Rabbit Liver Cytochrome P-450 LM_2: Roles of Substrates, Inhibitors, and Cytochrome b_5 in Modulating the Partition between Productive and Abortive Mechanisms

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Received January 30, 1987; Revised Manuscript Received May 29, 1987

ABSTRACT: Cytochrome b_5 (b_5) enhanced the rate of 7-ethoxycoumarin deethylation by rabbit liver cytochrome P-450 LM_2. The effect was saturable and can be analyzed as the sum of two effects: a decrease in the K_M for the substrate and an increase in the V_{max}. When two substrates were present simultaneously, they competed in a complex way depending on the presence of b_5. Various substrates at low concentrations inhibited 7-ethoxycoumarin deethylation in a competitive-like way. Only a part of the P-450 activity was found to be affected by this mode of inhibition. Higher inhibitor concentrations caused a new kind of inhibition characterized by much higher half-effect values. The pattern seemed dependent on the ability of the inhibitors to be metabolized and was dramatically changed by the addition of b_5. The relative rates of P-450-dependent NADPH oxidation and hydrogen peroxide and water formation were determined as well as their dependence on substrate and b_5. A steady-state kinetic model that includes two branch points for water, hydrogen peroxide, and product formation is proposed. The model allows a full prediction of the b_5 effects and seems consistent with most of the steady-state and rapid kinetic data available in the literature.

Cytochrome P-450 dependent monooxygenase activity can be obtained by using either NADPH[1] plus dioxygen or hydrogen peroxide as the source of oxidizing equivalents (White & Coon, 1980). The formation of large amounts of hydrogen peroxide generally observed during the dioxygen-supported reaction (Nordblom & Coon, 1977) led to the opinion that some kind of P-450–hydrogen peroxide complex may be an intermediate. However, several recent observations were not consistent with this opinion. First, a difference of regioselectivity for substrate oxidation was found from oxidant to oxidant with P-450 LM_2 (Mc Carthy & White, 1983), and no correlation between the rate of dioxygen-supported and hydrogen peroxide supported demethylation of amines was found in the case of rat liver P-450 (Estabrook et al., 1984). Second, a rapid and irreversible P-450 heme bleaching occurred during the peroxide-supported reaction (Blake & Coon, 1980) while little or no inactivation was observed with dioxygen plus NADPH. Third, denatured P-450 (P-420) that is inactive in the dioxygen-supported reaction still efficiently catalyzes some hydrogen peroxide dependent oxidations (Mc Carthy & White, 1983). Rapid kinetic studies on the spontaneous decay of the intermediate ferrous dioxygen complex of P-450 LM_4 demonstrated that this complex decomposed to give hydrogen peroxide in almost stoichiometric amounts (Oprian et al., 1983) without detectable substrate hydroxylation. These data suggested that formation of a P-450–hydrogen peroxide complex by dielectronic reduction of dioxygen may not represent an intermediate step of the dioxygen-supported monooxygenase activity.

Stopped-flow experiments (Pompon & Coon, 1984) have suggested the occurrence of a branch point at the level of the ferrous dioxygen complex between hydrogen peroxide formation and substrate oxidation. This work presents inhibition studies under steady-state conditions and stoichiometry determinations. In order to test a new kinetic model for P-450 turnover, the effects of the presence of cytochrome b_5 were analyzed.

Experimental Procedures

Purification of Enzymes. The purification of P-450 LM_2 from phenobarbital-treated rabbits was carried out according to previously published procedures (Haugen & Coon, 1976). The specific heme content of electrophoretically homogeneous preparations used was 16–17 nmol of P-450/mg of protein. The method of Strittmatter et al. (1978) was used for the purification of cytochrome b_5 from Tergitol NP-10 solubilized rabbit liver microsomes. The preparation was electrophoretically homogeneous and had a specific content of 55 nmol of heme/mg of protein. NADPH–cytochrome P-450 reductase was obtained from phenobarbital-treated rabbits as described by French and Coon (1979). Enzyme concentrations were calculated as previously described (Pompon & Coon, 1984; Gorsky et al., 1984).

Determination of 7-Ethoxycoumarin Deethylase Activity in the Presence of Effectors. The formation of 7-hydroxycoumarin was followed by fluorescence (excitation wavelength = 360 nm and emission wavelength = 460 nm). Calibration was performed with authentic pure 7-hydroxycoumarin. In order to minimize the causes of experimental errors associated with pre-steady-state effects on a multicomponent assay, the following procedure was designed: Typically, 100 μL of a solution of P-450 LM_2 (28 μM) was mixed with 100 μL of a solution of P-450 reductase (33 μM), 100 μL of sonicated 1 mg/mL dilauroylphosphatidylcholine in 0.12 M phosphate buffer, pH 7.4 and 100 μL of standard buffer (0.12 M phosphate buffer, pH 7.4, 30 μg/mL dilauroylphosphatidylcholine). The mixture was incubated 10 min at 25 °C and

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[1] Abbreviations: P-450 LM, liver microsomal P-450; b_5, cytochrome b_5; 7-EOC, 7-ethoxycoumarin; TCA, trichloroacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid. Other abbreviations are listed in the legend of Figure 6.

