Kinetics of Substrate Reaction in the Course of Hydroperoxide-Mediated Inactivation of Cytochrome P450 1A1[†]

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ABSTRACT: A schematic kinetic model is proposed for the hydroperoxide-mediated substrate reaction and cytochrome P450 inactivation. From the model, the relationships between the product concentration at infinite time (P_∞), the apparent rate constant of P450 inactivation (A), and the substrate concentrations are predicted, and the predictions were experimentally examined. The reciprocal of P_∞ is proportional to the reciprocal of the substrate concentration in both CuOOH- and H_2O_2-supported substrate reactions. The reciprocal of P_∞ is proportional to cumene hydroperoxide (CuOOH) concentration, but P_∞ is independent of H_2O_2 concentration, indicating different effects of CuOOH and H_2O_2 in P450 inactivation. The apparent rate constant (A) is proportional to the reciprocal of the substrate concentrations, suggesting substrate protection of P450 from hydroperoxide inactivation. The model also suggests that a second CuOOH molecule may compete with substrate for binding to the P450 substrate binding site. Simulated kinetics of production formation vs time are quite consistent with the experimental kinetics.

Cytochromes P450 comprise a family of hemoproteins which catalyze monooxidation of exogenous chemicals as well as endogenous compounds. During the monooxidation reaction, two electrons are transferred from NADPH to the P450[1] via P450 reductase (Ballou et al., 1974; Guengerich et al., 1975) and molecular oxygen is bound to the cytochrome P450 to form a oxyferro complex (Estabrook et al., 1971). Electron transfer to the oxygen results in production of an "activated oxygen" which is able to attack the substrate. H_2O_2 and organic hydroperoxides are also able to support substrate monooxidation in the absence of NADPH and P450 reductase (Nordblom et al., 1976; Blake & Coon, 1981a,b; Vaz et al., 1990). Since the hydroperoxide-supported P450 substrate reaction only involves a single protein and two substrate molecules, it provides a valuable and simple model system for investigating the nature of the activated oxygen intermediate involved in cytochrome P450 catalyzed oxidation (White et al., 1980; Blake & Coon, 1980; 1981a,b). Nordblom et al. (1976) proposed a mechanism for P450 interaction with hydroperoxides sharing common features with that of peroxidases. Binding of substrate and two-electron reduction followed by binding of oxygen and protonation generates a ternary peroxide complex. This complex may also be produced by direct interaction of substrate-bound ferric P450 with hydrogen peroxide. Koop and Hollenberg (1980) suggested an ordered Bi Bi mechanism for hydroperoxide-dependent dealkylation reaction catalyzed by rabbit liver microsomal P450. Both mechanisms focus on product formation but not on P450 inactivation, which was later recognized as both very common and important in the hydroperoxide-dependent reactions catalyzed by P450 (Ortiz de Montellano, 1986; Sligar & Murray, 1986; Guengerich, 1991). Prosthetic heme destruction and apocytochrome alkylation by heme fragments of other reactive intermediates are observed in varied situations (Guengerich, 1978; Shaefer et al., 1985; Correia et al., 1987; Davies et al., 1986; Yao et al., 1993). P450s were thought to be sacrificed in a process classified as mechanism-based or "suicide" inactivation. Shimizu et al. (1994) analyzed the kinetics of interactions between hydroperoxides and P450 1A2 by monitoring spectral changes of the Soret peak. Two phases of the reaction were proposed, in which the formation of an intermediate complex of heme iron and molecular oxygen comprised the first phase, and heme degradation or modification constituted the second phase of the spectral changes at 390 and 423 nm. However, the association between the spectral changes and P450 inactivation is somewhat uncertain because substrate binding also results in spectral changes. Thus, analysis of the kinetics of substrate reaction supported by hydroperoxides seems an appropriate mode for further study of P450 inactivation.

In this paper, we analyze the substrate reaction in the course of hydroperoxide-mediated P450 inactivation and propose a kinetic model of substrate reaction of P450 supported by hydroperoxides (Figure 1). This model considers P450 inactivation in addition to the substrate reaction. As proposed in the two earlier models mentioned above (Nordblom et al., 1976; Koop & Hollenberg, 1980), P450 binds to substrate and hydroperoxide and forms a ternary hydroperoxide complex. Protonation and subsequent loss of a molecular water or organic alcohol from this ternary hydroperoxide complex leave an activated oxygen species which rapidly attacks the substrate. On the other hand, hydroperoxide-bound P450 can be inactivated by various mechanisms. Using the model described herein, the relationships between the maximal amounts of product formation

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[1] Abbreviations: A, apparent rate constant of P450 inactivation; CuOOH, cumene hydroperoxide; P450, cytochrome P450; P_∞, product concentration at infinite time.

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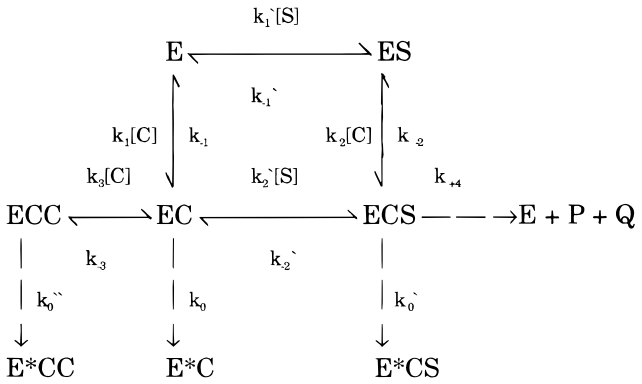

FIGURE 1: Kinetic model of substrate reaction of P450 supported by cumene hydroperoxide. C, CuOOH; E, free P450; S, substrates; P, deethylation product; Q, alcohol product of hydroperoxide.

