Kinetics of Ferric Cytochrome P450 Reduction by NADPH−Cytochrome P450 Reductase: Rapid Reduction in the Absence of Substrate and Variations among Cytochrome P450 Systems†

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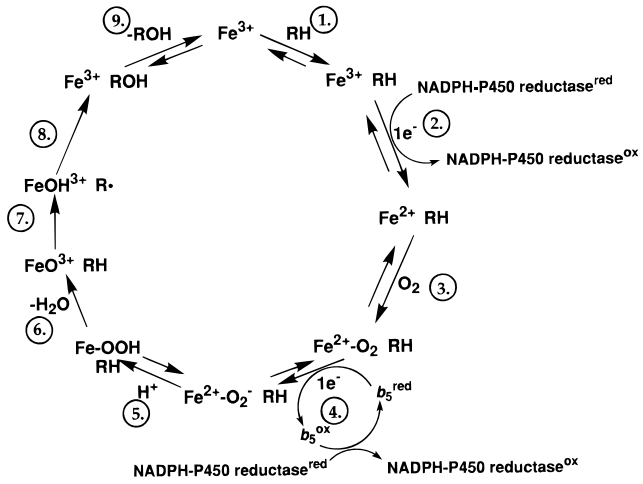
ABSTRACT: The reduction of ferric cytochrome P450 (P450) to ferrous is the first chemical step in almost all P450 reactions, and many characteristics of this step have been reported. Reduction kinetics of rabbit and human P450s were measured in a variety of systems. As reported earlier, P450 reduction is biphasic in microsomes and some purified P450 systems. However, this is not an inherent property of P450s, and some low- and high-spin iron P450s were reduced with single-exponential kinetics. Contrary to a generalized view, the presence of substrate is not necessary for rapid reduction of all P450s. Also, low-spin heme can be reduced as rapidly as high-spin in several P450s. P450s varied considerably in their reduction behavior, and even a single P450 showed remarkably different reduction kinetics when placed in various environments. P450 3A4 reduction was examined in liver microsomes, a reconstituted system, a fusion protein in which it was linked to NADPH−P450 reductase, and baculovirus and bacterial membranes in which P450 3A4 and NADPH−P450 reductase were coexpressed; the systems differed considerably in terms of the need for the substrate testosterone and cytochrome b_5 (b_5) for reduction and as to whether reduction was rate-limiting in the overall catalytic cycle. When b_5 was included in reconstituted systems, its reduction kinetics were linked with those of some P450s. This behavior could be simulated in kinetic models in which electrons flowed from the ferrous P450·CO complex to oxidized b_5. Overall, the kinetics of ferric P450 reduction cannot be generalized among different P450s in various systems, and concepts regarding influence of substrate, reaction sequence, and a rate-limiting step are not very universal.

P450[1] enzymes are rather ubiquitous in nature. These proteins are characterized by their cysteinyl−heme ligation and, generally, their monooxygenation reactions (*2*−*5*). Multiple forms of P450 are found in bacteria as well as humans (*6*). Their functions can be in specific and critical processes (*e.g.*, steroid anabolism in mammals) or rather unselective reactions (degradation of carbon sources for microorganisms, removal of ingested products for higher organisms).

The catalytic mechanisms of most of the monooxygenation reactions are considered to be relatively general and can be described by the paradigm shown (Scheme 1). However, the difficulties in elucidating mechanisms in such a multistep pathway are certainly considerable. The only stable intermediates are those designated Fe^{3+}, Fe^{3+} RH, and Fe^{3+} ROH in Scheme 1; Fe^{2+} RH is stable only in the absence of O_2. Much of the current dogma has been developed from biomimetic models (*7*) and from studies with simpler, more readily obtained bacterial P450s (*8*). Our own mechanistic focus has been given largely to latter events (steps 7 and 8 of Scheme 1), where we and others have utilized biomimetic models (*7*), kinetic hydrogen isotope effects (*9*−*11*), and

Scheme 1: General P450 Catalytic Cycle[a]



[a] RH = substrate, ROH = product. Step 4 can involve electron transfer from b_5 or directly from NADPH−P450 reductase in some cases.

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[1] Abbreviations: P450, microsomal cytochrome P450 (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1 (*1*); b_5, cytochrome b_5 (EC 4.4.2 group); di-12:0 GPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine; DCPIP, 2,6-dichlorophenolindophenol; αNF, α-naphthoflavone (7,8-benzoflavone); $E_{m,7}$, oxidation−reduction potential at pH 7.0, relative to hydrogen.

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substrates that undergo diagnostic rearrangements or conversions to inactivating species (*9, 12, 13*).

Recently we have directed our attention to step 2 of Scheme 1, in which the first electron enters the catalytic cycle. In the course of P450 research this reduction, as well as step 1, has been a source of much attention (*14*). Gillette and his associates (*15, 16*) first observed the biphasic nature of this step in rat liver microsomes, monitoring the formation of the Fe^{2+}·CO complex in microsomes mixed with NADPH. Diehl et al. (*17*) extended this approach with rat liver microsomes and the substrates cyclohexane and hexobarbital. Biphasic reduction kinetics were also observed, which could be described by rate constants of 46 and 10 min^{-1}.[2] The authors also used changes in the Soret spectrum as a measure of substrate binding and concluded that the extent of the faster phase of P450 reduction was similar to the fraction of substrate bound to P450. The authors conceptually linked the iron spin state, rate of reduction, and substrate binding. They also expressed the view that the rate of reduction of ferric P450 was rate-limiting. Extensive studies with bacterial P450 101 ($P450_{cam}$) showed a strong linkage between substrate binding, spin state, and $E_{m,7}$ (*8, 18*). Although bacterial P450 101 is reduced (by putidaredoxin) in a single-exponential reaction (*18*), many studies have been done with microsomal P450s in microsomes and after purification, and biphasic reductions have usually been observed (*14*−*17, 19*−*22*). At least three possible explanations have been presented: (i) The two phases correspond to reduction of the high- (faster rate) and low-spin (slower rate) P450 components (*17, 22*−*24*); (ii) biphasic reduction properties are due to aspects of intramolecular electron transfer between the two flavins in NADPH−P450 reductase (*20*); and (iii) biphasic reduction is due to reduction of two populations of spatially aggregated (*19*) or otherwise conformationally restricted populations of P450 (*14*).

Kominami and Takemori (*21*) observed biphasic reduction kinetics for P450 reduction in bovine adrenal microsomes and found that substrate was required for efficient reduction. However, they did not find any correlation of the high-spin state with rates of reduction of ferric P450. In a study with several isolated rat liver P450s in this laboratory, there was no general correlation between substrate binding, spin state, $E_{m,7}$, and rates of substrate oxidation (*25*). Backes and Eyer (*26*), in a series of studies with rabbit P450 2B4, were unable to correlate the high-spin content (induced by addition of substrates) with rates of P450 reduction. Although several key reviews point out that the P450 101 model of ligand/spin/redox equilibrium cannot be extended to mammalian P450s (*e.g.* refs 8 and 14), statements that low-spin P450 is not reducible are still common in the primary literature (*e.g.* refs 27 and 28) and texts and reviews (*e.g.* refs 29−31).

In the course of our studies on a series of recombinant human and other P450s we examined reduction kinetics in a variety of settings. We report here that some mammalian P450s clearly exhibit single-exponential reduction kinetics while others do not, that there is no general link between spin state and reduction kinetics, that the presence of substrate is not obligatory for rapid P450 reduction, and that a "linked" reduction of some P450s and b_5 postulated by Schenkman et al. (*32*) may provide a reasonable explanation for several observations regarding b_5.

[2] For consistency, all first-order rate constants are expressed in units of min^{-1}. We also refer to "biphasic" kinetics even though some may be more complex, if they can be fitted to biexponential plots.

