Kinetics of CO Binding to Cytochromes P450 in the Endoplasmic Reticulum

Aditya P. Koley,‡ Richard C. Robinson,‡ Allen Markowitz,§ and Fred K. Friedman*,‡

Laboratory of Molecular Carcinogenesis, National Cancer Institute, and Biomedical Instrumentation and Engineering Program, National Institutes of Health, Bethesda, Maryland 20892

Received October 14, 1993; Revised Manuscript Received December 22, 1993®

ABSTRACT: The kinetics of CO binding to cytochromes P450 in rat liver microsomes were examined using the flash photolysis technique. Modulation of the kinetics by P450 form-specific effectors such as anti-P450 monoclonal antibodies and substrates was used to elucidate the kinetic behavior of individual P450s within the endoplasmic reticulum. The problem of attributing a kinetic parameter to a single P450 in the presence of multiple microsomal P450s was overcome with a difference method that employs the difference of the kinetic profiles obtained in the presence and absence of a P450 effector. Applying this approach to study the conformation/dynamics of P450 2B1 in microsomes from phenobarbital-treated rats revealed that the substrate benzphetamine enhances while testosterone inhibits the rate of CO binding to this P450. Similar experiments with P450 1A1 in microsomes from 3-methylcholanthrene-treated rats showed that the substrate benzo[*a*]pyrene accelerates CO binding. These results show that the access channel between solvent and heme in the P450 interior can be altered in a substrate- and P450-dependent manner to either hinder or facilitate CO diffusion to the heme iron. These results also demonstrate that analytical difference methods may be employed to characterize the conformation of individual P450s in their native membrane environment in the endoplasmic reticulum.

The cytochromes P450 (P450)[1] of the microsomal mixed-function oxidase system metabolize a wide variety of xenobiotics such as drugs and carcinogens, as well as endogenous compounds such as steroids and prostaglandins (Lu & West, 1980; Ortiz de Montellano et al., 1986; Ryan & Levin, 1990). The multiple forms of P450 exhibit unique catalytic activity profiles toward different substrates. Studies of P450–substrate interactions fall into two general categories. First, one can examine a single purified P450 in a reconstituted system in which all components are defined but whose functional characteristics do not necessarily reflect those of the P450 in the endoplasmic reticulum. A second approach is to employ microsomes as the P450 source. Although closer to a native system, data interpretation is difficult owing to the multiplicity of microsomal P450s that potentially metabolize a particular substrate. For some P450s, this difficulty can be overcome by employing P450-specific probes such as monoclonal antibodies (Gelboin & Friedman, 1985; Friedman et al., 1985a; Ryan & Levin, 1990), substrates, and inhibitors (Murray & Reidy, 1990) to gauge the contribution of a given P450 to a microsomal-catalyzed reaction.

During the catalytic cycle, the ferrous form of P450 binds both substrate and an oxygen ligand. In order to clarify the mechanism of catalysis, it is important to elucidate the mechanism of ligand binding to P450, the interaction of substrate with the amino acids in the substrate binding site, and the interaction between ligand and substrate. Mechanistic studies of ligand binding to the P450 heme moiety yield important details about the heme environment and thus offer an approach to evaluating these interactions. The kinetics of CO binding to heme are a particularly useful probe since the heme–CO bond is readily photolyzed and re-formation of this complex can be spectrally monitored. Such studies have provided detailed information on structure/dynamics of myoglobin [see Frauenfelder et al. (1991) and references cited therein]. CO binding kinetics have also been an informative tool for probing the interactions of ligands and substrates with single P450s in reconstituted systems (Gray, 1982; Oertle et al., 1985; Davydov, 1986; Shimizu et al., 1991; Ohta et al., 1992) and expressed in yeast microsomes (Iwase et al., 1991). Although CO binding to rat liver microsomes has also been studied (Debey et al., 1973; Gray, 1978; Miura et al., 1991), the kinetics reflected those of the multiplicity of microsomal P450s and did not provide information on any individual P450.

In this study, we utilized P450 substrates and anti-P450 antibodies as effectors which alter the kinetics of CO binding to specific P450s in microsomes. The complexity of the microsomal system with its numerous differentially reactive P450s necessitated development of an analytic procedure that employed the difference profile of the kinetic progress curves obtained in the presence and absence of the P450 effector. Using this approach, we distinguished the kinetic behavior of individual P450s and evaluated the effect of substrates on CO binding to these P450s in their native membrane environment within the endoplasmic reticulum. Understanding such interactions between ligand, substrate, and P450 is essential for elucidating the mechanism of P450 catalysis.

MATERIALS AND METHODS

For preparation of rat liver microsomes, male Sprague-Dawley rats (6 weeks old) were injected intraperitoneally daily with phenobarbital (PB) (80 mg/kg of body weight for 3 days) to induce P450 2B1, or with 3-methylcholanthrene (MC) (40 mg/kg of body weight for 3 days) to induce P450 1A1. Microsomes were prepared by differential centrifugation and were suspended in 0.25 M sucrose and stored at −80 °C. The protein concentration was determined by the BCA protein

* Address correspondence to this author at the NIH Building 37, Room 3E-24, Bethesda, MD 20892. Telephone: 301-496-6365. FAX: 301-496-8419.

