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Kinetics of carbon monoxide binding to phenobarbital-induced cytochrome P-450 from rat liver microsomes: A simple bimolecular process

(heme proteins/ligand binding/cytochrome P-420/flash photolysis)

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ABSTRACT **The kinetics of carbon monoxide binding to phenobarbital-induced cytochrome P-450 (P-450$_{PB}$) and to its enzymatically inactive form P-420$_{PB}$ have been investigated by both stopped-flow and flash-photolysis spectrophotometry. When the simultaneous presence of both forms of the enzyme is taken into account, the binding of CO to these two proteins can be described in terms of two bimolecular processes with rate constants of 4.5×10^6 $M^{-1}\cdot s^{-1}$ and 4.7×10^5 $M^{-1}\cdot s^{-1}$ for P-450$_{PB}$ and 1.7×10^7 $M^{-1}\cdot s^{-1}$ and 1.5×10^6 $M^{-1}\cdot s^{-1}$ for P-420$_{PB}$. From kinetic studies of the binding of CO to P-450$_{PB}$ under different experimental conditions, investigations of the homogeneity of our P-450$_{PB}$ preparations, and comparative kinetic investigations of P-450s from different sources, we conclude that CO binding to reduced P-450$_{PB}$ is a simple bimolecular process and that the observed biphasic traces are due to heterogeneity of the proteins. This conclusion is in contrast with previous reports of complex reaction mechanisms for the binding of CO to P-450$_{PB}$. Optical spectroscopy studies indicate the existence of an equilibrium between P-450$_{PB}$ and P-420$_{PB}$, at least for the reduced carbonyl derivatives of the enzymes. The interconversion is strongly influenced by the aggregation state of the protein. Large differences between the CO binding properties of P-450$_{PB}$ and those of P-420$_{PB}$ are found. These are discussed in terms of possible effects of the proximal ligation state of the iron on heme reactivity.**

The two major protein components of the microsomal monooxygenase system are cytochrome P-450 (P-450) and its reductase. The spectral properties of P-450 indicate a thiolate anion as proximal ligand for the heme iron (1–3). One important step in the proposed physiological reaction cycle of P-450 (4) is the binding of one oxygen molecule to the ferrous form of the enzyme. Thus, understanding the mechanism of ligand binding to reduced P-450 is of great importance. Ferrous P-450 easily binds carbon monoxide, giving rise to a species with an unusual optical absorption spectrum having a Soret maximum around 450 nm. The kinetics of combination of CO with phenobarbital-induced ferrous cytochrome P-450 from rat liver microsomes (P-450$_{PB}$), both bound to microsomal membranes and in the solubilized form, have been investigated by Douzou and coworkers (5–7), Roesen and Stier (8), the group of Brunori [Galeotti, T., Dani, A., and Brunori, M. (1975) personal communication], and, more recently, by Gray (9–11). The above sets of work are not consistent with each other. In rapid-mixing measurements on purified P-450$_{PB}$ without substrate, the Douzou group reported a single bimolecular process, with a rate constant of 10^6 $M^{-1}\cdot s^{-1}$ at 20°C (5), and Brunori found two simultaneous processes, the first having a rate constant of 7×10^5 $M^{-1}\cdot s^{-1}$ at 20°C; Gray reported a three-step binding reaction, consisting of a bimolecular process ($k_1 = 3 \times 10^6$ $M^{-1}\cdot s^{-1}$ at 20°C) followed by a second one whose velocity has a hyperbolic dependence on CO concentration and a third, monomolecular one (refs. 10 and 11, work done with P-450$_{PB}$ as well as phenobarbital-induced P-450$_{LM2}$ or 5,6-benzoflavoneinduced P-450$_{LM4}$ from rabbit liver). For CO binding to microsomal membranes, measured by flash photolysis, Douzou found two bimolecular steps ($k_1 = 2.3 \times 10^6$ $M^{-1}\cdot s^{-1}$ and $k_2 = 4 \times 10^5$ $M^{-1}\cdot s^{-1}$ at 4°C; the same results were obtained with purified P-450$_{PB}$) (7); Roesen and Stier described a single exponential process with association and dissociation rate constants, at 25°C, of 1.3×10^6 $M^{-1}\cdot s^{-1}$ and 0.2 s^{-1}, respectively (8); and Gray reported three bimolecular processes ($k_1 = 1.2 \times 10^7$ $M^{-1}\cdot s^{-1}$, $k_2 = 2.3 \times 10^6$ $M^{-1}\cdot s^{-1}$, and $k_3 = 2.3 \times 10^5$ $M^{-1}\cdot s^{-1}$ at 20°C) and a fourth, very slow, monomolecular one (k_4 = 0.3–0.6 s^{-1}) when the measurements were done by stopped-flow techniques (9). From this rather confused picture, it is impossible to propose a reaction mechanism for the binding of ligands to ferrous P-450$_{PB}$. However, the diversity of the results suggests that the preparations used by the different groups varied in their protein composition.