EXPERIMENTAL PROCEDURES

Chemicals. (*S*)-Warfarin was a gift of Dr. L. S. Kaminsky, New York State Department of Health (Albany, NY). Tolbutamide was purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized from H_2O/CH_3OH. (*S*)-Mephenytoin was provided by Prof. G. R. Wilkinson, Department of Pharmacology, Vanderbilt University. All three of these compounds were dissolved as monosodium salts in aqueous solution prior to use.

Enzymes. P450 1A2 was purified from liver microsomes of rabbits treated with 7,8-benzoflavone using a modification (*33*) of the procedure of Alterman and Dowgii (*34*). Recombinant human P450s 1A2 (*33*), 2C9 (*35*), 2E1 (*36*), and 3A4 (*37*) were purified from *Escherichia coli* membranes as described. P450 2C19 was produced in a baculovirus system (*Trichoplusia ni* cells) by Dr. P. M. Shaw (PanVera Corp., Madison, WI) and purified using a modification of the procedure used to isolate P450 2C9 from *E. coli* (*35*). b_5 was purified from rabbit liver microsomes (*38, 39*). Recombinant rat NADPH−P450 reductase was prepared from *E. coli* using a vector obtained from Prof. C. B. Kasper, University of Wisconsin (Madison, WI) (*40*). All purified proteins were >95% homogenous as judged by electrophoresis. Baculovirus microsomes containing P450 3A4 were from Dr. Shaw of PanVera Corp. (*41, 42*); the molar ratio of NADPH−P450 reductase to P450 was 8. *E. coli* membranes containing equal amounts of P450 3A4 and human NADPH−P450 reductase were prepared using a bicistronic vector (*43*). The fused P450 3A4:rat NADPH−P450 reductase protein (*44*) was prepared as described (*45, 46*).

Reconstitution Systems. Purified rabbit P450 1A2 and human P450s 1A2 and 2E1 were mixed with a 2-fold molar excess of NADPH−P450 reductase and 30 μM di-12:0 GPC in 0.10 M potassium phosphate buffer (pH 7.4). Purified P450s 2C9, 2C19, and 3A4 were mixed with a 2-fold molar excess of NADPH-450 reductase, an equimolar amount of b_5, 0.25 mM sodium cholate, 30 mM $MgCl_2$, and a phospholipid mixture [20 μg mL^{-1} mixture (1:1:1, w/w/w) of di-12:0 GPC, L-α-dioleoyl-*sn-glycero*-3-phosphocholine, and bovine brain phosphatidylserine] (*47*) in 50 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (pH 7.4).

Spectroscopy. Absorbance spectra were recorded using modified Aminco DW2/OLIS and Cary 14/OLIS instruments (On-Line Instrument Systems, Bogart, GA). Second-derivative spectra were obtained using the manufacturer's software, with the application of the curve smoothing program. Analysis of second-derivative spectra involved the zero-baseline method (*48*).

Reduction Kinetics. Reduction of ferric P450 to the ferrous form was measured at 37 °C under an anaerobic CO environment using anaerobic techniques described elsewhere (*49*). All studies were done using an Applied Photophysics SX-17MV instrument (Applied Photophysics, Leatherhead, U.K.) and the manufacturer's computer system. P450 reductions were monitored near 450 nm (446−450 nm, depending upon the λ_{max} of each particular $P450^{red}$·CO complex) or at 390 or 415 nm (decrease in ferric P450). b_5 reduction was monitored by the increase at 424 nm, which

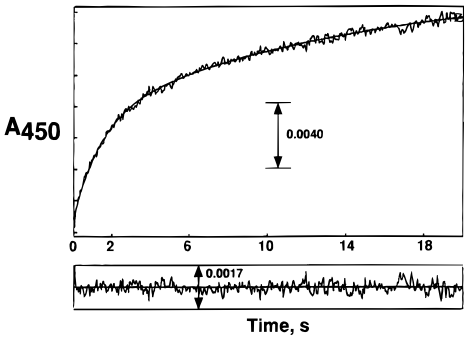


FIGURE 1: Kinetics of reduction of ferric P450 in human liver microsomes. Sample HL110 was used, which has been estimated to contain ~60% of its P450 as P450 3A4 (*55*).

is relatively distinct from changes in P450 spectral changes under these conditions (b_5 was usually equimolar with P450) as established in parallel experiments devoid of b_5.

Traces are presented as averages of several individual reactions (3−10) and the analysis of residuals is shown below the plots. The manufacturer's software supplied with the instrument also provides an estimate of the error in each trace (applied on the averaged traces). These estimates were $<\pm 10\%$ in every case and are not routinely presented except in a few selected cases.

Other Assays. Previously described procedures were used to monitor the oxidation of phenacetin (*50*), tolbutamide (*51*), (*S*)-warfarin (*52*), (*S*)-mephenytoin (*53*), and C_2H_5OH (*54*).

RESULTS

Reduction of Ferric P450 in Human Liver Microsomes. As expected from the literature describing other microsomal preparations, we found that apparently biphasic kinetics were observed when the ferric P450 in human liver microsomes was reduced using NADPH (Figure 1). In this particular case, global fitting of the results yielded an estimated 43% of the P450 reduced at a rate of 41 min^{-1} and the remaining 57% was reduced at a rate of 4 min^{-1}. Although the stopped-flow spectrophotometer used here was not specifically designed for use with turbid samples, the system could be used to obtain good data when the optical density was ≤ 1.0.

Monophasic Reduction of Human P450 1A2. The literature enumerates many cases of purified P450s showing biphasic reduction kinetics, and several explanations have been presented, including spin state populations and properties of the flavins in the reductase (*19*, *20*, *22*−*24*). Some of the purified P450s examined here showed biphasic reduction kinetics (*e.g.* ref 56), but others were clearly single-exponential, *e.g.* human P450 1A2 (Figure 2). A rapid drop in absorbance was seen due to the reduction of the flavins. The reduction of the flavins in NADPH−P450 reductase is complex and involves rapid internal electron transfers (*57*, *58*); the rate of decrease at 450 nm is at a rate similar to those reported by others (*57*). This drop in absorbance did not interfere appreciably with the analysis of P450 reduction in most cases. However, in some of the faster reductions (*e.g.*, Figure 2) the first 20−30 ms was deleted from the analysis or else a biexponential fit was applied for analysis.

The reduction rates measured at 446 and 390 nm were nearly identical (Figure 2). This result confirms the validity of both parameters and also that CO complexation is very fast and does not alter the analysis.

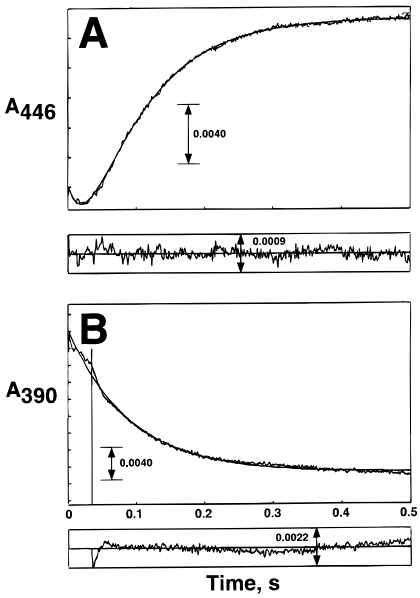


FIGURE 2: Reduction of ferric human P450 1A2. In part A (formation of Fe^{2+}·CO complex) the trace is fit to a biexponential plot with an initial decrease at 36 s^{-1} (2200 min^{-1}) and second exponential of 11.5 s^{-1} (690 min^{-1}). In part B (disappearance of Fe^{3+} form), the trace is fit to a single exponential of 11.2 s^{-1} (670 min^{-1}). Note the lag of ~20 ms in both parts, corresponding to electron transfer into the reductase.