‡ Laboratory of Molecular Carcinogenesis.

§ Biomedical Instrumentation and Engineering Program.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: P450, cytochrome P450; PB, phenobarbital; MC, 3-methylcholanthrene; PB-microsomes, microsomes from PB-treated rats; MC-microsomes, microsomes from MC-treated rats; Bz, benzphetamine; BP benzo[*a*]pyrene; MAb, monoclonal antibody.

0006-2960/94/0433-2484$04.50/0

assay (Pierce) using bovine serum albumin as a standard. The specific P450 contents for microsomes from PB- and MC-treated rats and for untreated rats were 2.4, 1.9, and 0.8 nmol/mg of protein, respectively.

Monoclonal antibodies 4-7-1 to P450 2B1 (Park et al., 1984) and 1-7-1 and 1-36-1 to P450 1A1 (Park et al., 1982) were obtained from Dr. S. S. Park (National Cancer Institute) and purified from ascites fluid (Stanker et al., 1985).

The apparatus and procedures for photodissociation of the P450–CO complex and monitoring of CO recombination were essentially as previously described (Markowitz et al., 1992). A microsomal suspension at 0.33 mg/mL was prepared under a nitrogen atmosphere using a 0.1 M sodium phosphate (pH 7.5)/20% glycerol (w/v) solution which had been deoxygenated by purging with nitrogen. A small volume of a saturated CO stock solution with a concentration of 0.93 mM (Miura et al., 1991) was added to yield the desired CO concentration. Excess (>20-fold) CO over P450 was used to ensure pseudo-first-order kinetics. Approximately 1 mg of sodium dithionite was then added to reduce the ferric heme to the ferrous state. When a substrate or MAb was present, these were preincubated with microsomes for 20 min at 23 °C before addition of CO and dithionite. When both MAb and substrate were present, microsomes were incubated with MAb for 20 min followed by incubation with substrate for 20 min. The final and stock concentrations of the substrates were 1 mM Bz (from a 0.1 M solution in water), 10 μM BP (from a 5 mM solution in DMSO), and 300 μM testosterone (from a 20 mM solution in methanol). The ratio of MAb to microsomal protein (w/w) was 1:4 as preliminary experiments showed that higher amounts of MAb had no further effect on the kinetics. To initiate the CO binding reaction, the microsomal sample was photolyzed at 532 nm with a laser flash, and absorbance changes due to formation of the P450–CO complex were measured at 450 nm. The sample temperature was maintained at 23 °C. In all experiments, reproducible progress curves were obtained upon at least five successive photolyses, and the final absorbances reverted to their initial values. Data from 3–5 photolyses were averaged to maximize the signal: noise ratio prior to data analysis.

Kinetic data were analzyed using the multiexponential model

$$\Delta A_t = \sum_{i=1}^{n} a_i e^{-k_i t} \qquad (1)$$

were ΔA_t is the total absorbance change observed at time t, a_i is the absorbance change for component i at $t = 0$, k_i is the observed pseudo-first-order rate constant for component i, and n is the number of independent components. Least-squares analysis was performed with RS/1 software (BBN Software Products, Cambridge, MA) on a Dell 450/ME microcomputer.

RESULTS AND DISCUSSION

The time course of CO binding to PB-microsomes at different CO concentrations is shown in Figure 1. The data at each CO concentration were well represented by three exponents (Figure 2 and Table 1) and did not fit a double-exponential model. The CO binding data thus demonstrate that PB-microsomes contain three kinetically distinguishable components. The rate constants varied linearly with CO concentration (Figure 2), indicating that each component corresponds to a bimolecular reaction. Although the data may most simply be interpreted in terms of three independently reacting P450s, microsomes contain a larger number of P450s,

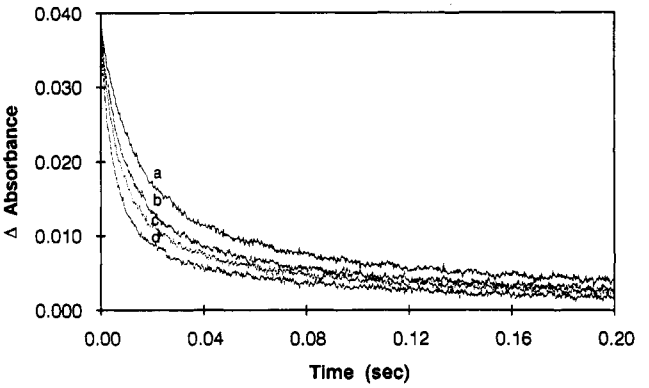

FIGURE 1: Binding of CO to PB-microsomes following photodissociation of the microsomal P450-CO complex. Absorbance changes following flash photolysis of the P450–CO complex were followed at 450 nm at CO concentrations of (a) 10, (b) 15, (c) 20, and (d) 25 μM. Microsomal concentration, 0.33 mg/mL in 0.1 M sodium phosphate buffer (pH 7.5) containing 20% (w/v) glycerol; temperature, 23 °C.