(P_∞), the apparent rate constant of inactivation (A), the initial velocities, and substrate concentrations were predicted and examined experimentally. All experimental results are consistent with the predictions from the model. The model provides a good basis from which to study cytochrome P450−substrate reactions supported by hydroperoxides.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma and were of analytical grade or better. Cytochrome P450 1A1 and NADPH-cytochrome P450 reductase were purified from β-naphthoflavone and phenobarbital-induced rat liver microsomes, respectively, as described previously (Saito & Strobel, 1981; Dignam & Strobel, 1977). Sodium cholate and the nonionic detergent Renex 690 used in the purification procedure were removed by chromatography on an hydroxylapatite column (Saito & Strobel, 1981).

Enzyme Assays. The fluorimetric assays for deethylation of 7-ethoxycoumarin and 7-ethoxyresorufin were modified from the methods reported by Aito (1978) and Lubet et al. (1985), respectively, and conducted using a Perkin-Elmer LS-5 spectrofluorimeter. For the 7-ethoxycoumarin deethylation assay, P450, 50−100 pmol, was mixed with the indicated concentrations of 7-ethoxycoumarin in 0.1 M potassium phosphate, pH 7.4. After addition of hydroperoxides, the time course of 7-hydroxycoumarin production was followed by monitoring the increase of emission fluorescence at 464 nm with excitation at 390 nm. Deethylation of 7-ethoxyresorufin was carried out in 50 mM Tris-HCl, pH 7.5, containing 25 mM $MgCl_2$. Resorufin production was followed by monitoring the increase of resorufin emission fluorescence at 586 nm with excitation at 522 nm. The presence of lipid did increase activity of P450 1A1 supported by hydroperoxides by 20%−30% but did not change the relative relationship of P_∞, A, and substrate concentrations in our experiments. Thus lipid was omitted for the activity assay to simplify the assay system.

Analytical Methods. Protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin as standard. Reduced CO difference spectra of cytochrome P450 1A1 were recorded in 0.1 M potassium phosphate buffer (pH 7.5) containing 2% glycerol using an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964).

Kinetic Analysis. Substrate reaction in the course of hydroperoxide-mediated P450 inactivation was analyzed according to the method of Tsou (1988) with the modification that hydroperoxides function both as the P450 inhibitors and substrates. To achieve pseudo-first-order conditions for P450 substrate reaction, substrate and oxidant concentrations are used at 1000 times greater than P450 concentration. Oxidant concentration is selected so that substrate concentrations did not change much before P450 is fully inactivated and product concentration is low enough to neglect product inhibition. The concentrations of product at infinite time (P_∞) were determined by monitoring the increase of product emission fluorescence. When the fluorescence remained unchanged for several hours, the concentration of the product (7-hydroxycoumarin or resorufin) was taken as P_∞. P_∞ was also calculated along with the rate constant of P450 inactivation (A) by fitting the data to the equation $P = P_\infty(1 - e^{-At})$, where product concentration (P) was calculated from product emission fluorescence and t was the reaction time.

Numerical Simulation. The kinetic model of hydroperoxide-mediated P450 inactivation and substrate reaction was converted into a set of equations for use with the simulation program HopKINSIM (Wachsstock & Pollard, 1994). The initial concentrations of P450, substrate, and hydroperoxide (legends to Figures 2, 3, and 5) and rate constants and dissociation constants calculated from the experimental data were defined for numerical simulation. The HopKINSIM 1.7.2 program was downloaded from the web site Wuarchive.wustl.edu by following Frieden's directions (1993).

RESULTS AND DISCUSSION

Kinetic Model for CuOOH-Supported Substrate Reaction and P450 Inactivation. Based on the proposed mechanism of Nordblom et al. (1976) and modified from the ordered Bi Bi model of Koop and Hollenberg (1980) for hydroperoxide-supported activity of P450, a schematic kinetic model taking into consideration hydroperoxide-induced P450 inactivation is proposed as illustrated in Figure 1. Implicit in this model is that P450 binds hydroperoxide and substrate and forms a ternary hydroperoxide complex. The scheme further suggests that, due to the hydrophobic cumene group in CuOOH and the hydrophobic characteristic of the substrate binding site, CuOOH may also bind to the substrate binding site.

In this model, we utilize the following definitions.

active enzyme:

$$[E_T] = [E] + [ES] + [EC] + [ECS] + [ECC]$$

inactivated enzyme:

$$[E^*_T] = [E^*C] + [E^*CS] + [E^*CC]$$

total enzyme: $[E_0] = [E_T] + [E^*_T]$

When [S] and [C] $\gg$ $[E_0]$, the rate of P450 inactivation is

$$-d[E_T]/dt = d[E^*_T]/dt = (k_0[EC] + k_0'[ECS] + k_0''[ECC]) \quad (1)$$

It is assumed that the steady state of the substrate reaction is rapidly achieved relative to the inactivation reactions. Consequently, although $[E_T]$ decreases, it does not disturb