Kinetics of Reduction of Various Purified Human P450s. Human P450s 1A2, 2C9, 2C19, and 2E1 were examined under various conditions [Table 1; some of the results, particularly the rates of substrate oxidations, have been presented elsewhere (*33*, *56*, *59*)].

In the absence of P450, NADPH−P450 reductase reduced b_5 in a biphasic process with rates of 490 and 80 min^{-1} (52% reduced in the faster phase).

Human P450 1A2 was reduced rapidly in a monophasic reaction, as already shown (Figure 2). The rate was rather invariant with regard to the absence or presence of the substrate phenacetin. Similar rates were measured in the presence of the substrate 17β-estradiol, although the data were not of as high quality because of turbidity induced by the substrate. When b_5 was present, it appeared to be reduced somewhat more rapidly than in the absence of P450, and the P450 reduction rate was slightly reduced. It should also be noted that the rates of ferric P450 reduction are much faster than rates of steady state NADPH or phenacetin oxidation.

P450 2C9 was reduced at only a very slow rate in the absence of substrate, as anecdotically reported elsewhere (*56*). In the presence of either (*S*)-warfarin or tolbutamide, reduction was more rapid, and 50−70% of the P450 was reduced at a rate of 160−250 min^{-1}. b_5 or apo-b_5 can enhance rates of substrate oxidation (*56*), but neither had a substantial effect on rates of P450 2C9 reduction. The reduction of b_5, observed at 424 nm, was very slow in the absence of P450 substrate, *i.e.* when P450 reduction was slow.

In contrast to P450 2C9, P450 2C19 was reduced at a nonlimiting rate even in the absence of substrate. The reactions were biphasic. The rate was substantially accelerated in the presence of the prototypic substrate (*S*)-mephenytoin.

P450 2E1 reduction was very rapid and was not significantly attenuated by the presence of the substrate C_2H_5OH

Table 1: Rates of Reduction of Ferric P450s and b_5 and of Substrate Oxidation

			pre-steady-state rate of reduction (min^{-1})				steady-state oxidation rate (min^{-1})	
P450	substrate	b_5	P450	% reduced in faster phase	b_5	% reduced in faster phase	NADPH	substrate
none	−	+			490/80	53		
1A2	−	−	800		−		41	−
1A2	−	+	690		2550		37	−
1A2	phenacetin	−	730		−		46	1[a]
1A2	phenacetin	+	510		1380		46	1[a]
2C9	−	−	<4		−		18	−
2C9	−	+	<4		26		11	−
2C9	(*S*)-warfarin	−	200/11	37	−		17	0.01[b]
2C9	(*S*)-warfarin	+	160/11	45	200/25	70	13	0.05[b]
2C9	tolbutamide	−	250/9	56	−		31	2.6[b]
2C9	tolbutamide	+	160/6	48			14	6.3[b]
2C9	−	(apo b_5)	<4		−		—[c]	5.5[b]
2C19	−	−	170/17		−		—[c]	1.7[d]
2C19	(*S*)-mephenytoin	−	960/120		−		—[c]	4.4[d]
2E1	−	−	1920/280	79	−		45	
2E1	−	+	400/14	20	800/70	52	24	−
2E1	C_2H_5OH[e]	−	1380/12	59	−		25	7.3[f]
2E1	C_2H_5OH[e]	+	100/17	38	210/26	36	43	10.4[f]
2E1	−	(apo-b_5)	1920/100	69	−		—[e]	

[a] Reference 33. [b] Reference 56. [c] Assays not done. [d] Results from Drs. H. Yamazaki, T. Shimada, and F. P. Guengerich (unpublished results). [e] 40 mM C_2H_5OH. [f] Results from ref 59.

or chlorzoxazone in this and repeated experiments (*59*). Reactions were biphasic. The apparent rates of P450 reduction were attenuated considerably in the presence of b_5, and rates of b_5 reduction exceeded the rates of P450 2E1 reduction. Apo-b_5, which has no electron transfer capability, did not affect the rates.

Effect of P450 Spin State on Reduction Kinetics. Human recombinant P450 1A2 is isolated as a nearly completely high-spin protein from *E. coli* (*33*). Rabbit (*60*) and rat (*25*) P450s 1A2 can be converted to low-spin P450 in the presence of 0.25 M 1-butanol. However, analysis of human P450 1A2 in the presence of 0.25 M 1-butanol indicated that it had been converted to cytochrome P420.

Rabbit P450 1A2, as isolated from liver microsomes of 7,8-benzoflavone-treated animals, is a mixture of low- and high-spin P450 (Figure 3). Addition of αNF converts this to a high-spin state (*33*). This is clearly shown by several spectral changes in Figure 3A, *i.e.*, blue shift of Soret to 390 nm, coalesced α,*β* peaks, and 646 nm band. The fractions of the individual spin states are readily estimated by second derivative analysis of the Soret region (Figure 3B) (*48*). The P450 1A2 was 84% low-spin in the absence of αNF and 87% high-spin in the presence of αNF. Upon addition of NADPH−P450 reductase and other components used to do reduction experiments, the spin state population did not change appreciably, as shown by the spectra in Figure 3B.

Rates of reduction of the low- and high-spin rabbit P450 1A2 components were monitored at 415 and 390 nm, respectively, in the absence of αNF. The rates were both determined to be ∼750 min^{-1} in this experiment, only slightly less than the rate estimated by the increase in A_{446} (for the Fe^{2+}·CO complex) (Figure 3D,E).

In a separate set of experiments done with a preparation of rabbit P450 1A2 (also predominantly low-spin), the rates of reduction as measured by the changes in absorbance at 446, 390, and 415 nm were ∼1600 min^{-1}, all effectively within experimental error (data not shown). In the same set of experiments, the assay was repeated in the presence of αNF (which was shown to convert the P450 to >85% high-spin iron, *vide supra*) and the rates of reduction were 1440 and 1860 min^{-1}, as estimated from the changes at 390 and 446 nm, respectively.

A similar set of experiments was done with a P450 2E1 preparation that contained a mixed-spin population, ∼80% low-spin as judged by second-derivative analysis (*cf.* ref 36). In these experiments the data could be fit to single-exponential plots and the rate constants were ∼1750 min^{-1} for traces of the absorbance at 450, 390, and 415 nm (data not presented).

Kinetics of Reduction of P450 3A4 in Baculovirus Microsomes. Previous studies indicated that very high rates of steady-state testosterone 6*β*-hydroxylation (up to 140 min^{-1}) could be achieved in baculovirus microsomes in which P450 3A4 was expressed in the presence of a large excess (8-fold) of NADPH−P450 reductase (*41*, *42*, *61*).

The baculovirus microsomes were very turbid, and spectral measurements were difficult (Figure 4A). However, the Soret region could be analyzed in such samples. The addition of the substrate testosterone produced a detectable shift in the spectrum, as shown in the expansion in the inset (Figure 4B). Second-derivative analysis indicated that the P450 3A4 was essentially completely in the low-spin state (417 nm band) in the absence of testosterone (Figure 4C). When testosterone was added, a strong shift to the high-spin form was seen in the second-derivative or the difference spectrum (Figure 4C,D).

Measurements of rates of P450 reduction with this system could be made when the optical density was ≤1.0. Although there was some noise in the data, rates could readily be measured (Figure 4E). In the absence of testosterone, the rate was 2200 min^{-1}. In the presence of 100 *μ*M testosterone the rate was 2300 min^{-1} (data not shown), within experimental error of the value measured in the absence of testosterone.