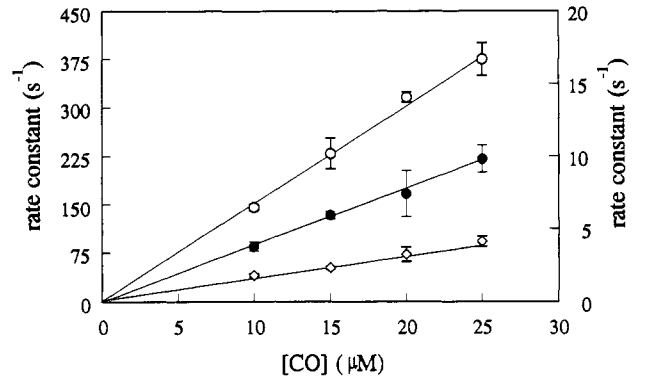

FIGURE 2: Pseudo-first-order rate constants for CO binding to P450s in PB-microsomes. The constants were obtained from least-squares multiexponential fits of the absorbance–time data at each CO concentration and correspond to k_1 (●) (right ordinate) and k_2 (◇) and k_3 (○) (left ordinate). Each point represents the average of at least four experiments.

and each of the three components must thus be comprised of one or more P450s which cannot be kinetically distinguished from one another.

In order to kinetically distinguish individual microsomal P450s, we next sought to evaluate the influence of specific P450 effectors on CO binding kinetics. Anti-P450 MAbs are an excellent effector for such studies and have proven useful for characterizing individual P450s in the presence of multiple microsomal P450s employing immunoassay (Song et al., 1984; Cheng et al., 1984; Robinson et al., 1989; Lorr et al., 1989), inhibition of P450-catalyzed activity (Fujino et al., 1984; Friedman et al., 1985b; Pelkonen et al., 1986; Pasanen et al., 1987), and substrate binding assays (Omata & Friedman, 1992a; Tsokos et al., 1992). Another class of effectors are P450 substrates which selectively bind certain forms of P450. Our subsequent work thus assessed the effect of MAbs and substrates with known P450 specificities on microsomal CO binding kinetics. Using this strategy, changes in the kinetics of a microsomal mixture of P450s were then attributed to the effector-specific P450.

We first evaluated the effect of MAb 4-7-1 to P450 2B1 (Park et al., 1984) on the kinetics of CO binding to PB-microsomes. The MAb clearly accelerated the overall rate (Figure 3A). Triexponential fits to these data (Table 1) reveal that the MAb altered not only the rate constants but also the absorbance amplitudes of the kinetically distinguishable components. The change in absorbances indicates that

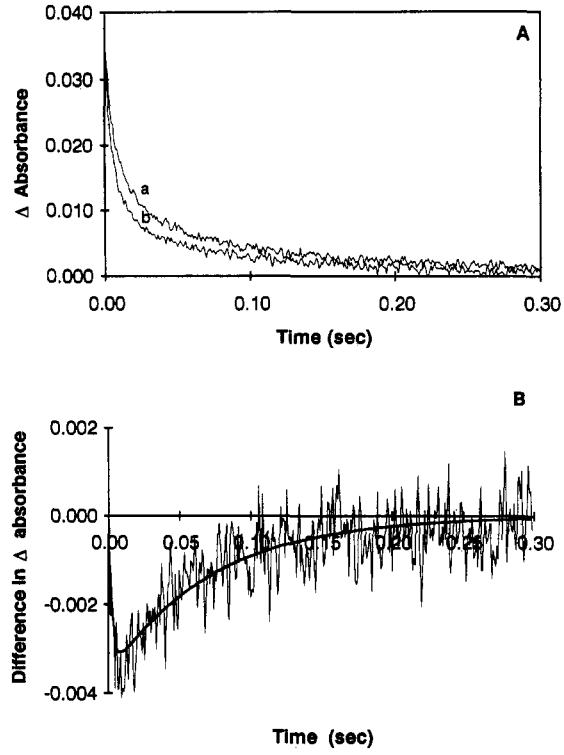

FIGURE 3: Effect of MAb 4-7-1 on the binding of CO to P450s in PB-microsomes. (A) Progress curves in the (a) absence and (b) presence of MAb. The CO concentration was 20 μM; MAb concentration was 0.08 mg/mL; remaining conditions and concentrations are the same as in Figure 1. (B) Difference between the traces in (A) and the best fit according to eq 5.

Table 1: Kinetic Parameters for CO Recombination with PB-Microsomes Obtained by Multiexponential Analysis: Effect of MAb 4-7-1 and Bz[a]

addition	a_1	k_1 (s^{-1})	a_2	k_2 (s^{-1})	a_3	k_3 (s^{-1})
none	0.0048	8.2	0.0155	69.3	0.0147	303
	(0.0007)[b]	(1.0)	(0.0009)	(7.2)	(0.0018)	(22)
MAb	0.0062*	7.8	0.0073*	54.2*	0.0215	192*
	(0.0004)	(1.2)	(0.0015)	(5.1)	(0.0053)	(20)
Bz	0.0072	18.8	0.0238	98.3	0.0040	235
	(0.0009)	(1.9)	(0.0040)	(10.2)	(0.0006)	(18)
MAb+Bz	0.0100*	19.1	0.0156*	72.5*	0.0089*	197*
	(0.0009)	(2.4)	(0.0019)	(9.9)	(0.0014)	(15)

[a] Measurements were performed at 23 °C with 20 μM CO. Pseudo-first-order rate constants are presented, and are the average of three experiments. Significant differences ($P < 0.05$) between the parameters for none and MAb, and for Bz and MAb+Bz, are denoted by an asterisk. [b] Standard deviations are given in parentheses.

the least-squares fitting procedure yielded components whose P450 compositions differ in the presence and absence of MAb. Such redistribution of P450 forms among several kinetic components is a general problem which prevents standard multiexponential analysis from yielding kinetic information on individual P450s. We thus cannot use this approach to derive kinetic parameters for P450 2B1 or associate a single component with a particular P450.