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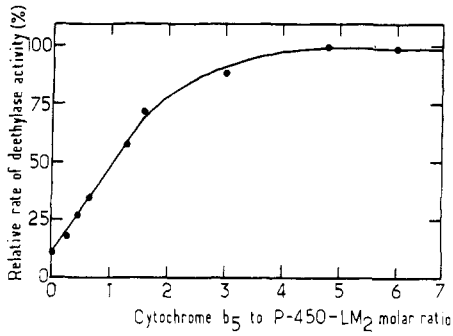


FIGURE 1: Effect of cytochrome b_5 on the rate of 7-ethoxycoumarin deethylation by cytochrome P-450 LM_2. Activities were determined at 30 °C in 0.12 M potassium phosphate buffer, pH 7.4, in the presence of 30 μg/mL dilauroylphosphadidylcholine. NADPH concentration was 0.1 mg/mL, and P-450 LM_2 concentration was 72 nM with a P-450 to P-450 reductase molar ratio of 1:1. Substrate concentration was 50 μM.

at least 1 h at 0 °C before use. The same enzyme stock solution was used in all the assays to be compared.

Two milliliters of standard buffer containing a suitable concentration of 7-EOC was pipetted into the fluorometer cuvette and allowed to equilibrate at 30 °C until no further change in the fluorescence intensity was observed. Twenty microliters of the enzyme mixture and 20 μL of a 10 mg/mL NADPH solution were then added. After 2-min incubation the linear trace was recorded during 1–3 min (slope S_1). One to twenty microliters of a concentrated inhibitor solution was then added to the cuvette, and the new stable slope (S_2) was recorded after a 15–60-s delay. Addition of 20 μL of a 33 μM b_5 solution followed by 30-s incubation allowed recording of a third linear trace (slope S_3). The normalized inhibition in the absence of b_5 was calculated as S_2/S_1 and the normalized inhibition on the b_5-dependent activity as $(S_3 - S_2)/(S_3^* - S_2^*)$, where $S3^*$ and $S2^*$ were values obtained when inhibitor was omitted and replaced by an equivalent volume of buffer. Absolute values can be calculated from the calibration.

Determination of Rate of Hydrogen Peroxide Formation. The same preincubated mixture of P-450, P-450 reductase, and lipid was used. A method was devised to correct for a transient overproduction of hydrogen peroxide that followed NADPH addition and for a lag time on the inhibitory effect of b_5 on the rate of hydrogen peroxide formation: Sixty microliters of enzyme mixture was added to 3 mL of standard buffer. The solution was equilibrated at 30 °C and divided into three tubes (1 mL each). NADPH (0.1 mg/mL) and b_5 (when used) were added at time 0, 1, and 2 min, respectively, to tube A, B, and C. At time 5 min, 200 μL of a 5% TCA solution was added to tube A. The same operation was done at 6 min to tube B, and a known amount of hydrogen peroxide was immediately added as an internal standard in this tube. The reaction in tube C was stopped after a 12-min incubation. Hydrogen peroxide in each tube was determined by the ferrithiocyanate method (Cochin & Axeroid, 1959). The amount of hydrogen peroxide was calculated from C minus A and the yield of hydrogen peroxide recovery from B minus A. Corrected rate of hydrogen peroxide formation was calculated from these values. Assays were performed in triplicate.

RESULTS

Effect of b_5 on Kinetic Parameters for 7-EOC Deethylation by P-450 LM_2. P-450 LM_2 catalyzed 7-ethoxycoumarin (7-EOC) deethylation has been chosen to monitor P-450 LM_2 monooxygenase activity. A 1 to 1 molar ratio of reductase to P-450 was used. The rate of product formation was stable for at least 10 min.

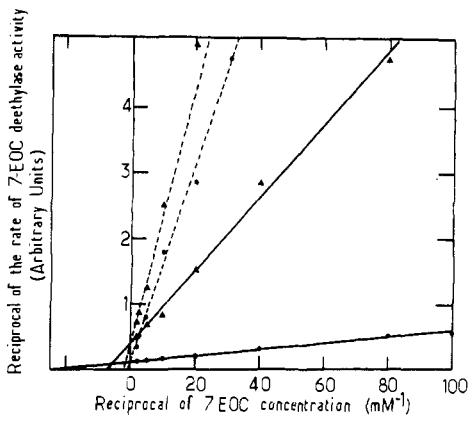


FIGURE 2: Determination of apparent kinetic parameters for the 7-ethoxycoumarin deethylase activity of cytochrome P-450 LM_2 in the presence or in the absence of cytochrome b_5. Effect of the presence of a second substrate. The cytochrome P-450 LM_2 to the P-450 reductase molar ratio was 1:1. Other experimental conditions were as described in the legend to Figure 1. When b_5 was added, the b_5 to P-450 molar ratio was 4.5:1. (●) b_5 added; (▲) b_5 absent; (---) 500 μM hexadeuteriocyclohexane added; (—) no cyclohexane.