Rates of Ferric P450 Reduction in a P450 3A4:NADPH−P450 Reductase Fusion Protein. Details of the reduction of a fusion protein were examined (Table 2). In the absence

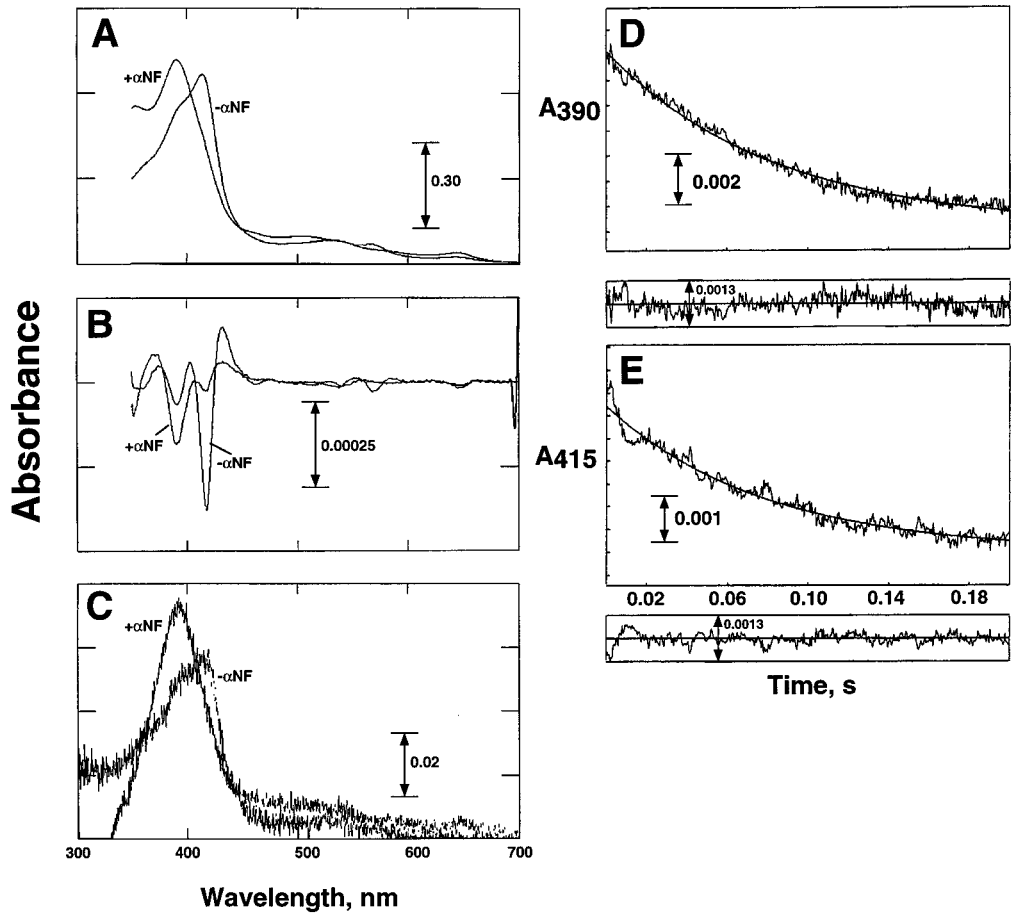


FIGURE 3: Reduction of ferric rabbit P450 1A2: (A) absolute spectra of rabbit P450 1A2 (7.4 mM in 0.10 M potassium phosphate buffer, pH 7.4), in the absence and presence of 50 μM αNF: (B) second-derivative spectra derived from spectra shown in part A; (C) absolute spectra of rabbit P450 1A2 recorded in the presence of NADPH−P450 reductase and other components used in reduction assays at the concentration used and blanked for contribution of other components; (D) kinetics of reduction, monitored at 390 nm; (E) kinetics of reduction, monitored at 415 nm.

Table 2: Rates of Reduction of Ferric P450 Heme in a P450 3A4:NADPH−P450 Reductase Fusion Protein

substrate	b_5	added NADPH−P450 reductase (μM)	reduction rate[a] (min^{-1})
testosterone[b]			98, 15[c]
	holo		1
testosterone	holo		9−12[d]
testosterone	apo		51, 15[c]
testosterone	holo	1.4 (4×)	55, 10[c]

[a] All rates were measured using a 0.35 μM concentration of the fusion protein, unless indicated otherwise. [b] 200 μM in all cases. [c] Biphasic. In all three cases, ∼45% was reduced in the faster phase. [d] The concentration of the fusion protein was varied from 0.055 to 0.65 μM. At the highest concentration (0.65 μM), a faster phase (32%) with a rate of 62 min^{-1} was observed.

of testosterone or an alternate substrate, only very low rates of reduction were observed. With testosterone present, biphasic reduction kinetics were seen in the absence of b_5. When b_5 was included, only monophasic reduction was observed, with apparent rates in the range of 9−12 min^{-1}. This rate was rather invariant over a fusion protein concentration range of 0.055−0.65 μM, although at the high concentration 32% of the P450 was reduced in a more rapid phase with a rate of 62 min^{-1}. The kinetics in the presence of apo-b_5 were similar to the reaction devoid of b_5 (although the rapid phase was slower). When excess NADPH−P450 reductase was added, a faster initial reduction phase was observed.

Table 3: Rates of Ferric P450 3A4 Reduction in Various Systems

system	substrate[a]	k (min^{-1})
reconstituted system (+ phospholipid mix, cholate, Mg^{2+}, b_5)	testosterone	1250
human liver microsomes		41 (± 2), 4 (±0.4)[b,c]
	testosterone	78 (±2), 7 (±0.2)[c,d]
baculovirus microsomes		2200 ± 70[c]
	testosterone	2300 ± 80[c]
E. coli membranes		7 ± 0.2[c]
	testosterone	12 ± 0.4[c]

[a] 200 μM. [b] Biphasic, 43% reduced in faster phase. [c] Error estimates are included in rates, which were sequentially measured on the same day. [d] Biphasic, 50% reduced in faster phase.

Rates of Ferric P450 Reduction in Other Systems. Rates of reduction of P450 3A4 in baculovirus microsomes have already been presented (Figure 4) and are listed in Table 3. We have also shown that in reconstituted systems (containing P450 3A4, NADPH−P450 reductase, a phospholipid mixture, cholate, and Mg^{2+}) that reduction is highly dependent upon the presence of substrate and, in several cases, either b_5 or apo-b_5 (*49*). In an experiment done here with the same NADPH−P450 reductase preparation used in the studies presented in Table 1, a (single-exponential) rate of 1250 min^{-1} was measured (Table 3).

A human liver microsomal sample (denoted HL 110) was estimated to contain ∼60% of its P450 as P450 3A4, as judged by immunoblotting (*62*) and by the extent of P450