The origin of this problem is that microsomes contain a finite yet unknown number of P450s whose concentrations are also unknown. The number of reactive P450 species is even greater when one considers that a single P450 form may be microheterogeneous and exhibit multiexponential CO binding kinetics (Gray, 1982; Oertle et al., 1985; Davydov et al., 1986). The capability to kinetically resolve a multiplicity of differentially reactive species is essentially limited by the sensitivity and signal/noise discrimination of the instrumentation, since these factors influence the error of the least-squares analysis and thus the maximum number of exponentials which reproducibly fit the data. Various laboratories with different instrumentation have thus reported that the kinetics of CO binding to microsomes are represented by one (Rosen & Stier, 1973), two (Debey et al., 1973), or three (Gray, 1978) kinetically distinct bimolecular processes. We thus sought to circumvent the major problem of fitting a minimum number of exponentials to a larger number of bimolecular reactions by developing a procedure which evaluates differences between the kinetic curves in the presence and absence of effector. This approach cancels the contributions from the majority of P450s which are unperturbed by the effector and generates a difference curve which only reflects the kinetic parameters of the perturbed P450. In contrast to the model in eq 1, whose measurable parameters correspond to components that are composed to multiple P450s, the parameters of the model we will henceforth use correspond to individual P450s. This analysis is derived as follows.

In the absence of effector, the absorbance progress curve is described by

$$\Delta A_t = \sum_{j=1}^{m} a_j^0 e^{-k_j^0 t} \quad (2)$$

where ΔA_t is the total absorbance change observed at time t, a_j^0 is the absorbance change at $t = 0$ and k_j^0 is the observed pseudo-first-order rate constant for the jth P450, and m is the total number of P450s. In the presence of effector, the equation is

$$\Delta A_t^* = \sum_{j=1}^{m} a_j^* e^{-k_j^* t} \quad (3)$$

where the modified parameters for each P450 are indicated by asterisks. Subtracting eq 2 from eq 3 yields

$$\Delta A_t^* - \Delta A_t = \sum_{j=1}^{m} a_j^* e^{-k_j^* t} - \sum_{j=1}^{m} a_j^0 e^{-k_j^0 t} \quad (4)$$

For the majority of P450s not perturbed by the effector, $a_j^0 = a_j^*$ and $k_j^0 = k_j^*$, and the corresponding terms in eq 4 are canceled. For the simplest case where only a single P450 is perturbed, eq 4 reduces to

$$\Delta A_t^* - \Delta A_t = a_1^* e^{-k_1^* t} - a_1^0 e^{-k_1^0 t} \quad (5)$$

and the time course of the difference between the absorbance changes in the presence and absence of effector yields a curve which is simply the difference between two exponential terms. Where more than one P450 is perturbed, terms representing differences between additional exponentials are added to eq 5.

Applying this analysis to the data in Figure 3A yielded a difference trace (Figure 3B) which is well-described by eq 5 and which thus indicates that the MAb changes the rate of a single P450. The resultant kinetic parameters (Table 2, first row) show that the MAb increased the CO binding rate of a MAb-specific P450 by about 10-fold (from 24.7 to 266 s^{-1}). In addition, the same absorbance amplitudes were obtained in the absence and presence of the MAb, which is also consistent with a change in kinetics for a single P450. The difference analysis thus yielded kinetic details beyond that provided by mere examination of the raw data (Figure 3A) which only showed that MAb increased the rate, or by standard

Table 2: Kinetic Parameters for CO Recombination to P450 Obtained with the Difference Model[a]

	effector	a_1*	k_1* (s^{-1})	a_1^0	k_1^0 (s^{-1})
PB-microsomes	MAb 4-7-1	0.0044	266	0.0039	24.7
		(0.0011)[b]	(23)	(0.0004)	(2.8)
PB-microsomes + Bz	MAb 4-7-1	0.0089	27.8	0.0092	109
		(0.0015)	(6.1)	(0.0018)	(21)
PB-microsomes + testosterone	MAb 4-7-1	0.0072	90.6	0.0080	17.4
		(0.0009)	(10.7)	(0.0005)	(1.6)
MC-microsomes	BP	0.0078	18.5	0.0075	1.63
		(0.0007)	(2.8)	(0.0006)	(0.20)

[a] Measurements were performed for the reactants in the first column in the absence and presence of the effector in the second column. Parameters were determined according to eq 5 and are the average of four experiments. a_1^0 and k_1^0 are the calculated parameters in the absence of effector while a_1* and k_1* correspond to those in the presence of the effector. [b] Standard deviations are given in parentheses.

multiexponential analysis (Table 1), which lacks the requisite precision to distinguish specific P450s.

