. For instance, the rate
measured for association of P450 3A4 and NADPH-P450 redu-
tase was 1.2 min-1 with P450 3A4 in the experiment of Fig. 6A
but 30 min-1 with human P450 IA2 in a separate experiment
(results not shown, reduction rate of 720 min-1 for preformed
complex). Such differences may have relevance when mixtures
of P450 s is considered in terms of competition for limiting
reductase (56, 57). When limiting NADPH-P450 reductase was
mixed with excess P450 3A4 and b5 and NADPH was added
from a second syringe, a rapid burst of reduction was seen for
each hemoprotein, followed by slow reduction dominated by
association/dissociation kinetics (Fig. 6). It should also be
pointed out that many of the kinetic traces of the reduction of
preformed P450 or P450/b5 complexes could be fit to first order
(single exponential) equations (e.g. Fig. 5). The first accounts
of the reduction kinetics of P450 s described biphasic kinetics (58,
59). Successive reports also present biphasic kinetics even with
purified P450 s (24, 25, 40–42, 49, 52, 60), although one of
these reports indicates that 90% of the reduction was done in
the fast phase (60). Most of these studies were done at 25°C or
less, and part of the reason we saw primarily single exponential
reduction kinetics may be due to the 37°C temperature we
used (for comparison to steady-state results). Lipid phase tran-
sitions and related phenomena may be operative at lower tem-
peratures. However, in systems where the amount of reductase
is limiting, diffusion is a likely explanation (42) rather than
inherent properties of the P450(s) or reductase.

The b5 stimulation of ferric P450 reduction does not seem to
be general. We have not seen this effect in other studies with
recombinant human P450 IA2 or 2E1. Reduction of P450 2E1
is slower in the presence of b5 (61), an effect reported for P450
2B4 by Golly et al. (49). Schenkenman et al. (52) also reported
slower reduction of ferric P450 2B4 in the presence of b5,
although the presence of P450 2B4 elevated the rate of

Fig. 6. Estimation of rates of interaction of P450 3A4 with
NADPH-P450 reductase and b5 as judged by reduction kinetics.
A, an experiment was set up in a manner similar to that of Fig. 4A,
with 0.35 µM P450 3A4, 0.35 µM b5, 200 µM testosterone, and other com-
ponents in one syringe; sufficient NADPH and NADPH-P450 reductase to
give final concentrations of 200 µM and 0.80 µM were included in
the other syringe. The solid line is a fit to a single exponential of 1.2 (± 0.02)
min-1 for the formation of the P450 3A4-CO complex (ΔA447). B, the
experiment of panel A was repeated, except that the P450 3A4 syringe
contained NADPH-P450 reductase (0.8 µM) and the b5 (0.35 µM) was
moved to the NADPH syringe. The kinetics of reduction were monitored
at 447 nm (Fe2+–CO complex) and the solid trace is a fit to a single
exponential of 0.18 (±0.006) min-1, with the residuals analysis shown at
the bottom.
Burst analysis of reduction of P450 3A4 and b5 in a system containing limiting NADPH-P450 reductase. A mixture containing 1.0 μM P450 3A4, 0.70 μM b5, and 0.40 μM NADPH-P450 reductase, 200 μM testosterone, and other typical components was mixed with NADPH (to give final concentration of 400 μM) under a CO atmosphere. Reduction of P450 3A4 was monitored at 447 nm (panels A and B) and reduction of b5 was monitored at 424 nm (panels C and D). The solid lines show single exponential fits to the indicated portion of the data, with residual fits at the bottom (in panels B and D, early segments were deleted from the analysis). Values of the fits were A, 920 ± 15 min⁻¹; B, 0.7 ± 0.005 min⁻¹; C, 830 ± 25 min⁻¹; and D, 3.8 ± 0.02 min⁻¹.

NADPH-P450 reductase-catalyzed reduction of b5. The postulate of “simultaneous” 2-electron reduction of ferric P450 and b5, presented in that article (i.e. sequential rapid reduction of ferric P450, transfer of an electron from ferrous P450 to ferric b5, and reduction of ferric P450) is of interest in light of the similarity of the rates of reduction of P450 3A4 and b5 seen in some of the experiments described in Table IV. However, this hypothesis does not seem to generally apply here. CO traps ferrous P450 but b5 has a similar rate of reduction as P450 in the presence of CO. Also, in other studies (not presented) with a recombinant P450 3A4:NADPH-P450 reductase fusion protein (62), reduction of b5 was faster than P450.

In conclusion, b5 has been shown to stimulate P450 3A4-catalyzed reactions without obligate electron transfer. In these cases (testosterone 6β hydroxylation, aflatoxin B1 oxidation) the mechanism of stimulation appears to involve enhancement of rates of ferric P450 reduction, a process which seems not to depend on the oxidation state of or transfer of electrons from b5. A mechanistic proposal has been presented previously (24) and still appears to be valid as a working model. As suggested by Lipka and Waskell (48), different substrates may preferentially bind to a P450, P450 3A4 in this case, to induce b5 binding. Alternatively, b5 may bind to the P450 in the absence of the substrate but the mode of interaction may be altered in the ternary P450-substrate-b5 complex. This binding evidently influences the transfer of electrons from NADPH-P450 reductase to the ferric enzyme and probably the ferrous-O2 complex.

However, some substrates such as ethylmorphine appear to be able to bind to P450 3A4 and induce rapid reduction in the absence of b5 (Table IV).

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