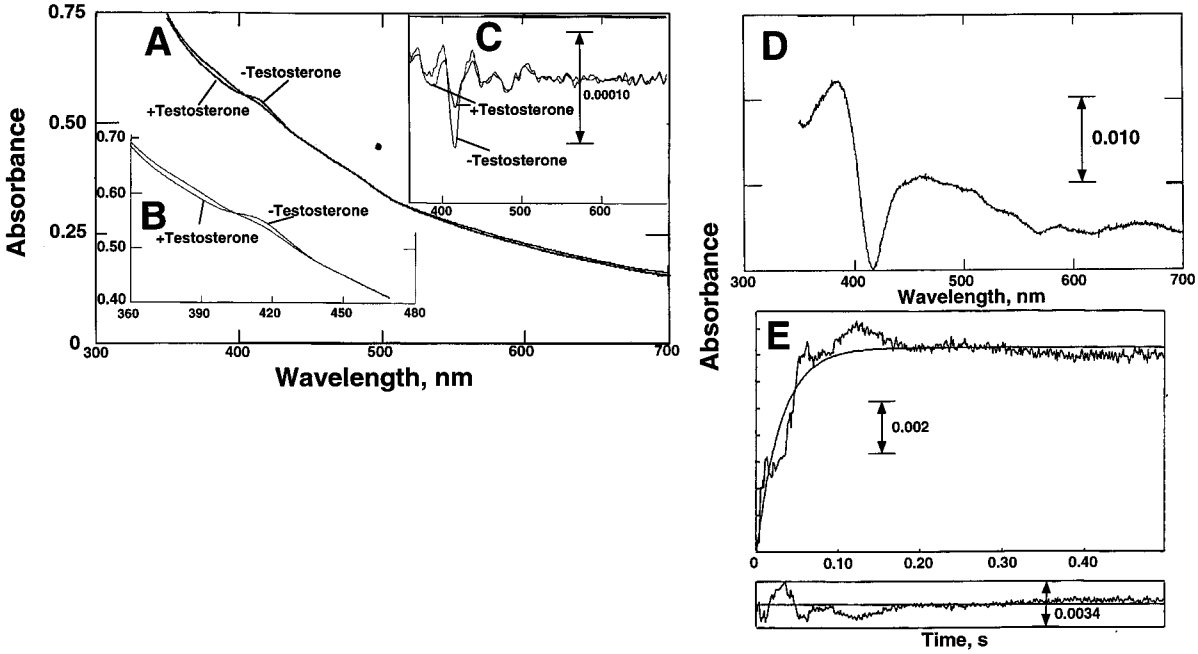


FIGURE 4: Spectra of P450 3A4 in baculovirus microsomes and reduction kinetics: (A) absolute spectra of baculovirus microsomes in which P450 3A4 was expressed, with the indicated trace recorded after adding 100 μM testosterone (all in 100 mM potassium phosphate buffer, pH 7.4); (B) expanded portion of the spectra of part A; (C) second-derivative spectra from part A; (D) difference spectrum from part A (minus testosterone spectrum subtracted from plus testosterone spectrum); (E) kinetics of anaerobic reduction of baculovirus microsomes (with P450 3A4 expressed) [the atmosphere was anaerobic CO, and the P450 concentration (0.5 μM) was the same as in parts A−D; the rate was 2100 min^{-1}].

destruction by the mechanism-based inactivator gestodene (*55*). Reduction kinetics were biphasic (Figure 1). The contributions of individual P450s in the reduction cannot be ascertained. The rate of the rapid reduction was increased in the presence of the P450 3A4 substrate testosterone (Table 3).

Another system involves *E. coli* membranes in which P450 3A4 and human NADPH−P450 reductase have been expressed at equimolar levels, utilizing a bicistronic expression construct (*43*). Reduction was monophasic. The rate was 7 min^{-1} in the absence of testosterone and increased to 12 min^{-1} in its presence (Table 3).

Simulation of Rates of Electron Transfer between P450 and b_5. There is a distinct decrease in the observed reduction rates of P450 2E1 and P450 1A2 when b_5 is present, while the observed reduction of b_5 in this system is much faster than that catalyzed by NADPH−P450 reductase in the absence of P450 (Table 1). To account for this behavior, we postulate that the rates observed are a composite of reactions that include a rapid reduction of b_5 by ferrous P450. Hence, we modeled the individual reaction kinetics mathematically using simulation software. The reactions are reduction of ferric P450 by reductase (experimentally measured), reduction of b_5 by reductase (experimentally measured), reduction of b_5 by ferrous P450, reduction of b_5 by CO-complexed ferrous P450, and binding of CO to P450 to form a binary complex. The following equilibria

$$\text{reductase}^{red} + \text{P450}^{ox} \rightleftarrows \text{reductase}^{ox} + \text{P450}^{red} \quad (1)$$

$$\text{reductase}^{red} + b_5{}^{ox} \rightleftarrows \text{reductase}^{ox} + b_5{}^{red} \quad (2)$$

$$\text{P450}^{red} + \text{CO} \rightleftarrows \text{P450}^{red}\cdot\text{CO} \quad (3)$$

$$\text{P450}^{red}\cdot\text{CO} + b_5{}^{ox} \rightleftarrows \text{P450}^{ox} + b_5{}^{red} \quad (4)$$

were considered using a KINSIM modeling program (*63*) to rationalize some of the effects of b_5 and P450s on the kinetics of reduction of each other. Reaction 3 was given a diffusion-limited forward rate constant and a K_{eq} of 1 μM (*64*), since CO association to ferrous P450 is usually considered to be effectively irreversible under a CO atmosphere. In each case considered, the rate of P450 reduction in the absence of b_5 was known (Table 1). The rate of b_5 reduction (eq 2) was measured (Table 1) in the absence of P450, and the reverse reaction was assumed not to contribute. Equation 4 is the major focus of our kinetic consideration in the modeling. The thermodynamics have some uncertainty. The $E_{m,7}$ of the b_5 is well-established at ~0 mV (*65*, *66*), but the $E_{m,7}$ values are not known for any of the human P450s under consideration here except P450 1A2 and 3A4 (*49*) and, because of the effect of CO on the equilibrium, the potential of the P450^{red}·CO ⇄ P450^{ox} couple is highly dependent upon the CO affinity.

The model calculates and displays the relationship of overall P450 and b_5 reduction to time (*e.g.* Figures 5 and 6). From this two-dimensional representation a rate of change can be estimated. This was then iteratively compared to the experimentally observed reaction rates, and rational adjustments in the estimates were made (Table 4). For the case of P450 2E1 a reduction rate of b_5 by P450^{red}·CO estimated at ~2700 min^{-1} will satisfy the observed rates of reduction. Similarly, the P450 1A2 case is satisfied by an estimate of ~2700 min^{-1}. The experimentally determined rate of b_5 reduction in the presence of P450 2C9 was much lower than in the absence of P450 (Table 1), and a model in which b_5 is partially "blocked" from the reductase was used. A k of 25 min^{-1} for reduction of b_5 by NADPH−P450 reductase yielded a satisfactory approximation to the experimental results (absence of substrate, Table 1); the simulated rate of

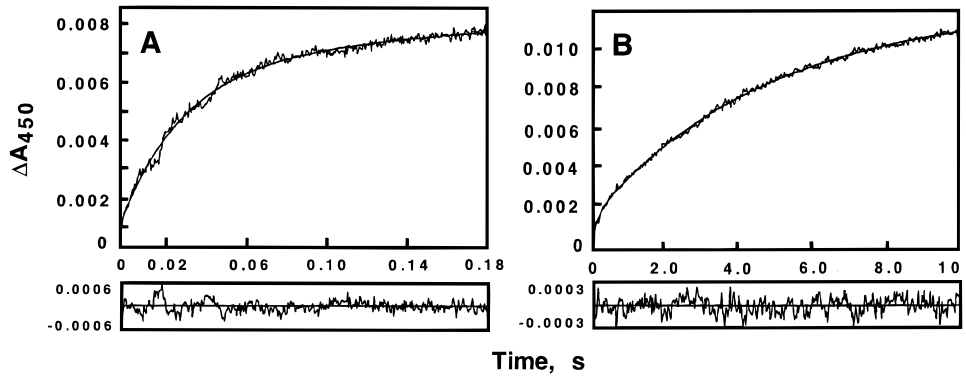


FIGURE 5: Effect of b_5 on apparent kinetics of reduction of P450 2E1. The methods are described under Experimental Procedures, and the rates (biphasic reduction) are presented in Table 1 for reduction in the (A) absence and (B) presence of b_5 (without substrate in both cases).