The next question concerns identification of the MAb-specific P450. Since MAb 4-7-1 recognizes 2B1/2 and 2A1 (Omata & Friedman, 1992a; Waxman, 1987), any of these may be responsible for the observed rate change. To determine whether this change is associated with the PB-induced P450s (2B1/2) or the constitutive P450 2A1, we carried out experiments with microsomes from untreated rats which contain negligible levels of P450s 2B1/2 (Guengerich et al., 1982). The MAb did not alter the rate of CO binding (data not shown), which indicates that the MAb-induced rate enhancement in PB-microsomes originated from P450s 2B1/2 and not constitutive P450s such as 2A1. To determine whether P450 2B1 or 2B2 was responsible for the rate change, we employed the MAb in conjunction with Bz as a complementary effector, since P450 2B1 metabolizes this substrate more efficiently than P450 2B2 (Guengerich et al., 1982). CO binding experiments using PB-microsomes equilibrated with Bz were thus performed and revealed that the MAb, in contrast to its effect on Bz-free microsomes, decreased the rate (Figure 4a). This result suggests that the MAb-induced changes in kinetics derive from the Bz-metabolizing P450 2B1 and agrees with a recent study which showed MAb 4-7-1 inhibition of Bz binding to PB-microsomes resulted from the specific action of this MAb on P450 2B1 (Omata & Friedman, 1992a). Although application of the multiexponential model to the data in Figure 4A yielded a triexponential fit, changes in the absorbance amplitudes of the three components (Table 1, addition of Bz versus addition of MAb+Bz) again precluded association of MAb- and Bz-specific P450 with any particular P450. We thus applied the difference method (Figure 4B). The analysis first shows that when Bz is bound to P450 2B1, the MAb decreases the CO binding rate about 4-fold (from 109 to 27.8 s^{-1}) (Table 2), in contrast to the MAb-induced rate acceleration observed for the Bz-free P450. In addition, comparison of the k_1^0 values in the absence and presence of Bz shows that Bz increased the rate for CO binding to P450 2B1 from 24.7 to 109 s^{-1}.

We also employed the P450 substrate testosterone as an additional probe and found that it decreased the rate of CO binding to both PB-microsomes and microsomes from untreated rats (data not shown). The latter observation indicates that the testosterone effect on PB-microsomes at least partially derives from constitutive P450s. To focus on a specific P450, we again employed MAb 4-7-1 and found that it increased the rate of CO binding to PB-microsomes equilibrated with testosterone (Figure 5A). The difference curve (Figure 5B) and its fit to eq 4 showed that MAb 4-7-1 increased the rate

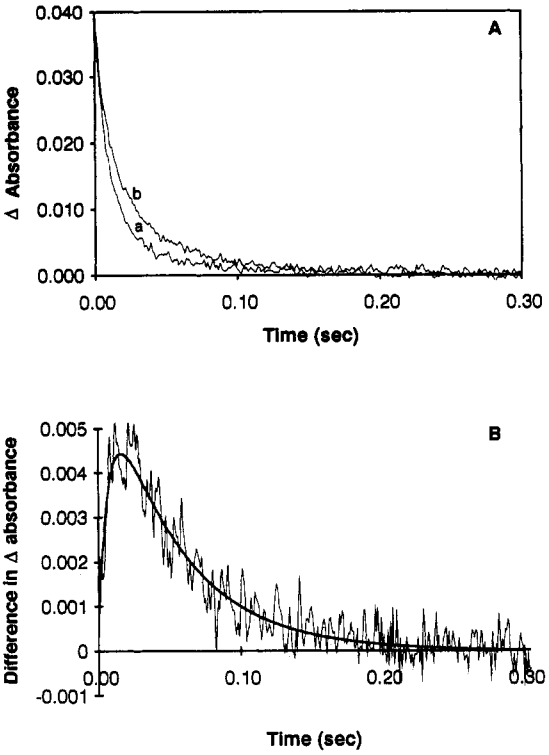

FIGURE 4: Effect of MAb 4-7-1 and Bz on the binding of CO to P450s in PB-microsomes. (A) Progress curves in the presence of (a) Bz and (b) Bz and MAb. Bz concentration was 1.0 mM; other conditions and concentrations are the same as in Figure 3. (B) Difference between the traces in (A) and the best fit according to eq 5.

for a MAb-specific P450 by 5-fold, from 17.4 to 90.6 s^{-1} (Table 2). MAb 4-7-1 did not have an effect on CO binding to control microsomes equilibrated with testosterone, which indicates that the changes in PB-microsomes do not derive from constitutive P450s and that the changes with PB-microsomes derive from P450s 2B1/2. Comparison of k_1^0 in the absence and presence of testosterone reveals that this substrate decreased the rate of MAb-specific P450 from 24.7 to 17.4 s^{-1} (Table 2). As was the case with the Bz binding experiments, the relatively high testosterone-hydroxylating activity of P450 2B1 relative to P450 2B2 (Guengerich et al., 1982) suggests that P450 2B1 is primarily responsible for the MAb-induced rate enhancement.