The effect of b_5 on the rate of 7-EOC deethylation is represented in Figure 1. Cytochrome b_5 increased the rate of the deethylation reaction about 10-fold. The effect was saturable, and the concentration of free b_5 for half-effect was 0.045 μM. This value is lower than the affinity value of cytochrome b_5 for P-450 LM_2 determined by other methods (Bonfils et al., 1981). Substitution of a cobalt heme reconstituted b_5 demonstrated that the analogue was only 2% as efficient as b_5 and gave a saturating concentration of 0.35 μM. The low residual effect observed can in fact be due to a slight contamination by some unresolved regular b_5. This experiment suggested that the b_5 effects were fully dependent on its electron exchange properties since cobalt b_5 was found to bind P-450 but to be incompetent for electron transfer to the P-450 ferrous dioxygen complex (D. Pompon, unpublished experiments).

The very large increase in the deethylase activity observed in the presence of regular b_5 can be considered as the sum of two effects: (i) increase in the V_{max} for the reaction and (ii) decrease of the apparent K_M for the substrate. Results of such an analysis are given in Figure 2. When 7-EOC was used as the sole substrate, addition of a saturating concentration of b_5 caused both a 4-fold increase in the V_{max} for the substrate deethylation and a 3.55-fold decrease in the apparent Michaelis constant. The combined effect of the two changes caused up to a 10-fold overall increase in the rate of deethylation at low substrate concentrations. These effects were reinvestigated when two substrates were present simultaneously. We found that the presence of 0.5 mM hexadeuteriated cyclohexane (Ch-d_6) did not change the V_{max} for 7-EOC deethylation either in the presence or in the absence of added cytochrome b_5. This phenomenon is to be expected for a simple competitive inhibitor role toward 7-EOC deethylation. Nevertheless, the effects of b_5 on the relative affinity of 7-EOC and Ch-d_6 for P-450 did not appear to be simple: b_5 decreased 3.5-fold the half-effect concentration of 7-EOC in the absence of Ch-d_6 but increased it 2-fold when Ch-d_6 was present.

Effect of b_5 on Inhibition of 7-EOC Deethylase Activity by the Presence of Other Substrates. Inhibition or activation of P-450 monooxygenase activity by effectors may in theory result from several microscopic events: (i) classical competition for the active site; (ii) modulation of the rate of electron transfer between the reductase or cytochrome b_5 and the P-450; (iii) shift in the ratio between abortive and hydroxylating mech-

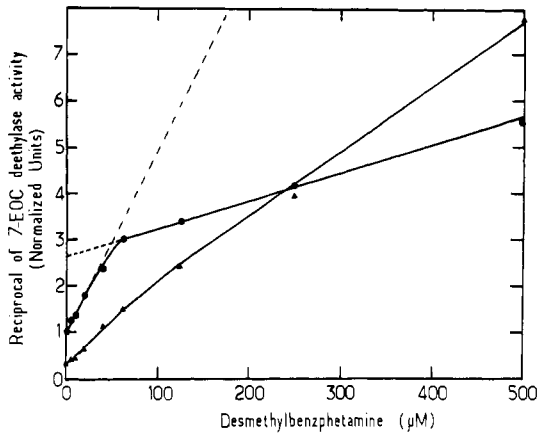


FIGURE 3: Inhibition of 7-EOC deethylase activity by demethylbenzphetamine. 7-EOC deethylase activity was normalized to 1 unit for the activity measured in the absence of both b_5 and demethylbenzphetamine. The standard procedure described under Experimental Procedures was used for activity measurements. (●) Reciprocal of the 7-EOC deethylase activity in the absence of cytochrome b_5; (▲) reciprocal of the increase of 7-EOC deethylase activity upon b_5 addition; (---) linear extrapolation for each phase of the trace. Experiments were carried out with the following final concentrations: P-450 LM_2, 0.084 μM; b_5, 0.33 μM; P-450 reductase, 0.1 μM; NADPH, 0.1 mg/mL; 7-EOC, 200 μM; dilauroylphosphatidylcholine, 30 μg/mL; potassium phosphate buffer, pH 7.4, 0.12 M; EDTA, 10 μM; glycerol, 0.2% v/v; methanol, 0–1% v/v.

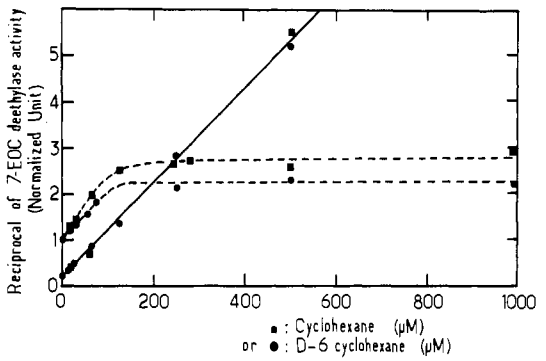


FIGURE 4: Inhibition of 7-EOC deethylase activity by cyclohexane and hexadeuteriocyclohexane. All conditions were identical with those described in the legend to Figure 3. (---) Reciprocal of the 7-EOC deethylase activity in the absence of b_5; (—) reciprocal of the increase of 7-EOC deethylase activity upon b_5 addition; (■) cyclohexane; (●) cyclohexane-d_6.

anisms at some step of the mechanism; (iv) depletion in the concentration of some intermediate oxygenated species, leading to a change of the limiting step. The study of the inhibition of the oxidation of a given substrate by various compounds ranging from a pseudosubstrate to a good substrate was used as an approach to distinguish between these possibilities. Figures 3–5 represent the more typical behaviors observed. In the absence of cytochrome b_5, plots of the reciprocal of the rate of 7-EOC deethylation versus the concentration of a second substrate were always bimodal, suggesting a complex effect. The unusual pattern is characterized by a first type of inhibition occurring with a low half-effect concentration (K_L). The maximal extent of this inhibition was surprisingly limited. Higher concentrations of inhibitor caused another kind of inhibition characterized by a much higher half-effect value (K_H) (see Figure 3 legend).