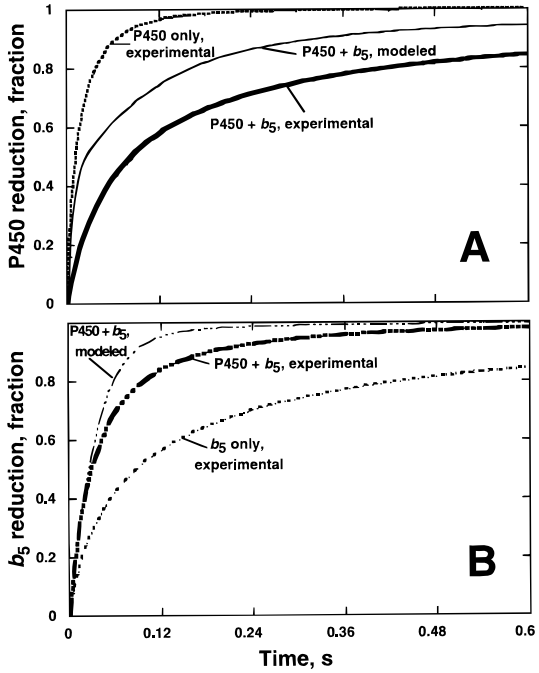


FIGURE 6: Simulation of kinetics of electron transfer among NADPH−P450 reductase, P450, and b_5. Kinetic simulation results are compared to experimental observations. Bold lines represent experimental results. Medium thickness lines represent simulation calculations. Thinnest lines represent the rate of reduction by NADPH−P450 reductase alone. The model estimates can be changed such that P450 reduction can replicate the experimental observations; however, the b_5 reduction agreement deteriorates. Similarly, if estimates are used that will mimic observations for b_5 reduction, then the P450 agreement deteriorates.

Table 4: Reduction Rate Estimates from Kinetic Simulation

P450	*k*, reduction of P450 by NADPH−P450 reductase (min^{-1})	*k*, reduction of b_5 by NADPH−P450 reductase (min^{-1})	*k*, reduction of b_5 by $P450^{red}$ (min^{-1})
2E1	1900[a]	490[a]	2700
1A2	800[a]	490[a]	2700
2C9	4[a]	25	1000

[a] P450 reduction by reductase is from experimental values (Table 1).

b_5 reduction is slower than in the cases of P450s 1A2 and 2E1 but still ~1000 min^{-1}.

DISCUSSION

Kinetics of reduction of the various P450s varied considerably with regard to several characteristics (Table 1). The reduction of P450s 1A2 and 3A4 (most conditions, Tables 2 and 3) was single-exponential, but P450s 2C9, 2C19 and 2E1 always yielded biphasic kinetics. The rates also varied considerably for the different P450s, by an order of magnitude under optimized conditions. The influence of substrates was also distinct for the different P450s (Table 1). Kinetic variation was seen not only among the different P450s but also among the different systems used; for example, P450 3A4 rates varied considerably (Tables 1, 3, and 5). Single-exponential reduction rates of 9−2100 min^{-1} were observed in the various systems. With P450 3A4 (Table 5) and the other P450s (Table 1), the degree to which this step limits the overall reaction (if at all) can vary considerably. With most of the P450s, the rate of reduction seen in the reconstituted system (composed of P450, NADPH−P450 reductase, and phospholipid) is much faster than substrate oxidation. This is also true for P450 3A4 in the reconstituted system and in baculovirus microsomes (Table 5). However, with the purified fusion protein, the bacterial membranes, and liver microsomes, this is not the case (Table 5).

Table 5: Summary of P450 3A4 Reduction Kinetics

	optimal reduction rate (min^{-1})	requirement for		steady-state testosterone 6β-hydroxylation rate (min^{-1})
		substrate	b_5	
purified, reconst system	700−1200	+	+	10−40 (~35)
purified 3A4−reductase fusion protein	9−12	+	−	12
baculovirus microsomes	2100	−	−	140
bicistronic *E. coli*	12	+	−	14
liver microsomes	(78/7)[a]	(+)	?	40[b]

[a] Biphasic. Rates based on total microsomal P450. [b] Rate based on calculation that 30% of total P450 is P450 3A4 (*42*).

Another point is the need for the presence of substrate for rapid reduction. P450s 1A2 and 2E1 showed no requirement for substrate in this regard. The lack of effect is not due to restriction of the consideration to only slowly oxidized substrates, in that the rates of reduction in the absence of substrate were >800 min^{-1} (considering only the faster phase of the P450 2E1 reduction). P450 2C9 clearly required a substrate to facilitate reduction, even though substrate oxidation rates were slow (Table 1). The faster phase of P450 2C19 reduction in the absence of substrate was not rate-limiting (Table 1), but the rate was accelerated in the presence of a substrate. We previously reported that reduction of P450 3A4 was only very slow in the absence of substrate (*49, 67*). These studies were repeated, and similar results were observed with P450 3A4 in other systems

(Table 3). However, in the baculovirus microsomes, in which the reductase is present at very high levels and the membrane environment appears rather optimal, rapid and complete reduction was observed without any substrate (Figure 4).[3] In bacterial membranes, in which P450 and the reductase were approximately equimolar (*43*), only ~2-fold stimulation by testosterone was seen (Table 3). There is clearly no generalized need for the presence of substrate for rapid P450 reduction. Even with bacterial P450 101, one study (*14*) indicates that the rate of reduction of camphor-free P450 101 was 30% that of the substrate-saturated form.

The literature evidence for a lack of association of $E_{m,7}$ with substrate binding and high-spin iron configuration is germane. In repeated studies no clear association was seen with several rabbit and rat liver P450s (*68, 69*), and a series of predominantly high-spin P450s had measured $E_{m,7}$ values varying from −170 to −360 mV (*70*). A correlation between rates of ferric P450 reduction and high-spin iron in rat liver microsomes using a series of amphetamine derivatives has been reported, but the range of (faster phase) reduction rates was only 26−44 min^{-1} (*71*). Preferential reduction of high-spin P450 in the faster phase was also a conclusion in other work with microsomes and a purified rat P450 (*22*−*24*). However, analysis of the results indicates less than a 2-fold difference in rates at wavelengths ascribed to different spin states (*23*), and the conclusions in a subsequent paper (*26*) are that high-spin P450 (P450 2B4) is not preferentially reduced.

Our own evidence for rapid reduction of low-spin P450 is fourfold: (i) In previous work (*49, 67*), P450 3A4 was rapidly reduced in the presence of the substrate ethylmorphine ($k = 660$ min^{-1}), under conditions in which the iron was ≥90% low-spin. (ii) In this work, the particular preparation of P450 2E1 used in the reduction kinetics work (Table 1) was >90% low-spin, as judged by second-derivative analysis. (iii) P450 3A4 was >90% low-spin in baculovirus microsomes (Figure 4). Under these conditions the protein was reduced, essentially completely, at a single-exponential rate of 2200 min^{-1}. The addition of testosterone to the microsomes shifted the equilibrium to high-spin (Figure 4), but the rate of reduction was not enhanced. (iv) Rabbit P450 1A2 was isolated in a mixed-spin population, in the absence of any substrate or ligand (Figure 3). Analysis of rates of change of absorbance signals for low- and high-spin iron showed the same rates (Figure 3). Further, when αNF was added to convert the P450 to a completely high-spin state, reduction was not enhanced.

In most of the systems nearly all of the P450 was reduced in the experiments presented, when careful measurements of the change in absorbance were analyzed. However, in three situations, only part of the P450 was reduced within a 2 min period. With P450 3A4 in the reconstituted system (reductase, phospholipid, cholate, Mg^{2+}), analysis indicated that ≥83% of the P450 was still intact in the tonometers after the kinetic measurements were completed. However, only 66% of this had actually been reduced during the reduction experiments. In the case of P450s 2C9 and 2C19, only 30−40% of the P450 was reduced. However, with P450 3A4, >90% was reduced in the studies with the enzyme in the baculovirus microsomes or bacterial membranes. Our current view is that the P450 that is not reduced either is spatially restricted from the reductase, in a kinetic sense, or is modified in its conformation during isolation and can be reduced by $Na_2S_2O_4$ but not the reductase.