One possible source of complication may be the ease by which P-450 converts to P-420 (12), a catalytically inactive form of the enzyme capable of binding CO and whose carbonyl derivative has an optical absorption spectrum with the Soret peak around 420 nm. For most of the previously reported kinetic studies on P-450$_{PB}$, the presence of P-420$_{PB}$ cannot be excluded. In fact, only Debey *et al.* (7) and Galeotti, Dani, and Brunori (1975, personal communication) seem to have taken heed of this problem. Therefore, we have investigated the kinetics of CO binding to P-420$_{PB}$ and to P-450$_{PB}$ under a variety of experimental conditions, with the ultimate aim of learning whether a complex model is required to explain ligand binding to ferrous P-450 from liver microsomes (10) or whether a simple bimolecular mechanism is sufficient, as it is for other P-450s (13, 14).

METHODS

Preparation of P-450$_{PB}$ and P-420$_{PB}$. P-450$_{PB}$ was purified from liver microsomes of phenobarbital-induced male Sprague–Dawley rats (150–170 g) as described (15). The P-450 content of the preparations was determined according to Gut *et al.* (15). Further purification of the isozymes P-450$_{PB4}$ and P-450$_{PB5}$ was achieved by hydroxylapatite chromatography (16). The P-450$_{PB}$ preparations, before and

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Abbreviations: P-450, cytochrome P-450; P-450$_{PB}$, phenobarbital-induced P-450 from rat liver microsomes; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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after the purification step introduced by Waxman and Walsh (16), were subjected to $NaDodSO_4$/PAGE (17) and isoelectric focusing (18). To avoid possible complications arising from P-450 aggregation during the isoelectric focusing (see below), we included 1% (wt/vol) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, a zwitterionic detergent; Pierce or Sigma) in the gel.

Carbonyl-P-450_{PB} was obtained by reducing the ferric form of the enzyme with dithionite in the presence of CO. The conversion of P-450_{PB} to P-420_{PB} was achieved by incubating the CO derivative of the enzyme at 37°C for 1 hr in the presence of dithionite. The process was monitored spectrophotometrically.

Dissociation of P-450_{PB} or P-420_{PB} Aggregates. To dissociate P-450_{PB} or P-420_{PB} oligomers into monomers, oxygen-free solutions of the carbonyl form of the protein were incubated overnight at 0°C with 1% CHAPS (19). The dissociation of P-450_{PB} aggregates, after incubation with 1% CHAPS, was detected by gel-permeation chromatography on Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala) (19).

CO Binding Kinetics. The measurements were performed with the stopped-flow apparatus described in ref. 20 and a flash-photolysis apparatus described in ref. 34. To obtain good resolution of the data points through the whole time range required to record the traces, a software-simulated logarithmic time base was used (21). Kinetic data were analyzed by least-squares fitting to the double-exponential model

$$\Delta OD_t = \Delta OD_0 - \{\Delta OD_0 \cdot [f_1 \cdot \exp(-k_1 t) + (1 - f_1) \cdot \exp(-k_2 t)]\},$$

where k_1 and k_2 are the rate constants for the fast and slow components of binding, respectively; t is the time; and f_1 is the fraction of fast-reacting sites (referred to hereafter as fraction of fast phase). This analysis, according to Levenberg (22) and Marquardt (23), was accomplished by use of the subroutine ZXSSQ (IMSL, Houston, TX).