The different inhibitors had relative K_L values of the same order of magnitude as their relative dissociation constants for P-450. A complementary experiment performed with two different concentrations of 7-EOC and dimethylaniline as inhibitor confirmed that inhibition observed at low inhibitor

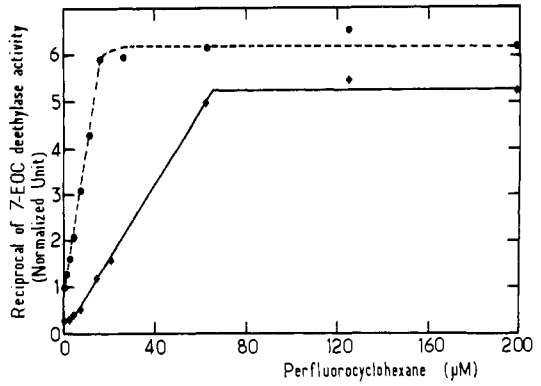


FIGURE 5: Inhibition of 7-EOC deethylase activity by perfluorocyclohexane. All conditions were identical with those described in the legend to Figure 3. (---) and (—) have the same meaning as in Figure 4.

Table I: Effect of Cytochrome b_5 on the Rate of 7-EOC Deethylation in the Presence of Various Other Substrates[a]

inhibitor	b_5 present[b]	% inhibition in first mode[c]	K_L (μM)[d]	K_H (μM)[d]
benzphetamine	−	38	10	320
	+	100	8	
cyclohexane	−	62	90	10000
	+	100	20	
demethylbenzphetamine	−	62	20	420
	+	100	20	
N,*N*-dimethylaniline	−	77	200	>15000
	+	100	100	
perfluorocyclohexane	−	83	3	>10000
	+	95	3–6	>10000

[a] 7-EOC deethylase activity considered was the absolute activity when b_5 was omitted and the b_5-dependent increase in the activity when b_5 was present. [b] "+" means present at a 4.5:1 b_5 to P-450 molar ratio. [c] Estimated by linear extrapolation to a null inhibitor concentration of the second mode of inhibition in a plot like Figures 3–5. [d] Increase in the inhibitory concentration that caused a 2-fold decrease in the rate of 7-EOC deethylation, using a linear fit of the first mode (K_L) or the second mode (K_H) of inhibition as in footnote *c*.

concentration seemed to result from the competition of both substrates for the same binding site (competitive like) or for two tightly interacting sites.

The second mode of inhibition that occurred at high inhibitor concentration seemed to result from a different mechanism. In particular, pseudosubstrates like perfluorinated cyclohexane completely lack this kind of inhibition (Figure 5) although they have some of the best affinities. Addition of b_5 to the incubation mixture caused a dramatic change in the pattern. The plot of the reciprocal of the absolute deethylation rate versus the inhibitor concentration was still not linear, but a nearly linear relationship can be obtained by plotting the reciprocal of the b_5-dependent increase in the 7-EOC deethylation rate versus the inhibitor concentration (Figures 3–5). Half-effect concentrations of inhibitor calculated from these plots were of the same order of magnitude as the K_L constants obtained in the absence of b_5.

K_H and K_L values determined for various inhibitors are summarized in Table I. In the absence of b_5 the fraction of activity inhibited in the low inhibitor concentration range was widely dependent on the inhibitor considered. Some correlation seems to exist between the ability of the inhibitor to be metabolized by P-450 LM_2 and the fact that only a little part of the activity was influenced along the first range of inhibition (for example, compare benzphetamine to demethylbenzphetamine and cyclohexane to perfluorocyclohexane). The ratio between K_H and K_L for a given inhibitor is very dependent

Table II: Effect of b_5 and 7-Ethoxycoumarin on the Stoichiometry of P-450-Catalyzed Oxygen Reduction[a]

substrate added (μM)	b_5 present[b]	NADPH oxidized [nmol/(nmol of P-450·min)][c]	product formed from substrate [nmol/(nmol of P-450·min)][c]	H_2O_2 formed [nmol/(nmol of P-450·min)][c]	total H_2O formed [nmol/(nmol of P-450·min)][d]	partition ratio k_1[e]	partition ratio k_2[f]
0	−	8.1	0	3.9	4.2	0.27	∞
0	+	8.1	0	3.3	4.8	0.36	∞
50	−	10.1	0.64	5.1	5.0	0.28	3.4
50	+	10.1	5.6	4.4	5.7	0.67	0.01
200	−	15.3	1.71	8.3	7.0	0.26	1.55
200	+	15.3	10.3	4.4	10.9	1.20	0.03