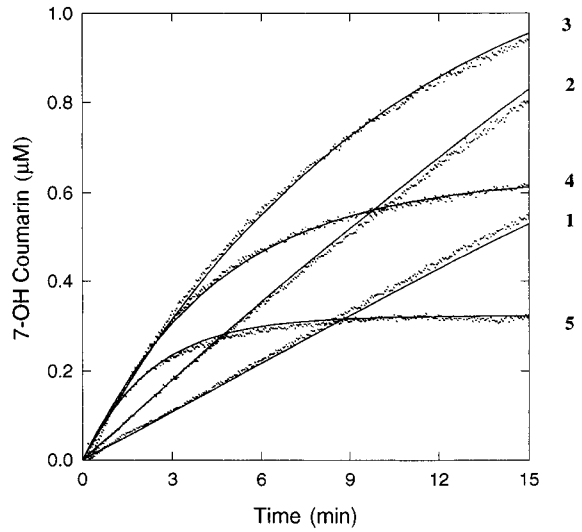

FIGURE 2: Time course of deethylation of 7-ethoxycoumarin. P450, 50 nM, was mixed with 0.2 mM 7-ethoxycoumarin in 0.1 M potassium phosphate buffer, pH 7.4. The increase of 7-hydroxycoumarin concentration was followed and calculated by monitoring the increase of emission fluorescence at 464 nm with excitation at 390 nm after addition of (1) 0.0625, (2) 0.125, (3) 0.5, (4) 1, and (5) 2 mM CuOOH. Dotted traces are the experimental data points, while solid lines are simulation results.

the steady state reached for the substrate reaction, and hence the relative relations among [E], [EC], [ES], [ECC], and [ECS] remain unchanged. The dissociation constants are defined as follows:

$$K_c''[ECC] = [EC][C] \qquad K_c'[EC] = [E][C]$$

$$K_c[ECS] = [ES][C] \qquad K_s'[ES] = [E][S]$$

$$K_S[ECS] = [EC][S]$$

$$K_c'K_s[ECS] = K_s'K_c[ECS] = [E][C][S]$$

These dissociation constants are the ratios of the respective backward and forward rate constants. Assuming the equilibrium is rapidly achieved, the rate of enzyme inactivation can be written as follows:

$$-d[E_T]/dt = A[E_T] \qquad (2)$$

where

$$A = (k_0K_sK_c''[C] + k_0'K_c''[C][S] + k_0''K_s[C]^2)/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2)$$

(See the appendix for a more extensive derivation.)

A is the apparent rate constant of P450 inactivation. When [C] is large enough, $A = k_0''$. Thus, *A* will increase when [C] increases and reach its maximum value at high [C]. Since

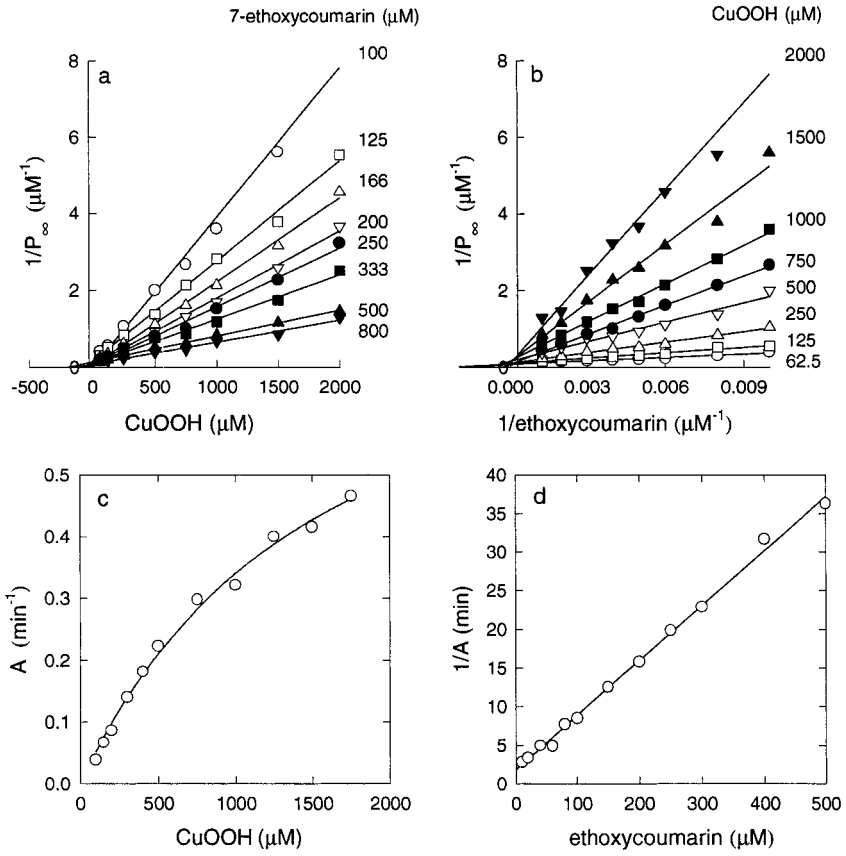

FIGURE 3: Relationship between product 7-hydroxycoumarin concentration at infinite time (P_∞) and CuOOH (a) or 7-ethoxycoumarin (b) concentration and relationship between apparent rate constant of P450 inactivation and CuOOH (c) or 7-ethoxycoumarin (d) concentration. (a,b) Indicated concentrations of CuOOH and 7-ethoxycoumarin were mixed with 50 nM P450 in 0.1 M potassium phosphate buffer, pH 7.4, and incubated at room temperature for 5 h (CuOOH > 0.5 mM) or 24 h (CuOOH < 0.5 mM). (c) P450, 50 nM, was incubated with 0.2 mM 7-ethoxycoumarin in 0.1 M potassium phosphate buffer, pH 7.4, at room temperature. The production of 7-hydroxycoumarin was followed after the indicated concentrations of CuOOH was added. (d) P450, 50 nM, was incubated with the indicated concentrations of 7-ethoxycoumarin in 0.1 M potassium phosphate buffer, pH 7.4, at room temperature. The production of 7-hydroxycoumarin was followed after 0.25 mM CuOOH was added. P_∞ and *A* were determined as described in Materials and Methods.