Previously we showed the requirement for b_5 or apo-b_5 in the rapid reduction of P450 3A4 under certain conditions (*49*). This result was very repeatable in the normal reconstituted system, but neither the bacterial membranes nor the baculovirus microsomes contain b_5 and reduction occurred, rapidly in the latter case (Table 3). In some of the systems, the presence of b_5 slowed the apparent rate of P450 reduction (*e.g.*, P450 2E1) and in other cases the rate of b_5 reduction was either facilitated (P450 1A2, 2E1) or retarded (P450 2C9). These results suggested a possible linkage of the reduction of b_5 with some P450s, as postulated earlier by Schenkman et al. (*32*) to explain some results on the salt dependence of P450 coupling efficiency. We considered a model in which electron transfer to b_5 was from ferrous P450, or actually the $P450^{red}$·CO complex. Such a transfer would reoxidize the P450 and require a "second" reduction of the P450 iron atom. Thus, b_5 reduction would appear to be faster than reduction of P450, as observed here (Table 1). The simulations were consistent with such a scheme (Figure 6). A rapid rate of electron transfer from ferrous P450 to b_5 is necessary. However, there is precedent for this in the work of Pompon and Coon (*72*). For instance, biphasic b_5 reduction was observed at 5 °C with $k = 660$ and 120 min^{-1}. The $\Delta E_{m,7}$ for transfer from the $P450^{red}$·CO complex to b_5 is probably relatively favorable. An effective $E_{m,7}$ for rabbit P450 2B4 in the presence of CO was reported to be −150 mV (*68*). This is probably reasonable, since the change in $E_{m,7}$ due to the presence of CO, ΔE, is expressed in the form

$$\Delta E = (RT/nF) \ln(K_d/[CO])$$

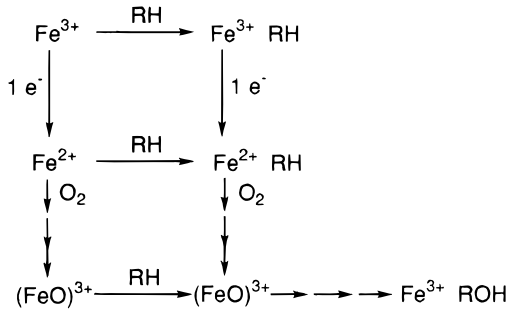
where R is the gas constant, T the absolute temperature, n the number of electrons involved, F the Faraday constant, and K_d the dissociation constant for the $P450^{red}$·CO complex (*64, 73*). The K_d for P450 101^{red}·CO is 0.8×10^{-6} M (*64*) and, if we assume a K_d of 10^{-6} M and $E_{m,7}$ of ~−300 mV for each P450 (*25, 49*), then at 1 mM CO (saturation) the $E_{m,7}$ for the $P450^{ox}$/$P450^{red}$·CO couple should be ~−150 mV in each case, which can be compared to an $E_{m,7}$ of ~0 mV for b_5 (*65, 66*).

Another point is that there seems to be little control of the substrate over the rate of ferric P450 reduction in several experimental settings used here. This is not really unusual, and a bacterial methane monooxygenase exhibits similar properties (*74*). Rapid reduction of ferric P450 in the absence of substrate is a problem in itself, in that production of partially reduced oxygen species occurs. The decrease in NADPH oxidation due to the presence of b_5 with several P450s (Table 1) may be explained by the ability of P450s to rapidly deliver electrons to b_5; for example, b_5 can act as a reservoir of electrons, accepting them (from P450) when ferric P450 is reduced rapidly and then donating them (to $P450^{red}$·O_2) after ferrous b_5 accumulates.

Why are reduction kinetics multiphasic? In microsomes, the answer is probably that different P450s are being reduced

[3] The baculovirus results observed with P450 3A4 are probably not general. P450 2C9 was also expressed in a baculovirus system, in the presence of excess NADPH−P450 reductase, but the rate of ferric P450 reduction was 10-fold greater in the presence of the substrate tolbutamide than in its absence and appeared to be rate-limiting for the overall reaction.

Scheme 2: Possible Steps in P450 Reduction and Substrate Binding



at different rates. The matter of a step involving transfer to b_5 must also be considered, for under the proper circumstances this can change a single-exponential reduction of P450 into an apparently biphasic result (Figure 6). With regard to individual P450s, the various possibilities cited in the literature may be considered again. Since some P450s are reduced with single-exponential kinetics, the answer cannot be an intrinsic property of the reductase. Nor is the biphasic reduction due to differential kinetics for spin states, for the reasons cited above. Morover, rates of interconversion of spin states are too rapid to account for the differential, as discussed earlier (*26*, *75*). The answer is probably most akin to that of Peterson et al. (*19*) (see also ref 14), who postulated an ordering due to clustering of the P450 in aggregates. Whether the differential of pools of P450 being reduced at different rates is due to a spatial effect or to individual populations of different conformational isomers of P450 is unknown at this time.

In concluding, then, one may ask the question of why substrate binding is shown as the first step in P450 catalytic cycles. In many cases binding is not obligatory for reduction, contrary to a common dogma. We have shown that P450 reduction can occur rapidly in the absence of substrate in many cases (Scheme 2). Nevertheless, substrate binding probably is the first step in most P450 cycles, at least under laboratory experimental conditions in which it is in excess, simply because substrate binding is *faster* than reduction. For instance, if substrate binding occurs at a diffusion-limited rate of $\sim 10^8$ M^{-1} s^{-1}, then at a substrate concentration of 10^{-6} M the on-rate is ~ 100 s^{-1}, or 6000 min^{-1}, very competitive with reduction. In some cases substrate binding may be needed for reduction, presumably to shift the P450 to a conformation more favorable for reduction. There is no reason that substrate binding cannot occur later in the reaction cycle, if not required for reduction (Scheme 2). In the case of reaction supported by oxygen surrogates, substrate binding may even be preceeded by the formation of an oxygenating species.