In addition to PB-microsomes, CO binding kinetics were also examined in another system containing a different mix of P450s, that of microsomes from MC-treated rats. Addition of BP, a substrate for the MC-induced P450 1A1 in these microsomes, increased the rate of CO binding (Figure 6A). Application of the difference method yields a good fit (Figure 6B) whose parameters revealed a rate enhancement of about 11-fold, from 1.63 to 18.5 s^{-1} (Table 2). To identify the BP binding P450 responsible for this change, we evaluated the effect of BP on the CO binding kinetics of microsomes from untreated rats which lack 1A1 (Guengerich et al., 1982). BP had no effect (data not shown), which indicates that the BP-accelerated rate osberved in MC-microsome P450s derives from 1A1. We also evaluated the effect of MAbs 1-7-1 and 1-36-1 to P450 1A1 (Park et al., 1982). Neither MAb affected the rate of CO binding either in the presence or in the absence of BP (data not shown). Although MAb 1-7-1 inhibits P450 1A1 hydroxylation of BP (Park et al., 1982), our finding that it did not reverse the BP-induced rate enhancement indicates that this MAb does not inhibit BP binding to P450 1A1 in microsomes. This result agrees with recent work which showed

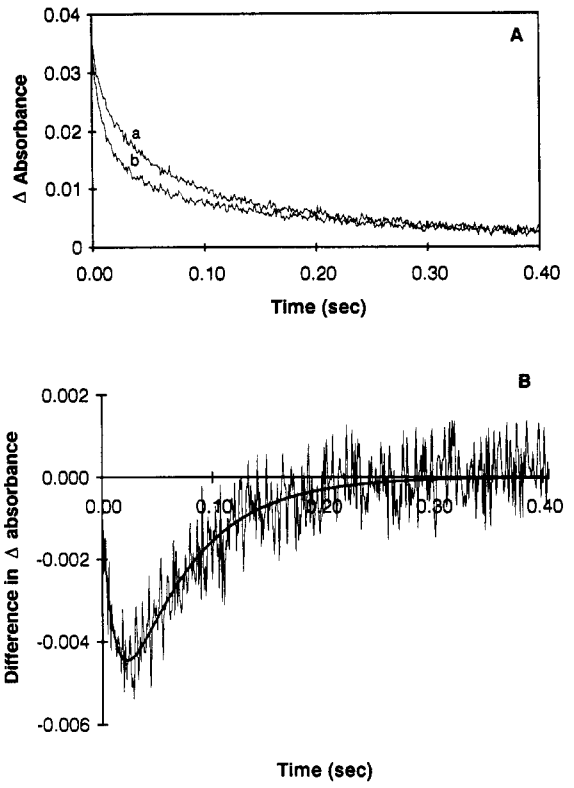

FIGURE 5: Effect of MAb 4-7-1 and testosterone on the binding of CO to P450s in PB-microsomes. (A) Progress curves in the presence of (a) testosterone and (b) testosterone and MAb. Testosterone concentration was 0.3 mM; other conditions and concentrations are the same as in Figure 3. (B) Difference between the traces in (A) and the best fit according to eq 5.

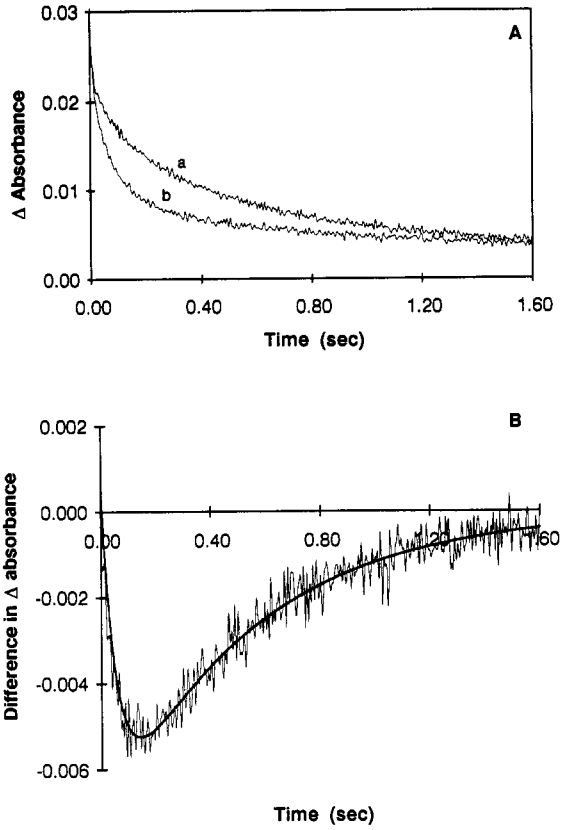

FIGURE 6: Effect of BP on the binding of CO to P450s in MC-microsomes. (A) Progress curves in the absence (a) and presence (b) to 10 μM BP. Conditions and concentrations are the same as in Figure 3. (B) Difference between the traces in (A) and the best fit according to eq 5.

that MAb 1-7-1 does not inhibit BP binding to purified P450 1A1 (Omata & Friedman, 1991, 1992b) but rather inhibits catalytic activity by interfering with the interaction between P450 1A1 and NADPH–cytochrome P450 reductase.