Table 1: Simulation Parameters for the Model

dissociation constants (μM)		rate constant (min^{-1})	
K_s'	12.9	k_{+4}	9.9
K_s	275	k_0	0.0091
K_c'	29.5	k_0'	0.055
K_c	628	k_0''	1.48
K_c''	2810		

the active oxygen and electron were easily passed to the substrate in the ternary complex ECS, the inactivation of ECS could be much less important than that of ECC, which cannot bind more substrate and has no natural electron acceptor. If this is true, the expression for the apparent inactivation rate constant can be taken approximately as eq 3. The reciprocal of A is expected to be proportional to substrate concentrations, if the inactivation of the ternary hydroperoxide complex ECS is indeed much less important than that of ECC.

$$A = (k_0K_sK_c''[C] + k_0''K_s[C]^2)/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (3)$$

With the boundary condition $t = 0$, $[E_T] = [E_0]$, the expression for $[E_T]$ is

$$[E_T] = [E_0]\, e^{-At}$$

and the velocity for substrate reaction can be expressed as eq 4

$$d[P]/dt = k_{+4}[ECS] = B\, e^{-At} \quad (4)$$

where

$$B = k_{+4}K_c''[C][S][E_0]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2)$$

(See the appendix for a more extensive derivation.)

B is the initial rate of substrate reaction. Rearrangement of the expression for B yields eqs 5 and 6.

$$1/B = (K_sK_c'K_c'' + K_sK_c''[C] + K_s[C]^2)/(k_{+4}K_c''[C][E_0][S]) + (K_c + [C])/(k_{+4}[C][E_0]) \quad (5)$$

$$1/B = (K_sK_c' + K_c[S])/(k_{+4}[S][E_0][C]) + (K_c''K_s + K_c''[S] + K_s[C])/(k_{+4}K_c''[S][E_0]) \quad (6)$$

From eq 5, the double-reciprocal plots of B vs [S] would give straight lines, with a fixed [C]. The relationship between B and [C] is more complicated, as is shown in eq 6. When $K_c''[S] \gg K_s[C]$; however, the reciprocal of B will also be approximately proportional to the reciprocal of [C].

With the boundary conditions $t = 0$, $[P] = 0$, the concentration of product can be expressed as eq 7

$$[P] = B\,(1 - e^{-At})/A \quad (7)$$

$$[P_\infty] = B/A = k_{+4}K_c''[E_0][S]/(k_0K_sK_c'' + k_0'K_c''[S] + k_0''K_s[C]) \quad (8)$$

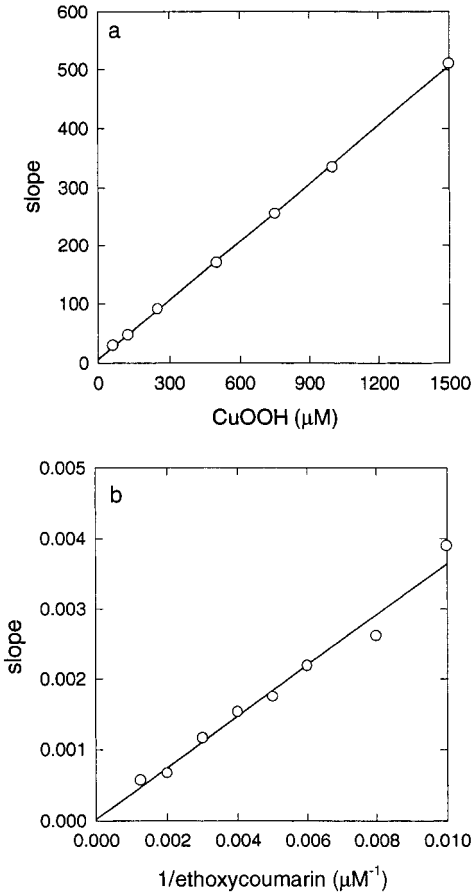

FIGURE 4: Secondary plots of Figure 3a,b. Slope was calculated from Figure 3a,b.

Rearrangement of eq 8 yields eq 9.

$$1/[P_\infty] = (k_0K_s/[S] + k_0')/(k_{+4}[E_0]) + k_0''K_s[C]/(k_{+4}K_c''[E_0][S]) \quad (9)$$

At a fixed [S], a plot of the reciprocal of P_∞ vs [C] should give a straight line. The slope was proportional to the reciprocal of [S] as shown in eq 10.

$$\text{slope} = k_0''K_s/(k_{+4}K_c''[E_0][S]) \quad (10)$$

Equation 8 can also be restated as eq 11.

$$1/[P_\infty] = (k_0K_sK_c'' + k_0''K_s[C])/(k_{+4}K_c''[E_0][S]) + k_0'/(k_{+4}[E_0]) \quad (11)$$

At a set of fixed [C], the double-reciprocal plots of P_∞ vs [S] should give a set of straight lines, with the same value of the intercepts on the Y-axis. The slopes of this set of lines should be proportional to [C] as expressed in eq 12.