For CO binding studies by stopped-flow methods, concentrated solutions (≈0.1 mM) of ferric P-450 were diluted anaerobically with argon-saturated buffer, and a few grains of sodium dithionite were added to reduce the enzyme and completely remove oxygen. CO solutions of the desired concentration were prepared by anaerobically mixing argon-saturated buffer with water equilibrated with pure CO gas at 20°C and atmospheric pressure (24). Again, complete removal of oxygen was attained by addition of a few grains of dithionite.

For the flash-photolysis measurements, the samples were prepared by diluting ferric P-450 with argon-saturated buffer and subsequently adding known amounts of CO solution and solid dithionite.

RESULTS

Electrophoretic Analysis of P-450_{PB}. Our preparations contain about 12 nmol of P-450/mg of protein and are homogeneous as judged by $NaDodSO_4$/PAGE. However, isoelectric focusing analysis, both before and after hydroxylapatite chromatography, shows, in addition to minor bands, the presence of three major ones having similar intensities and pIs all close to 7.

CO Binding Kinetics. Wavelength-dependent flash-photolysis studies of CO binding to different P-420_{PB} preparations show a quasiisosbestic point around 455 nm, with significant fluctuations from case to case. Thus, monitoring the combination of CO with P-450_{PB} at this wavelength (to be determined each time) excludes any contribution by P-420_{PB} in the sample. Under these conditions, the kinetics of CO binding to P-450_{PB} can be represented by a two-exponential function, as shown in Fig. 1*A*, which depicts the time course

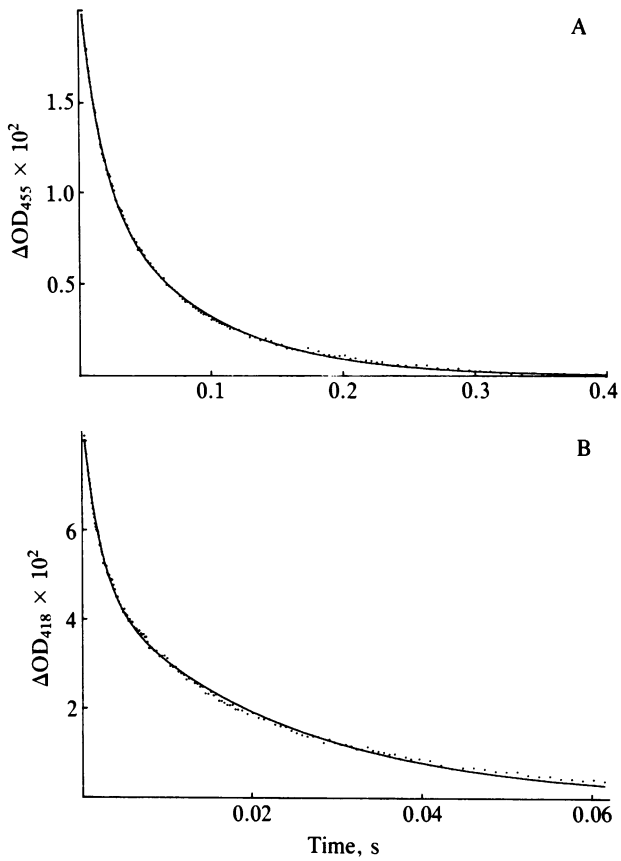

FIG. 1. Flash-photolysis progress curves for the binding of CO to P-450_{PB} (*A*) and to P-420_{PB} (*B*). Both experimental points and fitted curves are given. Conditions were as follows: buffer, 0.15 M potassium phosphate (pH 7.4) containing 20% (for P-420_{PB}) or 50% (wt/vol) glycerol (for P-450_{PB}); protein concentration, ≈1.8 μM; CO concentration, ≈20 μM; temperature, 10°C.