[a] NADPH oxidation, 7-ethoxycoumarin deethylation, and hydrogen peroxide formation were determined in duplicate during separate incubations. The same premixed solution of cytochrome P-450, cytochrome P-450 reductase, and dilauroylphosphatidylcholine was used the same day for the three kinds of determinations. Other conditions are described under Experimental Procedures. [b] b_5 to P-450 LM_2 molar ratio: (−) 0; (+) 4/1. [c] See Experimental Procedures. [d] Calculated from electron balance as $e = b - d$ (see text). [e] Ratio for the partition of the ferrous dioxygen complex $Fe^{II}O_2$ between branch A and branch B in Scheme I. If the contribution of step 6 is neglected, k_1 = (flow-through, branch A)/(flow-through, branch B) = $[c + (e - c)/2]/2d = (c + b - d)/4d$. [f] Ratio for the partition of the "active oxygen" Fe=O between reduction to water only and product formation: $k_2 = [(e - c)/2]/c = (b - d - c)/2c$. b = rate of NADPH oxidation; e = rate of water formation; c = rate of product formation; d = rate of H_2O_2 formation.

on the inhibitor considered. The lowest ratio (32) was obtained with the best substrate (benzphetamine) while a value of at least 3000 was obtained with a pseudosubstrate (Table I). These observations suggest (i) that inhibition of 7-EOC deethylase activity by other substrates can be described as a partially competitive inhibition only at low inhibitor concentration, (ii) that the presence of b_5 dramatically changed the inhibition pattern, and (iii) that the inhibition pattern seemed dependent on the ability of the inhibitor to be metabolized, suggesting that inhibition resulted from a mechanism much more complex than a simple competition for binding at the active site.

Role of b_5 as a Modulator for Hydrogen Peroxide Formation. Hydrogen peroxide was always found as a byproduct of P-450 turnover in a reconstituted system (Nordblom & Coon, 1977; Gorsky et al., 1984; Morgan et al., 1982). Autoxidation of the P-450 reductase can only account for a little part of the rate of hydrogen peroxide formation. In the absence of b_5 and substrates, we found that the rate of hydrogen peroxide formation, in a system omitting the P-450 LM_2 or including a P-450 LM_2 inactivated by the suicide substrate *N*-hydroxy-*d*-amphetamine, was only 16% of the rate observed with the complete system.

The influence of b_5 and substrates on the hydrogen peroxide production by P-450 LM_2 in a reconstituted system is illustrated in Figure 6. In the absence of substrate, the addition of b_5 caused a slight decrease of the rate of hydrogen peroxide formation. No decrease occurred in the rate of NADPH oxidation, suggesting some shift from a $2e^-$ toward a $4e^-$ reduction of the dioxygen molecule (Gorsky et al., 1984; Staudt et al., 1974; Zhukov & Archakov, 1982). In the absence of b_5, the addition of a good substrate (Eoc, Bpt, Ch) caused an increase in hydrogen peroxide production while addition of a poor substrate (Pfch, Npt) caused no increase or decreased the rate of hydrogen peroxide formation. The comparison between a good and a poor substrate (Ch and Pfch, Bpt and Npt) having related structures is particularly striking. On the contrary, in the presence of b_5 good and bad substrates had similar and limited effects on the rate of hydrogen peroxide formation. This rate remained unchanged or decreased below the reference level (no substrate, no b_5).

Effect of b_5 on Relative Stoichiometries of Product, Water, and Hydrogen Peroxide Formation. Simultaneous measurements of the rates of NADPH oxidation, 7-EOC deethylation, and hydrogen peroxide formation were performed for various substrates and b_5 concentrations. By use of these data, total water formation was calculated from the overall electron balance, assuming that the sole stable products formed during

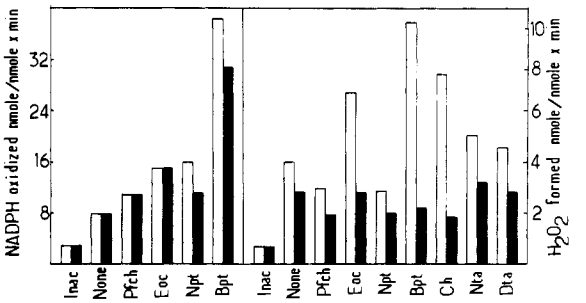


FIGURE 6: Effects of cytochrome b_5 on rates of NADPH oxidation and hydrogen peroxide formation in the presence of various effectors. Experiments were performed at 30 °C at a P-450 to reductase ratio of 1:1; the b_5 to P-450 ratio was 4:1 in standard conditions (see Figure 3 legend) except that glycerol was omitted. Filled bars, b_5 added; open bars, no b_5. Abbreviations: None, no substrate; Inac, P-450 fully inactivated by pretreatment with *N*-hydroxy-*d*-amphetamine; Pfch, 50 μM perfluorocyclohexane; Eoc, 200 μM 7-ethoxycoumarin; Npt, 200 μM demethylbenzphetamine; Bpt, 200 μM benzphetamine; Ch, 150 μM cyclohexane; Nta, 250 μM *p*-nitroanisole; Dta, 250 μM trideuterio-*p*-nitroanisole.

the reaction were water, hydrogen peroxide, oxidized substrate, and $NADP^+$. The calculation is valid both in the presence and in the absence of b_5 because NADPH was the primary electron source for both P-450 and b_5 reduction in the reconstituted system. Substrate contribution did not appear in the final electron balance because the formal reaction RH + $^1/_2O_2 \rightarrow$ ROH is electron balanced by itself.