$$\text{slope} = (k_0K_sK_c'' + k_0''K_s[C])/(k_{+4}K_c''[E_0]) \quad (12)$$

Time Course of Deethylation of 7-Ethoxycoumarin. The time course of deethylation of 7-ethoxycoumarin supported by CuOOH was followed by monitoring the production of 7-hydroxycoumarin. The concentrations of both CuOOH and 7-ethoxycoumarin were maintained at 1000 times higher than that of P450 so that pseudo-first-order kinetic conditions were maintained. As shown in Figure 2, at low concentrations of CuOOH (<0.1 mM), the initial velocity of 7-hydroxycoumarin production is almost constant in the first 15 min,

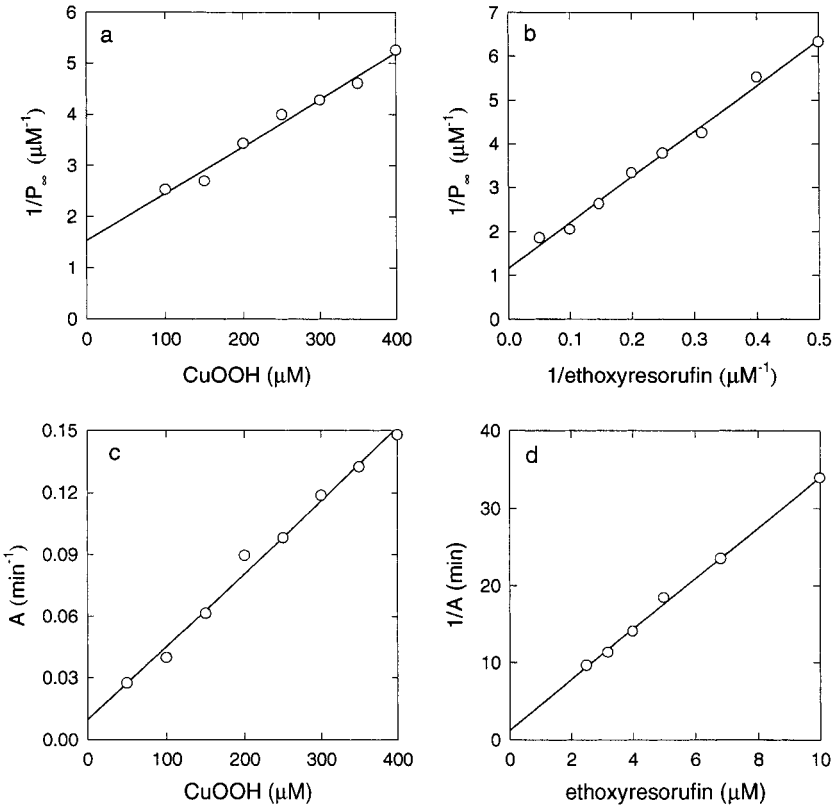

FIGURE 5: Relationship between product resorufin at infinite time (P_∞) and CuOOH (a) or 7-ethoxyresorufin (b) concentrations and relationship between rate constant of P450 inactivation and CuOOH (c) or 7-ethoxyresorufin (d) concentration. Due to the high background fluorescence of 7-ethoxyresorufin, concentrations of CuOOH and 7-ethoxyresorufin are lower than those of CuOOH and 7-ethoxycoumarin used in the experiments to Figure 3. (a,c) P450, 100 nM; 7-ethoxyresorufin, 0.004 mM; (b,d) P450, 100 nM; CuOOH, 0.2 mM. The production of resorufin was followed by monitoring the resorufin fluorescence at 586 nm with excitation at 522 nm. P_∞ and A were calculated by fitting the data to the equation $P = P_\infty (1 - e^{-At})$.

indicating the P450 inactivation was not significant and could be neglected when only the initial velocity is considered. At high concentrations of CuOOH (>1 mM), the initial velocity dropped drastically and the concentration of 7-hydroxycoumarin reached its maximum in 30 min, suggesting that the inactivation of P450 had become an important consideration during this time. The P450 present in the reaction mixture was completely destroyed in 30 min.

Relationship between the P_∞, Apparent Rate Constant of P450 Inactivation (A), and Substrate Concentrations. Using CuOOH and 7-ethoxycoumarin as substrates, the relationships between P_∞, A, and substrate concentrations were determined and are shown graphically in Figure 3. As predicted by eqs 9 and 11, the reciprocal of P_∞ is proportional to concentrations of CuOOH (Figure 3a) and to the reciprocal of 7-ethoxycoumarin concentrations (Figure 3b), respectively. The data of Figure 3b also confirm the prediction that all the straight lines have the same intercept on the Y-axis. The value of the intercept ($k_0'/(k_{+4}[E_0])$) in Figure 3a is about 0.11 μM^{-1} and $k_0'/k_{+4} = 0.0056$ (also see Table 1), suggesting that the rate constant of substrate reaction (k_{+4}) is about 200 times greater than rate constant of ECS inactivation (k_0'). This seems logically consistent since the active oxygen is easily passed to the substrate in the ternary complex ECS. The assumption that the inactivation of the ternary complex ECS is less important than that of EC or ECC was also confirmed by the data of Figure 3d, which show that the reciprocal of A was proportional to 7-ethoxycoumarin concentrations as expected in eq 3. But the inactivation of ECS does exist, because k_0' is not zero. Moreover, substrate-mediated P450 inactivation has been directly observed and reported (Ortiz de Montellano, 1986; Sligar & Murray, 1986; Guengerich, 1991).