of CO combination with P-450_{PB}. The same result is obtained in rapid-mixing experiments (data not shown). The data of Gray (9) can be reproduced by following the reaction at 448 nm. Changing the monitoring wavelength reveals an isosbestic point between unliganded and CO-bound P-450_{PB} around 418 nm. We thus chose this wavelength to monitor CO binding to P-420_{PB} and found that this reaction is also biphasic (Fig. 1*B*). The ligand concentration linearly affects CO binding both to P-450_{PB} as well as to P-420_{PB}. This is shown in Fig. 2, where plots of the apparent pseudo-first-order rate constants are plotted against the total CO concentrations. The values were obtained by iterative approximation and thus exhibit relatively large fluctuations, as shown by the error bars. The rate constants obtained from the slopes and intercepts of the linear regression lines through the points are given in the figure legend.

Lowering the intensity of the photodissociating light pulse does not affect the velocity of CO binding nor does it produce a change in the fraction of fast phase (f_1, defined in *Methods*) for either P-450_{PB} or P-420_{PB} (data not shown).

We have also investigated the effects of solvent, substrates, and aggregation state of the protein on the kinetics of CO binding to P-450_{PB}. Changing the glycerol concentration in the buffer from 0 to 50% produces a significant increase in both rate constants without affecting the fraction of fast phase (Table 1). Furthermore, the conversion of P-450_{PB} to P-420_{PB} slows when the glycerol concentration is increased. The addition of 1.25 mM phenobarbital to the reaction mixture does not appreciably influence the CO binding process, in agreement with previous observations (ref. 11; T. Galeotti, A. Dani, and M. Brunori, personal communication), whereas