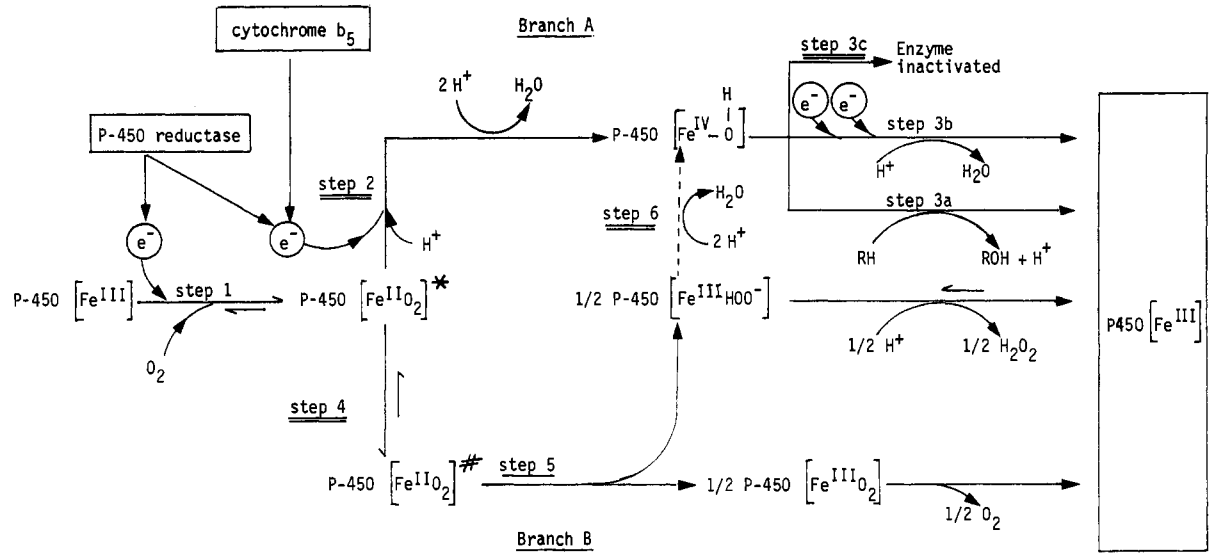
Thus the final fully balanced reaction can only be written as

$$b\mathrm{H}^+ + b\mathrm{NADPH} + c\mathrm{RH} + [(b + d + c)/2]\mathrm{O}_2 \rightarrow b\mathrm{NADP}^+ + c\mathrm{ROH} + d\mathrm{H}_2\mathrm{O}_2 + (b - d)\mathrm{H}_2\mathrm{O}$$

This formula clearly demonstrates that, independently of the P-450 mechanism, the total rate of water formation is fully determined when both the rate of NADPH oxidation and the rate of hydrogen peroxide formation are known. The formula contains three independent parameters if we do not take account of the energy balance. The results of experimental determinations are summarized in Table II, which also contains values for two partition ratios (k_1 and k_2) calculated by assuming the hypothetical Scheme I. Ratio k_1 described the postulated partition of the ferrous dioxygen intermediate between product or water formation in one way and hydrogen peroxide release in the other. It was calculated by assuming that water was formed only on the productive branch A and that hydrogen peroxide formation was restricted to branch B.

With b_5 omitted, the calculated ratio k_1 was found to keep a constant value close to 0.27, independent of the substrate

Scheme I: Kinetic Scheme for P-450 LM_2[a]



[a] P-450 $[Fe^{II}O_2]^*$ and $[Fe^{II}O_2]^\#$ represent two conformational states of the ferrous dioxygen complex that differ in their reactivity toward b_5.

concentration. Addition of b_5 did not modify the rate of NADPH oxidation but caused a clear change in product stoichiometries. In particular, the rate of hydrogen peroxide formation in these conditions was nearly independent of substrate concentration. As a consequence, the partition ratio between the rate of productive and hydrogen peroxide forming mechanisms (ratio k_1) increases up to 4 times when substrate concentrations increase from low values to a saturating level.

The second partition ratio k_2, which measured the postulated partition of the species carrying the "active oxygen" between deactivation by full reduction to water and a substrate hydroxylating pathway, also appeared to be dependent on the presence of both b_5 and substrate. In the absence of b_5 this ratio decreased with increasing substrate concentrations but always kept relatively high values. At saturating level of substrate the calculation suggested that only 25% of the active oxygen was used for substrate oxidation and that 75% of this species was still reduced to water. On the contrary, in the presence of b_5, the ratio k_2 decreased from large values to zero when substrate was added even at a nonsaturating concentration (Table II). This means that when b_5 was present, reduction of the active oxygen to water was completely turned off by the presence of substrates and that most of the active oxygen carrying species was used for substrate oxidation.