The plot of A vs CuOOH concentrations seemed to be hyperbolic (Figure 3c), and A reached a maximum value of 1.5 at 20 mM CuOOH when the concentration of 7-ethoxycoumarin was 0.2 mM (data not shown). A double-reciprocal plot of A against CuOOH concentration, however, did not give a straight line.

Secondary plots of the data for Figure 3a,b give the relationship between the value of the slope and substrate concentrations. As predicted by eq 12, the slopes of the lines in Figure 3b are proportional to the CuOOH concentrations as shown in Figure 4a. The ratio of the slope to the intercept on the Y-axis in the new plot gives the ratio of k_0''/k_0K_c''. This value is 0.058 μM^{-1} as calculated from the data of Figure 4a (also see Table 1), suggesting that the most important inactivation of P450 is the inactivation of complex ECC. The plot of slopes of the lines in Figure 3a vs the reciprocal of 7-ethoxycoumarin concentrations gives a straight line as shown in Figure 4b. As predicted by eqs 10 and 12, the lines in Figure 4a,b should have the same values of slope ($k_0''K_s/(k_{+4}K_c''[E_0])$. These values as calculated from Figure 4a,b are 0.33 and 0.34 μM^{-1}, respectively, so the value of $k_0''K_s/(k_{+4}K_c'')$ is about 0.0165.

These results seem independent of the nature of the primary substrate; when 7-ethoxycoumarin is replaced by 7-ethoxyresorufin, the relationship between P_∞, A, and concentrations of substrates appears the same as those with 7-ethoxycoumarin as shown in Figure 5. The value of the

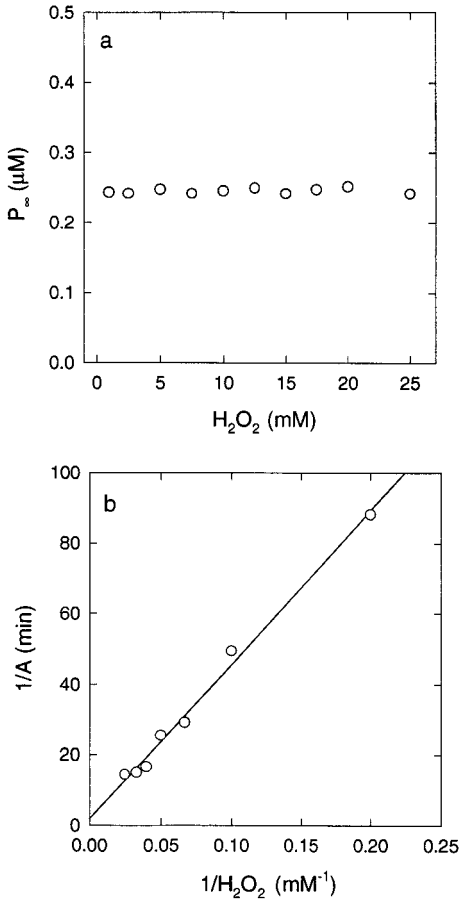

FIGURE 6: Relationship between P_∞, rate constant of P450 inactivation (A), and H_2O_2 concentrations. P450, 50 nM, was mixed with 0.2 mM 7-ethoxycoumarin in 0.1 M potassium phosphate buffer, pH 7.4. 7-Hydroxycoumarin fluorescence at 460 nm was followed after addition of the indicated concentrations of H_2O_2.

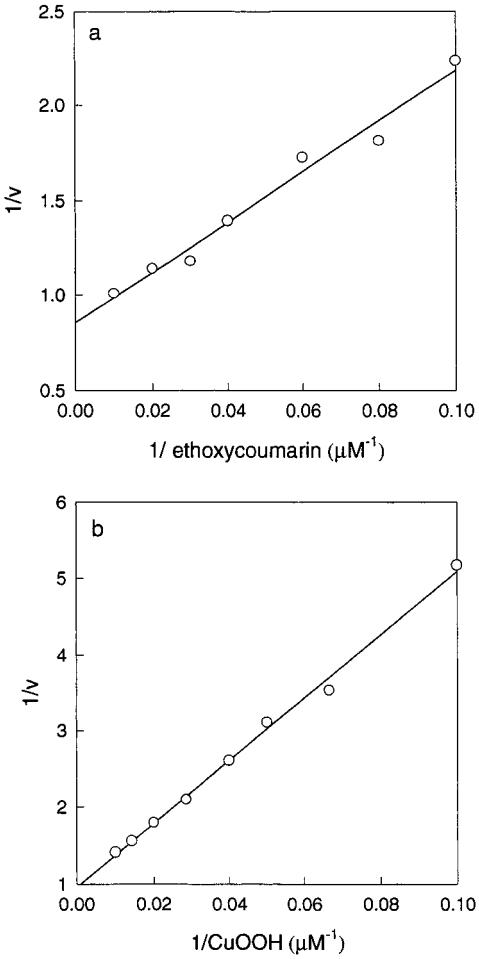

FIGURE 7: Double-reciprocal plots of initial velocity against substrate concentrations with a fixed concentration of 0.05 mM CuOOH (a) or against CuOOH concentrations with a fixed concentration of 0.2 mM 7-ethoxycoumarin (b). P450, 50 nM, was mixed with indicated concentrations of 7-ethoxycoumarin in 0.1 M potassium phosphate buffer. The increase of emission fluorescence of 7-hydroxycoumarin was followed in the first minute after CuOOH was added. Velocity is expressed as nanomoles of 7-hydroxycoumarin liberated per nanomole of P450 1A1 per minute.