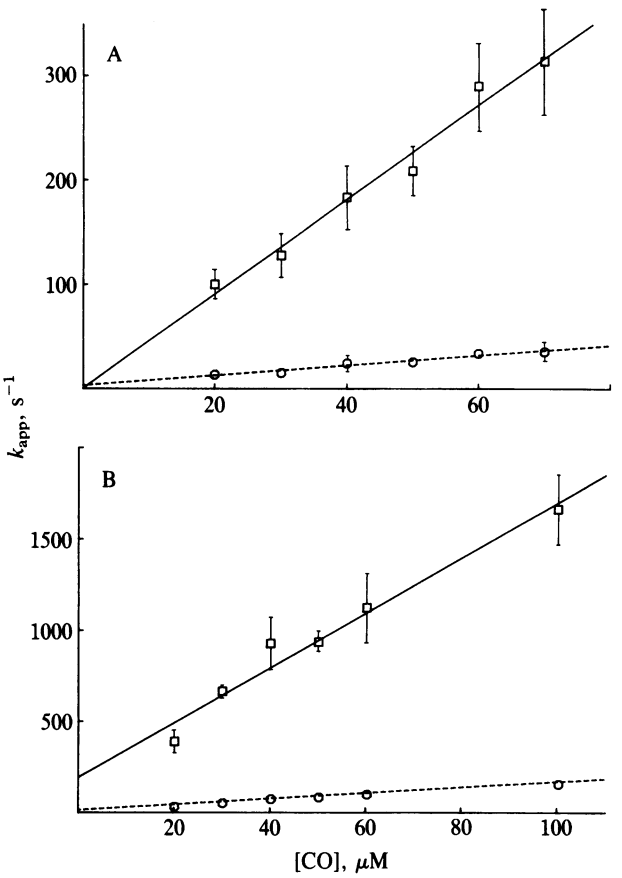

FIG. 2. Plots of the apparent pseudo-first-order constants (k_{app}) for CO binding constants to P-450$_{PB}$ (*A*) and P-420$_{PB}$ (*B*), obtained by least-squares fitting to the flash-photolysis data, as a function of ligand concentration for the fast (—) and slow (----) phases. The ligand binding ($M^{-1}\cdot s^{-1}$) and dissociation (s^{-1}) rates obtained from the regression lines are as follows: (*A*, P-450$_{PB}$) $k_1 = 4.5 \times 10^6$; $k_2 = 4.7 \times 10^5$; $k_{-1} = 1.2$; $k_{-2} = 4.0$. (*B*, P-420$_{PB}$) $k_1 = 1.7 \times 10^7$; $k_2 = 1.5 \times 10^6$; $k_{-1} = 138.2$; $k_{-2} = 13.9$. No direct measurement of the dissociation velocities has been made. Buffer, protein concentration, and temperature were as described in the legend to Fig. 1.

1 mM benzphetamine or ethoxycoumarin, which induce the type I substrate spectrum in ferric P-450$_{PB}$ (25), produce appreciable effects on the reaction rates as well as on the fraction of fast phase (Table 1). Incubation overnight with 1% CHAPS dissociates P-450$_{PB}$ aggregates, as is the case for rabbit P-450$_{LM4}$ (19). Such disaggregation does not alter the velocities of the two processes but slightly modifies their relative amplitudes (Table 1). In the case of P-420$_{PB}$, the addition of 1 mM ethoxycoumarin produces no effect on the CO binding kinetics (data not shown). In the pH range 7.4–8.1, the reaction velocities, as well as the fraction of fast phase, remain constant. In contrast, lowering the pH from 7.4 to 6.1 produces significant increases in the reaction rates but leaves the fraction of fast phase unchanged (Table 2).

Arrhenius plots for the binding of CO to P-450$_{PB}$ and P-420$_{PB}$ are linear for both the fast and the slow phase (Fig. 3). From the slopes of the regression lines through the experimental points, the activation energies given in the figure legend were calculated. No effect of temperature on the fraction of fast phase was observed.

Optical spectra of P-420$_{PB}$, before and after the temperature-dependent kinetic determinations, showed a substantial reconversion of P-420$_{PB}$ to P-450$_{PB}$. This phenomenon was much more pronounced when the protein was incubated overnight with 1% CHAPS (data not shown).

The kinetics of CO binding to P-450$_{CAM}$ was also investigated. In keeping with a previous report (13), it was found to be a process that could be represented by a single-exponential function (data not shown).

Table 1. Kinetics of CO binding to P-450$_{PB}$ under various experimental conditions

Experimental condition*	f_l †	Apparent pseudo-first-order rate constant, s^{-1}	
		$k_{1\,app}$	$k_{2\,app}$
Group 1			
0% Gro	0.68 ± 0.01	39.1 ± 6.8	3.8 ± 0.2
20% Gro	0.69 ± 0.01	48.8 ± 2.0	4.4 ± 0.4
Control	0.69 ± 0.01	65.2 ± 6.0	5.8 ± 0.6
Group 2			
1.25 mM PB‡	0.65 ± 0.06	58.6 ± 8.0	10.4 ± 3.2
Control	0.64 ± 0.02	70.4 ± 4.2	9.2 ± 1.6
Group 3			
1 mM BzP§	0.79 ± 0.05	102.2 ± 8.4	19.8 ± 8.0
Control	0.67 ± 0.01	67.2 ± 8.0	7.2 ± 2.0
Group 4			
1 mM EC‖	0.72 ± 0.04	100.6 ± 14.2	8.0 ± 3.8
Control	0.68 ± 0.05	73.6 ± 11.2	6.4 ± 2.8
Group 5			
1% CHAPS	0.72 ± 0.02	73.4 ± 5.4	7.4 ± 1.8
Control	0.66 ± 0.03	57.0 ± 7.6	7.2 ± 1.2

Data were obtained from flash-photolysis measurements and are presented as mean ± SD. At least three parallel experiments were performed for each group in the table. The differences in the control between groups may be ascribed to experimental errors, mainly variations of the ligand concentrations.

*For each group, 50% (wt/vol) glycerol (Gro) was used as control condition, except when the effect of ethoxycoumarin was studied (Group 4); since 1% ethanol was added to dissolve the substrate, 50% glycerol plus 1% ethanol was used as control in this case. Other conditions were as described in the legend to Fig. 1.