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REFERENCES

1. Palmer, G., and Reedijk, J. (1992) *J. Biol. Chem. 267*, 665−677.
2. Guengerich, F. P. (1991) *J. Biol. Chem. 266*, 10019−10022.
3. Porter, T. D., and Coon, M. J. (1991) *J. Biol. Chem. 266*, 13469−13472.
4. Ortiz de Montellano, P. R., Ed. (1995) *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Plenum Press, New York.
5. Guengerich, F. P. (1997) in *Biotransformation, Vol. 3, Comprehensive Toxicology* (Guengerich, F. P., Ed.) pp 37−68, Elsevier Science Ltd., Oxford, U.K.
6. Fulco, A. J. (1991) *Annu. Rev. Pharmacol. Toxicol. 31*, 177−203.
7. McMurry, T. J., and Groves, J. T. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., Ed.) pp 1−28, Plenum Press, New York.
8. Mueller, E. J., Loida, P. J., and Sligar, S. G. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 83−124, Plenum Press, New York.
9. Groves, J. T., McClusky, G. A., White, R. E., and Coon, M. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 154−160.
10. Gelb, M. H., Heimbrook, D. C., Mälkönen, P., and Sligar, S. G. (1982) *Biochemistry 21*, 370−377.
11. Guengerich, F. P., Yun, C.-H., and Macdonald, T. L. (1996) *J. Biol. Chem. 271*, 27321−27329.
12. Ortiz de Montellano, P. R. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 245−303, Plenum Press, New York.
13. Bondon, A., Macdonald, T. L., Harris, T. M., and Guengerich, F. P. (1989) *J. Biol. Chem. 264*, 1988−1997.
14. Peterson, J. A., and Prough, R. A. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., Ed.) pp 89−117, Plenum Press, New York.
15. Gigon, P. L., Gram, T. E., and Gillette, J. R. (1969) *Mol. Pharmacol. 5*, 109−122.
16. Sasame, H. A., and Gillette, J. R. (1969) *Mol. Pharmacol. 5*, 123−130.
17. Diehl, H., Schädelin, J., and Ullrich, V. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 1359−1371.
18. Fisher, M. T., and Sligar, S. G. (1985) *J. Am. Chem. Soc. 107*, 5018−5019.
19. Peterson, J. A., Ebel, R. E., O'Keeffe, D. H., Matsubara, T., and Estabrook, R. W. (1976) *J. Biol. Chem. 251*, 4010−4016.
20. Oprian, D. D., Vatsis, K. P., and Coon, M. J. (1979) *J. Biol. Chem. 254*, 8895−8902.
21. Kominami, S., and Takemori, S. (1982) *Biochim. Biophys. Acta 709*, 147−153.
22. Backes, W. L., Sligar, S. G., and Schenkman, J. B. (1982) *Biochemistry 21*, 1324−1330.
23. Backes, W. L., Tamburini, P. P., Jansson, I., Gibson, G. G., Sligar, S. G., and Schenkman, J. B. (1985) *Biochemistry 24*, 5130−5136.
24. Tamburini, P. P., Gibson, G. G., Backes, W. L., Sligar, S. G., and Schenkman, J. B. (1984) *Biochemistry 23*, 4526−4533.
25. Guengerich, F. P. (1983) *Biochemistry 22*, 2811−2820.
26. Backes, W. L., and Eyer, C. S. (1989) *J. Biol. Chem. 264*, 6252−6259.
27. Narasimhulu, S. (1993) *Biochemistry 32*, 10344−10350.
28. Narasimhulu, S. (1996) *Biochemistry 35*, 1840−1847.
29. Testa, B. (1995) *Biochemistry of Redox Reactions*, Academic Press, London, U.K.
30. Lewis, D. F. V. (1996) in *Cytochromes P450: Metabolic and Toxicological Aspects* (Ioannides, C., Ed.) pp 355−398, CRC Press, Boca Raton, FL.
31. Lewis, D. F. V. (1996) *Cytochromes P450: Structure, Function and Mechanism*, Taylor and Francis, Bristol, PA.
32. Schenkman, J. B., Voznesensky, A. I., and Jansson, I. (1994) *Arch. Biochem. Biophys. 314*, 234−241.
33. Sandhu, P., Guo, Z., Baba, T., Martin, M. V., Tukey, R. H., and Guengerich, F. P. (1994) *Arch. Biochem. Biophys. 309*, 168−177.
34. Alterman, M. A., and Dowgii, A. I. (1990) *Biomed. Chromatogr. 4*, 221−222.
35. Sandhu, P., Baba, T., and Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 306*, 443−450.
36. Gillam, E. M. J., Guo, Z., and Guengerich, F. P. (1994) *Arch. Biochem. Biophys. 312*, 59−66.

37. Gillam, E. M. J., Baba, T., Kim, B.-R., Ohmori, S., and Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 305*, 123−131.
38. Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978) *Methods Enzymol. 52*, 97−101.
39. Shimada, T., Misono, K. S., and Guengerich, F. P. (1986) *J. Biol. Chem. 261*, 909−921.
40. Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. (1989) *J. Biol. Chem. 264*, 7584−7589.
41. Guengerich, F. P., Parikh, A., Johnson, E. F., Richardson, T. H., von Wachenfeldt, C., Cosme, J., Jung, F., Strassburg, C. P., Manns, M. P., Tukey, R. H., Pritchard, M., Fournel-Gigleux, S., and Burchell, B. (1997) *Drug Metab. Dispos.* (in press).
42. Shaw, P. M., Hosea, N. A., Thompson, D. V., Lenius, J. M., and Guengerich, F. P. (1997) *Arch. Biochem. Biophys.* (in press).
43. Parikh, A., Gillam, E. M. J., and Guengerich, F. P. (1997) *Nat. Biotechnol. 15*, 784−788.
44. Shet, M. S., Fisher, C. W., Holmans, P. L., and Estabrook, R. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11748−11752.
45. Guengerich, F. P., Martin, M. V., Guo, Z., and Chun, Y.-J. (1996) *Methods Enzymol. 272*, 35−44.
46. Ueng, Y.-F., Kuwabara, T., Chun, Y.-J., and Guengerich, F. P. (1997) *Biochemistry 36*, 370−381.
47. Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) *Biochemistry 31*, 6063−6069.
48. O'Haver, T. C., and Green, G. L. (1976) *Anal. Chem. 48*, 312−318.
49. Yamazaki, H., Johnson, W. W., Ueng, Y.-F., Shimada, T., and Guengerich, F. P. (1996) *J. Biol. Chem. 271*, 27438−27444.
50. Distlerath, L. M., Reilly, P. E. B., Martin, M. V., Davis, G. G., Wilkinson, G. R., and Guengerich, F. P. (1985) *J. Biol. Chem. 260*, 9057−9067.
51. Knodell, R. G., Hall, S. D., Wilkinson, G. R., and Guengerich, F. P. (1987) *J. Pharmacol. Exp. Ther. 241*, 1112−1119.
52. Kaminsky, L. S., Fasco, M. J., and Guengerich, F. P. (1979) *J. Biol. Chem. 254*, 9657−9662.
53. Shimada, T., Shea, J. P., and Guengerich, F. P. (1985) *Anal. Biochem. 147*, 174−179.
54. Brady, J. F., Lee, M. J., Li, M., Ishizaki, H., and Yang, C. S. (1988) *Mol. Pharmacol. 33*, 148−154.
55. Guengerich, F. P. (1990) *Chem. Res. Toxicol. 3*, 363−371.
56. Yamazaki, H., Gillam, E. M. J., Dong, M.-S., Johnson, W. W., Guengerich, F. P., and Shimada, T. (1997) *Arch. Biochem. Biophys. 342*, 329−337.
57. Oprian, D. D., and Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935−8944.
58. Bhattacharyya, A. K., Lipka, J. J., Waskell, L., and Tollin, G. (1991) *Biochemistry 30*, 759−765.
59. Bell, L. C., and Guengerich, F. P. (1997) *J. Biol. Chem. 272*, in press.
60. White, R. E., and Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073−3083.
61. Lee, C. A., Kadwell, S. H., Kost, T. A., and Serabjit-Singh, C. J. (1995) *Arch. Biochem. Biophys. 319*, 157−167.
62. Guengerich, F. P. (1988) *Mol. Pharmacol. 33*, 500−508.
63. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.
64. Lau, S. M. C., Harder, P. A., and O'Keefe, D. P. (1993) *Biochemistry 32*, 1945−1950.
65. Velick, S. F., and Strittmatter, P. (1956) *J. Biol. Chem. 221*, 265−275.
66. Seetharaman, R., White, S. P., and Rivera, M. (1996) *Biochemistry 35*, 12455−12463.
67. Yamazaki, H., Ueng, Y.-F., Shimada, T., and Guengerich, F. P. (1995) *Biochemistry 34*, 8380−8389.
68. Guengerich, F. P., Ballou, D. P., and Coon, M. J. (1975) *J. Biol. Chem. 250*, 7405−7414.
69. Bäckström, D., Ingelman-Sundberg, M., and Ehrenberg, A. (1983) *Acta Chem. Scand. 37*, 891−894.
70. Huang, Y. Y., Hara, T., Sligar, S., Coon, M. J., and Kimura, T. (1986) *Biochemistry 25*, 1390−1394.
71. Blanck, J., Rein, H., Sommer, M., Ristau, O., Smettan, G., and Ruckpaul, K. (1983) *Biochem. Pharmacol. 32*, 1683−1688.
72. Pompon, D., and Coon, M. J. (1984) *J. Biol. Chem. 259*, 15377−15385.
73. Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, MD.
74. Liu, Y., Nesheim, J. C., Lee, S. K., and Lipscomb, J. D. (1995) *J. Biol. Chem. 270*, 24662−24665.
75. Ziegler, M., Blanck, J., and Ruckpaul, K. (1982) *FEBS Lett. 150*, 219−222.

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