intercept on the Y-axis ($k_0'/(k_{+4}[E_0])$) was 1.16 μM^{-1} and $k_0'/k_{+4} = 0.058$, suggesting that the protection of P450 inactivation by 7-ethoxyresorufin is less than that by 7-ethoxycoumarin. To lower the background fluorescence at 586 nm, concentrations of both 7-ethoxyresorufin and CuOOH are lower than those used in the study of deethylation of 7-ethoxycoumarin. CuOOH concentrations used in Figure 5c only cover the concentrations in the linear range of the hyperbolic-like curve in Figure 3c, and A seems to be proportional to CuOOH concentration.

Kinetic Model for H_2O_2-Mediated Deethylation of 7-Ethoxycoumarin and 7-Ethoxyresorufin. Replacing CuOOH with H_2O_2 allows us to test this scheme and its prediction of CuOOH binding to the substrate binding site, since H_2O_2 cannot bind to the substrate binding site, the hydrophobic group of cumene being absent. So k_3 becomes 0, and then the expressions for A and P_∞ can be restated as follows:

$$A = (k_0K_s[C] + k_0'[C][S])/(K_sK_c' + K_s[C] + K_c[S] + [C][S])$$

$$P_\infty = B/A = k_{+4}[E_0][S]/(k_0K_s + k_0'[S])$$

P_∞ has the same relationship to 7-ethoxycoumarin concentrations (data not shown), but P_∞ is independent on H_2O_2 concentrations (Figure 6a). The reciprocal of A is proportional to the reciprocal of H_2O_2 concentrations (Figure 6b). When substrate concentrations are low (<0.05 mM), $k_0K_s \gg k_0'[S]$, the reciprocal of A is proportional to [S]. At high [S] (>0.2 mM), [EC] is small, the inactivation of the ternary complex ECS becomes important compared with that of EC, and the linear relationship of $1/A$ vs [S] is not maintained (data not shown).

Relationships between Initial Velocities and Substrate Concentrations. As shown in Figure 2, when CuOOH concentration was lower than 0.1 mM, the rate constant of inactivation was small enough to be neglected if only the initial velocities were considered. So the velocities were determined in the first minute after the reaction was initiated by the addition of CuOOH to the P450 and substrate solution. When the CuOOH concentration was fixed, a double-reciprocal plot of velocities vs 7-ethoxycoumarin concentrations gave a straight line (Figure 7a) as predicted by eq 5.

When 7-ethoxycoumarin concentrations were fixed and higher than 0.1 mM, the double-reciprocal plot of velocities against CuOOH concentrations also gave a good straight line as shown in Figure 7b. But the good linear relationship between the double reciprocals of velocities and CuOOH concentration could not be maintained when 7-ethoxycoumarin concentration was lower than 0.025 mM. This was expected by the present model but is not consistent with an

ordered Bi Bi mechanism (Koop & Hollenberg, 1980). In the ordered Bi Bi mechanism, hydroperoxide binds to P450 after substrate binding, thus the P_∞ would be expected to be independent of substrate concentration when the steady state condition is maintained. The present modified random mechanism seems to be a more reasonable mechanism for the CuOOH- or H_2O_2-supported deethylation reactions. The ordered Bi Bi model proposal was based on data from liver microsomal P450 (Koop & Hollenberg, 1980). The lipid or other proteins may prevent P450 from the binding of hydroperoxides directly with the consequence that only substrate-bound P450 is accessible to hydroperoxide. The membrane protection of P450 from denaturation by exposure to guanidine hydrochloride or urea was previously discussed (Yu et al., 1995). An alternative explanation is based on the fact that the hydroperoxide used earlier in the proposal for an ordered Bi Bi mechanism was *tert*-butyl hydroperoxide. *tert*-Butyl hydroperoxide has been shown to be much less effective in heme degradation than CuOOH or H_2O_2 (Shimizu et al., 1994) and may have a lower affinity for microsomal P450. The spectral changes of heme destruction by *tert*-butyl hydroperoxide are monophasic, while those by CuOOH and H_2O_2 are biphasic.

The data presented by Nordblom et al. (1976) also showed that a double-reciprocal plot of initial velocity vs CuOOH or H_2O_2 concentrations give a good straight line at a fixed substrate concentration. The concentrations of *N*-methylaniline and benzphetamine used in their study were 6.7 or 1 mM, respectively, which probably also maintained the condition $K_c''[S] \gg K_s[C]$. The reason CuOOH is more effective in substrate catalysis and P450 inactivation than is H_2O_2 is probably that CuOOH contains an hydrophobic cumene group which favors binding to P450.

Comparison of Experimentally Determined and Simulated Kinetics of Product Formation. The rate constants and dissociation constants were calculated from the primary and secondary plots shown in Figures 3−7 and other similar plots not shown. The catalytic parameters for CuOOH-supported deethylation of 7-ethoxycoumarin are shown in Table 1. Comparison of the rate constants of P450 inactivation, k_0, k_0', k_0'', suggested that the most important inactivation occurs in the ternary complex ECC. These parameters and the initial concentrations of P450, 7-ethoxycoumarin, and CuOOH were given for numeric integration using the program HopKINSIM 1.7.2. The simulated kinetics is shown as solid lines in Figure 2, which are in good agreement with the experimental data (dotted traces). The simulated time course of deethylation of 7-ethoxyresorufin are also in good agreement with the experimental kinetics (data not shown).