The mechanism of substrate and ligand migration from solvent through the protein matrix to the heme is an intriguing question in heme protein dynamics. This problem has been extensively addressed with numerous dynamic studies of myoglobin (Frauenfelder et al., 1991). For P450cam, dynamic studies (Atkins & Sligar, 1989; Stayton et al., 1989; Di Primo et al., 1990, 1993) and crystallographic structures of its complexes with various substrates, ligands, and inhibitors [see Raag and Poulos (1993) and references cited therein] have provided insight into the complex interactions among protein, heme, substrate, and ligand. However, comparable information is unavailable for the mammalian P450s whose tertiary structure is unknown and which exhibit only limited homology with P450cam.

The effect of substrate on CO binding kinetics offers a unique tool to probe the conformation/dynamics of mammalian P450s as well as the interaction of substrate with heme and protein. A substrate whch binds P450 near the heme or in the ligand access channel in the protein interior can sterically hinder ligand diffusion to the heme iron. For example, the rate of CO recombination with P450cam was greatly decreased when the substrate camphor was bound in the active site (Peterson & Griffin, 1972). Camphor binding also changed the infrared CO stretching frequencies of P450cam (O'Keefe et al., 1978; Shimada et al., 1979; Jung et al., 1992), which suggests that camphor directly interacts with the bound CO. These results are supported by crystallographic analysis of the P450cam–camphor–CO complex which revealed that CO displaces and remains in contact with camphor (Raag & Poulos, 1989). For P450cam, bound camphor thus imposes steric constraints which decrease the CO recombination rate.

In addition to sterically hindering CO binding, a substrate can alter the kinetics by inducing conformational changes in distal regions of the P450 molecule, including those in the ligand access channel. In addition, the substrate may not change the time-averaged conformation but instead alter the dynamics of the residues in the access channel; for example, binding of camphor substrate to P450cam alters the dynamics but not the average structure of the substrate binding region (Poulos et al., 1986). In contrast to a steric effect which would reduce the rate of CO binding, these substrate-induced conformational effects can either hinder or facilitate ligand diffusion from solvent to heme. Our results demonstrate that the effect is P450- and substrate-dependent: addition of Bz to PB-microsomes or BP to MC-microsomes increased the CO binding rate while addition of testosterone to PB-microsomes decreased the rate. The rate changes reflect substrate-induced changes in the CO access channel of specific P450s. Testosterone thus constrains while Bz enlarges the CO access channel of P450 2B1 while BP enlarges the CO access channel of P450 1A1. However, since we do not have a good model for these P450s it would be premature to speculate on the specific amino acid residues responsible for the observed changes.

The kinetics of CO binding have been an informative probe of ligand and substrate interactions with individual P450s in reconstituted systems (Gray, 1982; Oertle et al., 1985; Davydov, 1986; Shimizu et al., 1991; Ohta et al., 1992) and in yeast microsomes (Iwase et al., 1991). The liver microsomal system is more complex as it contains multiple P450s, some of which may display conformational microheterogeneity and

thus further increase the number of distinctly reactive P450 components. In addition, P450–P450 interactions and P450 interactions with microsomal membrane lipids and mixed-function oxygenase proteins may modulate the CO binding kinetics. For example, coexpression of the NADPH–cytochrome P450 reductase with P450 1A1 in yeast microsomes has been shown to alter the kinetics (Iwase, 1991). Thus, the kinetic behavior of a particular P450 in its purified state may not represent that of its native milieu. While we and others (Debey et al., 1973; Gray, 1978; Miura et al., 1991) have applied classical multiexponential analysis to microsomal kinetic data, the parameters thus obtained derive from a mixture of P450s and are uninterpretable in terms of individual P450s. In contrast, we have shown that a difference analysis method yields parameters that correspond to individual P450s. Using this approach, the contribution of a given P450 to the overall kinetics can be gauged in the presence of a microsomal mixture of P450s, by simply performing measurements in the absence and presence of specific P450 effectors. This approach to characterization of individual P450s in a native membrane environment should also be applicable to other parameters of interest, such as enzymatic activities.

ACKNOWLEDGMENT

We thank Dr. Yoshiaki Omata for his critical reading of the manuscript and valuable suggestions.

REFERENCES

Atkins, W. M., & Sligar, S. G. (1989) *J. Am. Chem. Soc. 111*, 2715–2717.

Cheng, K. C., Gelboin, H. V., Song, B. J., Park, S. S., & Friedman, F. K. (1984) *J. Biol. Chem. 259*, 12279–12284.

Davydov, R. M., Khanina, OIu., Iagofarov, S., Uvarov, VIu., & Archakov, A. I. (1986) *Biokhimiya* (Moscow) *51*, 125–129.

Debey, P., Balny, C., & Douzou, P. (1973) *FEBS Lett. 35*, 86–90.

Di Primo, C., Hui Bon Hoa, G., Douzou, P., & Sligar, S. G. (1990) *J. Biol. Chem. 265*, 5361–5363.

Di Primo, C., Hui Bon Hoa, G., Deprez, E., Douzou, P., & Sligar, S. G. (1993) *Biochemistry 32*, 3671–3676.

Frauenfelder, H., Sligar, S. G., & Wolynes, P. G. (1991) *Science 254*, 1598–1603.

Friedman, F. K., Park, S. S., & Gelboin, H. V. (1985a) *Rev. Drug Metab. Drug Interact. 5*, 159–192.

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., Crespi, C. L., Marletta, M. A., & Gelboin, H. V. (1985b) *Mol. Pharmacol. 27*, 652–655.