†"Fraction of fast phase," see *Methods* for definition.

‡Phenobarbital.

§Benzphetamine.

‖Ethoxycoumarin.

DISCUSSION

The results reported in Table 1 and Figs. 1 and 2 show that CO binding to P-450$_{PB}$ is a much simpler process than previously reported by some authors (6, 9–11). It is clear that the three bimolecular processes observed by Gray, monitoring at 448 nm, result from the simultaneous combination of CO to P-420$_{PB}$ and P-450$_{PB}$. In fact, the slow CO binding process for P-420$_{PB}$ and the fast one for P-450$_{PB}$ have similar reaction rates (Fig. 2). In contrast, the nondependence on CO concentration found by Gray for his third phase does not find an immediate explanation in our results. The flash-photolysis data reported by the group of Douzou (7) are fully consistent with our findings. These investigators kept P-450$_{PB}$ always at very low temperatures, largely preventing the conversion to P-420$_{PB}$ (7). Our data are also in agreement with what was

Table 2. Rate constants ($M^{-1}\cdot s^{-1}\cdot 10^{-6}$) for CO binding to P-450$_{PB}$ at various pH values

pH	f_l*	k_1	k_2
6.1	0.68 ± 0.03	3.1 ± 0.3	0.30 ± 0.06
7.4	0.69 ± 0.02	2.3 ± 0.4	0.19 ± 0.05
8.1	0.69 ± 0.03	2.3 ± 0.4	0.18 ± 0.03

Rate constants were obtained by linear regression from measurements at CO concentrations ranging between 10 and 100 μM and are presented as mean ± SD. Experimental conditions were as follows: buffers, 0.15 M potassium phosphate containing 20% glycerol; protein concentration, ≈1.8 μM; temperature, 10°C.

*Fraction of fast phase (see *Methods*).

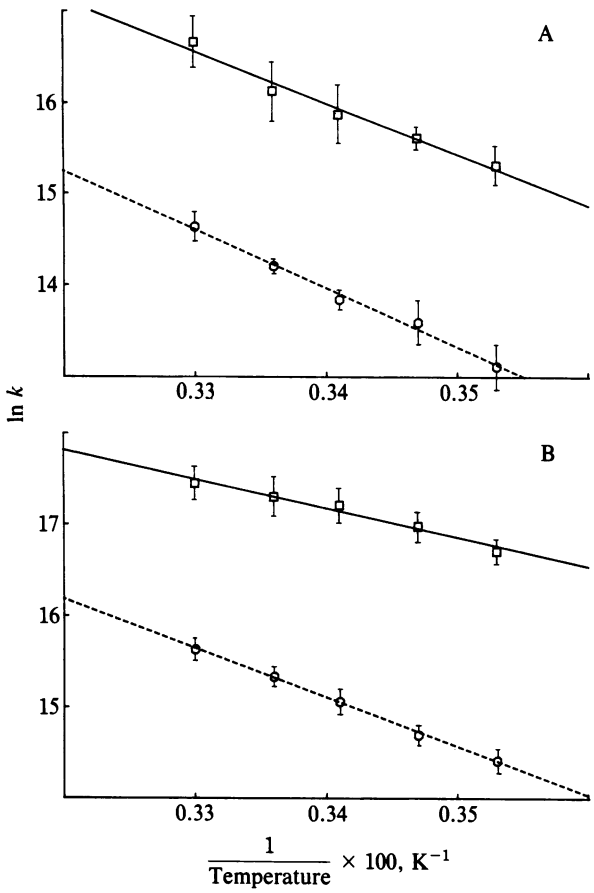

FIG. 3. Arrhenius plots for the binding of CO to P-450$_{PB}$ (*A*) and P-420$_{PB}$ (*B*) for the fast (—) and slow (----) phases. The activation energies (in kJ/mol) calculated from the slopes of the regression lines are 45.2 and 51.2, respectively, for the fast and slow phases in *A* (P-450$_{PB}$) and 25.6 and 43.2 for the fast and slow phases in *B* (P-420$_{PB}$). Protein concentration and buffer were as described in the legend to Fig. 1; CO concentration was ≈70 μM for P-450$_{PB}$ and ≈100 μM for P-420$_{PB}$.