The facts that H_2O_2 is produced as a by-product of uncoupled reactions (Hanukoglu et al., 1993; Karuzina & Archakov, 1994; Rapoport et al., 1995) and that the presence of the oxygen acceptor can reduce but cannot eliminate H_2O_2 production in some cases strongly suggests that the formation of oxyferro complex is independent of the existence of substrate, thus supporting a random mechanism.

In the present model, EC is proposed as a simple complex of P450 and hydroperoxide. In the reaction supported by CuOOH, the cumene group of CuOOH is probably released from EC before it binds substrate or an additional CuOOH. A common oxyferro complex is believed to exist in the substrate reaction of P450 supported by NADPH and NADPH P450 reductase and in that supported by hydroperoxide (Rahimtula & O'Brien, 1975; Blake & Coon, 1980, 1981a,b). This P450 oxyferro complex can bind and pass the oxygen and electron to the substrate. If our assumption that an additional CuOOH can compete with substrate to bind to this P450 intermediate is true, then we would then expect an inhibition of P450 activity by the CuOOH analogue, 2-phenyl-2-propanol. Determined by difference binding spectra, 2-phenyl-2-propanol shows a high affinity for P450 1A1 with an apparent K_d value of 4.3 μM. The apparent K_d value for 7-ethoxycoumarin is about 16.2 μM as calculated from difference binding spectra. 2-Phenyl-2-propanol was, in fact, found to be a very good inhibitor of P450 activities supported by NADPH P450 reductase or hydroperoxides when the ratio of 2-phenyl-2-propanol concentration to 7-ethoxycoumarin concentration is greater than 0.5 (data not shown). The binding of 2-phenyl-2-propanol to P450 may affect the interaction between P450 and reductase in addition to the competition with substrate. A radiolabeled photosensitive analogue of CuOOH, azidocumene, has also been synthesized to identify the proposed two binding sites of CuOOH in P450. Azidocumene is also a good inhibitor of P450 activity as is 2-phenyl-2-propanol. The inhibitions are not simple competitive or noncompetitive inhibitions, and the mechanism of inhibition and the identification of the CuOOH binding sites in P450 are under further study.

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APPENDIX

From the definition of the dissociation constants in the results section, [EC], [ES], [ECS], and [ECC] can be expressed as follows:

$$[EC] = [E][C]/K_c'$$

$$[ES] = [E][S]/K_s'$$

$$[ECS] = [E][C][S]/(K_cK_s') = [E][C][S]/(K_sK_c')$$

$$[ECC] = [EC][C]/K_c'' = [E][C]^2/(K_c'K_c'')$$

Thus,

$$[E_T] = [E] + [EC] + [ES] + [ECS] + [ECC] = [E](1 + [C]/K_c' + [S]/K_s' + [C][S]/(K_sK_c') + [C]^2/(K_c'K_c'')) = [E](K_sK_c'K_c'' + K_sK_c''[C] + K_sK_c'K_c''[S]/K_s' + K_c''[C][S] + K_s[C]^2)/(K_sK_c'K_c'') \quad (13)$$

When $K_sK_c' = K_s'K_c$ is applied to eq 13, $[E_T]$ can be expressed as

$$[E_T] = [E](K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2)/(K_sK_c'K_c'')$$

[E], [EC], [ES], [ECS], and [ECC] can be expressed as follows:

$$[E] = [E_T]K_sK_c'K_c''/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (14)$$

$$[EC] = [E][C]/K_c' = [E_T]K_sK_c''[C]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (15)$$

$$\begin{aligned}[ES] &= [E][S]/K_s' \\ &= [E_T](K_sK_c'K_c''[S]/K_s')/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \\ &= [E_T]K_cK_c''[S]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (16)\end{aligned}$$

$$\begin{aligned}[ECS] &= [E][C][S]/(K_sK_c') \\ &= [E_T]K_c''[C][S]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (17)\end{aligned}$$

$$\begin{aligned}[ECC] &= [E][C]^2/(K_c'K_c'') \\ &= [E_T]K_s[C]^2/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \quad (18)\end{aligned}$$

Applying eqs 15, 17, and 18 to eq 1 yields eq 19.

$$\begin{aligned}-d[E_T]/dt &= (k_0[EC] + k_0'[ECS] + k_0''[ECC]) \\ &= [E_T](k_0K_sK_c''[C] + k_0'K_c''[C][S] + k_0''K_s[C]^2)/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \\ &= A[E_T] \quad (19)\end{aligned}$$

Thus

$$A = (k_0K_sK_c''[C] + k_0'K_c''[C][S] + k_0''K_s[C]^2)/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2)$$

With the boundary conditions $t = 0$, $[E_T] = [E_0]$, the expression for $[E_T]$ is

$$[E_T] = [E_0]\, e^{-At}$$

The velocity of substrate reaction can be expressed as

$$\begin{aligned}d[P]/dt &= k_{+4}[ECS] \\ &= k_{+4}K_c''[C][S][E_T]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \\ &= k_{+4}K_c''[C][S][E_0]\, e^{-At}/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2) \\ &= B\, e^{-At}\end{aligned}$$

$$B = k_{+4}K_c''[C][S][E_0]/(K_sK_c'K_c'' + K_sK_c''[C] + K_cK_c''[S] + K_c''[C][S] + K_s[C]^2)$$

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