Fujino, T., West, D., Park, S. S., & Gelboin, H. V. (1984) *J. Biol. Chem. 259*, 9044–9050.

Gelboin, H. V., & Friedman, F. K. (1985) *Biochem. Pharmacol. 34*, 2225–2234.

Gray, R. D. (1978) *J. Biol. Chem. 253*, 4364–4369.

Gray, R. D. (1982) *J. Biol. Chem. 257*, 1086–1094.

Guengerich, F. P., Ghazi, A. D., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Iwase, T., Sakaki, T., Yabusaki, Y., Ohkawa, H., Ohta, Y., & Kawato, S. (1991) *Biochemistry 30*, 8347–8351.

Jung, C., Hui Bon Hoa, G., Schroder, K., Simon, M., & Doucet, J. P. (1992) *Biochemistry 31*, 12855–12862.

Lorr, N. A., Bloom, S. E., Park, S. S., Gelboin, H. V., Miller, H., & Friedman, F. K. (1989) *Mol. Pharmacol. 35*, 610–616.

Lu, A. Y. H., & West, S. B. (1980) *Pharmacol. Rev. 31*, 277–295.

Markowitz, A., Robinson, R. C., Omata, Y., & Friedman, F. K. (1992) *Anal. Instrum. 20(4)*, 213–221.

Miura, Y., Kawato, S., Iwase, T., Ohta, S., & Hirobe, M. (1991) *Biochemistry 30*, 3395–3400.

Murray, M., & Reidy, G. F. (1990) *Pharmacol. Rev. 42*, 85–101.

Oertle, M., Richter, C., Winterhalter, K. H., & DiIorio, E. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4900–4904.

Ohta, Y., Kawato, S., Tagashira, H., Takemori, S., & Kominami, S. (1992) *Biochemistry 31*, 12680–12687.

O'Keefe, D. H., Ebel, R. E., Peterson, J. A., Maxwell, J. C., & Caughey, W. S. (1978) *Biochemistry 17*, 5845–5852.

Omata, Y., & Friedman, F. K. (1991) *Biochem. Pharmacol. 42*, 97–101.

Omata, Y., & Friedman, F. K. (1992a) *Biochemistry 31*, 8862–8867.

Omata, Y., & Friedman, F. K. (1992b) *FEBS Lett. 309*, 249–252.

Ortiz de Montellano, P. R., Ed. (1986) *Cytochrome P-450: Structure, Mechanism and Biochemistry*, Plenum Press, New York.

Park, S. S., Fujino, T., West, D., Guengerich, F. P., & Gelboin, H. V. (1982) *Cancer Res. 42*, 1798–1808.

Park, S. S., Fujino, T., Miller, H., Guengerich, F. P., & Gelboin, H. V. (1984) *Biochem. Pharmacol. 33*, 2071–2081.

Pasanen, M., Pelkonen, O., Kauppila, A., Park, S. S., Friedman, F. K., & Gelboin, H. V. (1987) *Dev. Pharmacol. Ther. 10*, 125–132.

Pelkonen, O., Pasanen, M., Kuha, H., Gachalyi, B., Kairaluoma, M., Sotaniemi, E. A., Park, S. S., Friedman, F. K., & Gelboin, H. V. (1986) *Br. J. Clin. Pharmacol. 22*, 125–134.

Peterson, J. A., & Griffin, B. W. (1972) *Arch. Biochem. Biophys. 151*, 427–433.

Poulos, T. L., Finzel, B. C., & Howard, A. J. (1986) *Biochemistry 25*, 5314–5322.

Raag, R., & Poulos, T. L. (1989) *Biochemistry 28*, 7586–7592.

Raag, R., & Poulos, T. L. (1993) *Biochemistry 32*, 4571–4578.

Robinson, R. C., Shorr, R. G. L., Varrichio, A., Park, S. S., Gelboin, H. V., Miller, H., & Friedman, F. K. (1989) *Pharmacology 39*, 137–144.

Rosen, P., & Stier, A. (1973) *Biochem. Biophys. Res. Commun. 51*, 603–611.

Ryan, D. E., & Levin, W. (1990) *Pharmacol. Ther. 45*, 153–239.

Shimada, H., Iizuka, T., Ueno, R., & Ishimura, Y. (1979) *FEBS Lett. 98*, 290–294.

Shimizu, T., Ito, O., Hatano, M., & Fujii-Kuriyama, Y. (1991) *Biochemistry 30*, 4659–4662.

Song, B. J., Fujino, T., Park, S. S., Friedman, F. K., & Gelboin, H. V. (1984) *J. Biol. Chem. 259*, 1394–1397.

Stanker, L. H., Vanderlaan, M., & Juarez-Salinas, H. (1985) *J. Immunol. Methods 76*, 157–169.

Stayton, P. S., Poulos, T. L., & Sligar, S. G. (1989) *Biochemistry 28*, 8201–8205.

Tsokos, D. C., Omata, Y., Robinson, R. C., Krutzsch, H. C., Gelboin, H. V., & Friedman, F. K. (1992) *Biochemistry 31*, 7155–7159.

Waxman, D. J., Lapenson, D. P., Park, S. S., Attisano, C., & Gelboin, H. V. (1987) *Mol. Pharmacol. 32*, 615–624.