found by Brunori [Galeotti, T., Dani, A. and Brunori, M. (1975), personal communication]. Thus, the presence of denatured forms of the enzyme (P-420$_{PB}$) in P-450$_{PB}$ preparations has played a dominant role in producing inconsistency in the results reported in the literature, as postulated in the Introduction. Once we determined the conditions under which the contribution of P-420$_{PB}$ to the reaction of P-450$_{PB}$ with CO can be eliminated, an analysis of this process aimed at the understanding of its mechanism was possible.

The linear dependence of the pseudo-first-order binding rates on CO concentration, as obtained by least-squares fitting of the experimental data, shows that both processes are bimolecular. This finding and the fact that no difference in the CO binding kinetics is observed, either between rapid mixing and flash photolysis measurements or between fully and partially photodissociated material, exclude monomolecular structural rearrangements of the protein during ligand binding (see ref. 26, pp. 208–214). If P-450$_{PB}$ were in a fast (as compared to CO binding) equilibrium between two conformers with different CO binding properties, and this conformational change depended on the ligation state of the heme, the stopped-flow and the flash-photolysis investigations would have given different answers. In the first case, the protein would have been, at time zero, completely in the unliganded conformation, whereas in the flash-photolysis measurement the opposite (or almost the opposite) would have been true. Furthermore, in the case of a slow (as compared to CO binding) equilibrium between conformers, partially photodissociating the sample would have preferentially produced one unliganded conformational species and this would have resulted in a discrepancy between kinetic measurements performed at different energies of the photolysing light pulse.

A fundamental question remains: Why is CO binding to P-450$_{PB}$ biphasic? Other P-450s react with CO in a simple manner; this is the case for P-450$_{CAM}$ (P-450 from *Pseudomonas putida* grown on camphor as carbon source), as shown by Mims *et al.* (13) and confirmed by us, and for adrenal P-450 (14). The first and simplest possibility that one may consider is that P-450$_{PB}$ exists in an oligomer–monomer equilibrium and that the two aggregation states differ in their ligand binding properties. Such an explanation is supported by the observation that solubilized microsomal cytochrome P-450 forms macromolecular aggregates (19). Incubation of P-450$_{PB}$ with 1% CHAPS completely disaggregates the oligomers. Parallel measurements done on samples incubated with and without CHAPS clearly show (Table 1) that the biphasic CO binding cannot be ascribed to aggregation phenomena.

Another reasonable explanation for the biphasic kinetics of CO binding to P-450$_{PB}$ is an equilibrium between two protonation states of the protein. As shown in Table 2, the relative amounts of rapidly and slowly reacting species are pH-independent, thus this possibility is also ruled out. However, the influence of pH on the reaction rates may indicate the presence of a residue, with a slightly acidic pK_a, in the vicinity of the heme and thus able to influence its reactivity. Temperature, like pH, does not influence the fraction of fast phase, a fact that also speaks against a conformational equilibrium as a reason for biphasic CO binding to P-450$_{PB}$.