Discussion

Many papers have described an important but complex role for b_5 in P-450-dependent monooxygenase activity (Lu et al., 1974; Imai & Sato, 1977; Schenkman et al., 1976; Chiang, 1981; Bösterling et al., 1982; Vatsis et al., 1982; Sugiyama & al., 1980; Kuwahara & Omura, 1980; Canova-Davis & Waskell, 1984). The effects of b_5 can be either a stimulation or an inhibition depending on the substrate and on the isoenzyme considered. The electron-transfer properties of b_5 play an important role in its effects (Morgan & Coon, 1984; Gorsky & Coon, 1986). A mechanistic interpretation based on rapid kinetic data has been proposed (Pompon & Coon, 1984) that involved a branch point at the level of the ferrous dioxygen complex between a conformational and/or electronic rearrangement and a reduction by cytochrome b_5. The rapid kinetic approach gave a good description of the kinetic parameters at the level of this branch point (Pompon & Coon, 1984; Coon et al., 1985), but it did not show how these two pathways are related to the substrate oxidation step. Recent work by Gorsky and Coon (1986) clearly verifies a main prediction of the model deduced from rapid kinetic experiments. They found that, during demethylation of dimethylaniline by P-450 LM_2, addition of b_5 depressed the formation of hydrogen peroxide and increased the amount of product formation in a 1 to 1 molar ratio. A similar effect was observed in our experiment with 7-EOC. Nevertheless, the ratio was found to be significantly lower than 1:1 and dependent on the substrate concentration. The observation that a transient overproduction of hydrogen peroxide and underproduction of product immediately followed the addition of NADPH led us to measure the steady-state rate of NADPH oxidation and H_2O_2 and product formation instead of average values determined by an end-point method in a previous paper (Gorsky & Coon, 1986).

Which Steady-State Model for P-450 LM_2? In order to be compatible with the results, a model must satisfy the following conditions: (i) Hydrogen peroxide formation and substrate oxidation belong to parallel pathways and do not occur directly from a common immediate precursor. The presence of substrates enhanced both NADPH oxidation and hydrogen peroxide formation; the presence of a pseudosubstrate (not metabolized) enhanced NADPH oxidation but not hydrogen peroxide formation. (ii) Water formation by $4e^-$ reduction of dioxygen and substrate hydroxylation must occur as competitive pathways for the degradation of a common oxygenated species. (iii) The addition of b_5 increased the V_{max} but decreased the K_M for substrate oxidation and decreased the dependence of the rate of hydrogen peroxide formation on the concentration of substrate. (iv) Two simultaneously present substrates interacted both in partially competitive ways and in noncompetitive ways in the absence of b_5. The b_5-dependent increase in the activity is inhibited only in a competitive way.

Scheme I is a reasonable working model, taking into consideration the preceding rules and available literature data. The model includes two branch points. The first stands at the level of the ferrous dioxygen species (P-450 $[Fe^{II}O_2]$). This first branch point has been suggested by rapid kinetic studies on electron transfer between cytochrome b_5 and the P-450 ferrous dioxygen complex as well as by steady-state data on the balance of product and hydrogen peroxide formation [this

study and Gorsky and Coon (1986)]. In branch A, step 2 represents the one-electron reduction of the ferrous dioxygen complex by b_5 or, in its absence, by the P-450 reductase. Step 4 in branch B represents an internal electronic rearrangement inside the ferrous dioxygen complex documented in a previous paper, in the case of P-450 LM_2 and L_{M4} (Pompon & Coon, 1984). Step 5 (branch B) is less documented and represents the decomposition of the rearranged ferrous dioxygen complex into oxidized P-450 and hydrogen peroxide. This process may be roughly described as a bimolecular dismutation reaction between two molecules of this complex. Rapid kinetic data (Oprian et al., 1983; Pompon & Coon, 1984) suggested that the overall rate of step 5 is 1–2 orders of magnitude slower that the rate of step 4. Step 5 thus represents the rate-limiting step for the recycling of the enzyme fraction that enters branch B. Step 6 is hypothetical and represents a possible alternative to hydrogen peroxide dissociation. This step was introduced in order to account for the hydrogen peroxide supported substrate hydroxylation (Mc Carthy & White, 1983; Estabrook et al. 1984). Formation of the species carrying the active oxygen (represented as P-450 [$Fe^{IV}OH$]) takes place mainly through the nearly irreversible step 2 (Harada et al., 1984). We assume that this species can decay in only three ways, namely, substrate oxidation (step 3a), reduction to water (step 3b), and suicide (enzyme inactivation step 3c).

Interpretation of Stoichiometries. Following Scheme I, hydrogen peroxide formation and product plus water formation occur in two independent pathways. The observation of a large substrate dependence of the rate of NADPH oxidation suggests that substrates increase the overall flow through the common branch AB, both with and without b_5. The rate of step 2 (branch A) is limiting in the absence of b_5; consequently, the predictable effect of substrate addition is an increase in the steady-state concentration of the ferrous dioxygen complex and in turn in the rate of hydrogen peroxide formation (branch B). These effects were observed.