Having ruled out the possibility that different conformers of the same protein are responsible for the biphasic binding kinetics, we must consider the possibility that chemically different P-450s exist in the preparations. One possible source of this heterogeneity could be a partial saturation of the enzyme with substrate. This hypothesis seems unlikely because the fraction of fast phase is the same for different preparations and is not influenced by changing experimental conditions such as temperature or pH, which would most likely influence the affinity of P-450$_{PB}$ for substrates. However, we have investigated the effect of various substrates on the CO binding kinetics. The data in Table 1 show that the kinetic behavior of reduced P-450$_{PB}$ cannot be ascribed to substrate binding. The small change in the fraction of fast phase, observed in the presence of either ethoxycoumarin or benzphetamin, is clearly related to changes in the spectral properties of the enzyme. Once this possible source of intermolecular heterogeneity is ruled out, the only likely explanation for the biphasic kinetic behavior of P-450$_{PB}$ and P-420$_{PB}$ is that at least two species of the enzyme are present in the preparations. This conclusion is not only reached by exclusion but is also validated by some direct experimental evidence. Our isoelectric focusing analysis as well as the report of Bansal *et al.* (27) show that P-450$_{PB}$ preparations contain three distinct forms of the enzyme in roughly equivalent amounts. One may speculate that two of the three forms of P-450$_{PB}$ react rapidly with CO, since the fraction of fast phase is found to be consistently around 0.68. This would not be unique to P-450$_{PB}$ since in other cases, structurally different proteins show indistinguishable functional properties (as for human adult hemoglobins A and A_2). Unfortunately, our efforts to separate the different P-450$_{PB}$ species in a native form have failed; thus no kinetic investigation of homogeneous preparations could be made.

Another interesting aspect emerging from our investigation is the relatively rapid reconversion of P-420$_{PB}$ to P-450$_{PB}$ in the presence of 1% CHAPS, a process also occurring in the absence of the detergent, but to a much lower extent.

Apparently, protein disaggregation shifts the equilibrium between P-450$_{PB}$ and P-420$_{PB}$ toward the native form of the enzyme. One possible explanation for this phenomenon is that the interconversion involves structural modifications of areas of the molecule away from the heme. Incidently, the equilibrium between P-450$_{PB}$ and P-420$_{PB}$ may be the explanation for the very slow monomolecular process described by Gray in rapid-mixing experiments (10).

From the results reported in this work, some speculations on the geometry of the heme pocket in P-450 can be made. The value of the fast rate constant for the binding of CO to P-450$_{PB}$ is consistent with an open heme crevice (13, 28, 29). The same does not apply to the slow phase. This difference may be related to modifications of the structure of the protein at either the distal or the proximal side of the heme. In the first case, the different CO binding velocities would be ascribed to steric phenomena; in the second, a regulation of heme reactivity from the proximal ligand would be implied (30, 34). The second hypothesis seems to be favored by the results obtained in the presence of ethoxycoumarin and benzphetamin, which showed an increase in the CO binding velocities upon substrate binding.

An analogous explanation may be proposed for the large differences in the CO dissociation rates found between P-450$_{PB}$ and P-420$_{PB}$. The nature of the structural differences between P-450 and P-420 is not known. The position of the Soret peak in the optical spectrum of carbonyl-P-420 suggests the presence of an axial nitrogenous ligand bound to the iron. On the other hand, the very high CO dissociation rates found in P-420$_{PB}$ are not consistent with this hypothesis, because in heme-proteins in which a histidine residue is known to be bound to the heme iron as proximal ligand, such high CO dissociation rates have not been observed (ref. 26, pp. 226, 310; ref. 31). In contrast, for a heme model compound having a sulfur atom as axial ligand (32), a very low CO affinity has been reported. A plausible explanation for the big difference in CO dissociation rates between P-450$_{PB}$ and P-420$_{PB}$ may be a different position of the iron atom relative to the heme plane, resulting from a more or less loose bond with the proximal ligand. Such an explanation has been invoked for other heme proteins (30).

We have shown that CO binding to P-450$_{PB}$ is a simple bimolecular process, which implies that other ligands of the ferrous enzyme, such as oxygen, also bind in the same way. The observed biphasicity of ligand binding to reduced P-450$_{PB}$ may be due to heterogeneity of the preparations, which may imply that the biphasic reduction kinetics of the ferric enzyme by NADPH, through P-450 reductase (3), are not related to a complex reaction mechanism. Further investigations are needed to clarify this point. However, preliminary studies on the reduction of P-450$_{CAM}$ by sodium dithionite indicate that this process is also monophasic, in contrast with the biphasic process found for P-450$_{PB}$ by Davydov and Kurganov (33).

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