Consideration of results in Table II suggests that the partition ratio k_1 between branch A and B is independent of the substrate concentration in the absence of b_5 but markedly dependent when b_5 was present. A suitable interpretation can be found by considering the work of Tamburin and Gibson (1983). They described a cooperative binding of substrate and b_5 due to the higher affinity of both species for the high-spin form of P-450. Under these conditions the substrate dependence of the partition factor k_1 can be interpreted as a substrate dependence of the b_5 binding affinity that caused in turn an indirect substrate dependence for the rate constant of step 2. This may offer a substrate-dependent way to regulate the rate of formation of the active oxygen species. The rate of step 4 seemed to be independent of the presence of b_5 (Pompon & Coon, 1984), so addition of b_5 will increase the partition ratio k_1. The predictable and observed effect will be a decrease in the rate of hydrogen peroxide formation and a balanced concomitant increase in the rate of product formation (this work; Sugiyama et al., 1980; Gorsky & Coon, 1986).

Pathway A of Scheme I contains a second branch point at the level of the active oxygen species. Ratio k_2 which describes the flow ratio between substrate oxidation and water formation was also found to be dependent on b_5 and substrate. The water-forming step 3b was turned on in the absence of an easily metabolized substrate. Electrons for this step may be given by ferrocytochrome b_5 (Staudt et al., 1974) or by default by the reductase (Gorsky et al., 1984). The fact that steps 2 and 3b are both dependent on an electron transfer from b_5 may contribute to avoid a large accumulation of the active oxygen species when electron availability is limited (for example, no b_5).

It was experimentally observed (dependence of the partition ratio k_2 on the substrate concentration) that the presence of a good substrate very efficiently competed with the reduction of the active oxygen species to water. Consequently, the best coupling between NADPH oxidation and substrate oxidation was observed when both b_5 and substrate were in saturating amounts. Roles of b_5 may thus be (i) to shift the ratio k_1 toward the productive branch A, into a substrate-dependent manner, and (ii) to coregulate the rate of steps 3b and 2 in order to avoid any accumulation of active oxygen species. Such accumulation may cause a rapid enzyme inactivation during organic hydroperoxide supported mechanisms as observed by Blake and Coon (1980).

Interpretation of Competition between Substrates. As documented, increasing but low concentrations of a pseudosubstrate caused a partially competitive inhibition of 7-EOC deethylase activity both in the presence or in the absence of b_5. The use of a true substrate as inhibitor was expected to produce a similar pattern if competitive-like inhibition fully accounts for the effect. A bimodal inhibition was in fact observed in the absence of b_5. A possible explanation is the occurrence of two independent oxidation mechanisms. Examination of Scheme I and of a paper of Estabrook et al. (1984) suggests that a true P-450 pathway (branch A) and a peroxidase-like pathway (branch B + step 6) may both contribute to the overall oxidation rate with a relative proportion depending on the substrate and/or inhibitor present.

Let us assume that the fraction of the 7-EOC deethylase activity inhibed at low inhibitor concentration was associated to the contribution of branch A and the fraction inhibited at higher concentration was associated to the contribution of branch B. We found that under the conditions used the addition of b_5 did not change or decrease the flow through branch B but markedly increased the flow through branch A. Thus only branch A is expected to contribute to the b_5-dependent rate of oxidation, and this increase was found to be effectively inhibited in a monophasic way. On the contrary, both branches are expected to contribute to the activity observed without b_5, and experimentally, two inhibition constants were found. Estabrook et al. demonstrated the absence of a correlation between the dioxygen-supported and the hydrogen peroxide supported rate of hydroxylation. Taking in account these results, true substrates are expected to inhibit branch A and branch B with a relative efficiency dependent on their structure and their ability to be metabolized through branch A or B. This may offer an explanation for the dependence of the inhibition pattern on the kind of inhibitor used.

Most of the steps in Scheme I can probably be decomposed into several microscopic steps (binding, electron transfer, rearrangement), so the scheme certainly neglects several interactions between different species. Nevertheless, Scheme I represents a reasonable framework for the interpretation of the role of b_5 and for the effects of factors controlling the coupling between NADPH oxidation and substrate hydroxylation. Probably the most critical question to solve is to know if a unique active oxygen species belongs to the so-called O_2-dependent mechanism (branch A) and to the hydrogen peroxide dependent mechanism (branch B + step 6). The apparent absence of correlation between the rate of substrate hydroxylation by the two pathways may be due to differential substrate effects on different limiting steps for branch A, branch B, and step 6, and not to the existence of distinct forms of active oxygen (Estabrook et al., 1984).

ACKNOWLEDGMENTS

Most of the results presented in this paper were obtained during a stay in Dr. M. J. Coon's laboratory (University of Michigan, Ann Arbor, MI). I thank Dr. M. J. Coon and Dr. L. D. Gorsky for discussions throughout the course of this work and Dr. M. Tegoni, Dr. F. Labeyrie, and Dr. C. Herbert for suggestions about the manuscript.

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