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Effects of Oxygen on the Relative Photodissociability of Cytochrome P-450·CO Complex in Rat Liver Microsomes†

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Received October 17, 1990

ABSTRACT: Flash photolysis of cytochrome P-450 in phenobarbital-induced rat liver microsomes was examined by observing the decay of absorbance change, $A(t)$, at 450 nm after photolysis of the heme·CO complex by a depolarized laser flash. The relative photodissociability, $A(0)/Ab$, is significantly decreased upon an increase in oxygen concentration. $A(0)/Ab$ is 96%, 86%, and 51% at oxygen concentrations of 90, 115, and 155 μM when compared with $A(0)/Ab$ at 0 μM O_2. In the presence of aminopyrine, the oxygen-dependent decrease in the relative photodissociability was enhanced about 2 times. The rate of CO recombination with cytochrome P-450 was decreased by increasing O_2 concentration. By going from 0 to 155 μM O_2, about 17% decrease and 58% decrease in the recombination rate were observed in the absence and presence of aminopyrine, respectively. In the absence of CO, no absorbance change $A(t)$ at 450 nm was observed even at 155 μM O_2, excluding a possible contribution of P-450·O_2. The present observation leads to the speculation that oxygen molecules around the heme·CO complex affect the electronic state of the heme, resulting in an increase in Fe–CO bond strength.

The liver microsomal monooxygenase system containing cytochrome P-450 catalyzes the metabolism of a wide variety of endogenous and xenobiotic compounds. Cytochrome P-450 receives two electrons from NADPH–cytochrome P-450 reductase and cytochrome b_5 and activates molecular oxygen by donating these electrons, resulting in N-demethylation or aromatic hydroxylation of substrates (Lu & Coon, 1968; Hildebrandt & Estabrook, 1971).

Oxygen concentration is essentially very important for the drug oxidation activity of cytochrome P-450. In vitro biochemical studies have usually been performed under an air-saturated high oxygen concentration of about 220 μM, although the practical oxygen concentration in liver is very low, about 35 μM (Erickson et al., 1982). It is therefore very important to investigate a possible difference in the catalytic mechanism of cytochrome P-450 depending on the oxygen concentration between in vivo and in vitro conditions (Jones, 1981).

So far, little is known about the oxygen concentration dependence for drug oxidation activity of cytochrome P-450 (Fujii et al., 1981; Tsuru et al., 1982; Holtzman et al., 1983; Pohl et al., 1984; Stevens et al., 1984; Webster et al., 1985). We have reported that the amount of metabolites from a

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0006-2960/91/0430-3395$02.50/0

substrate (e.g., anisole or *N*-methylaniline) formed by rat liver microsomes varied as a function of oxygen concentration (Takahara et al., 1986; Soma et al., 1989). To date, however, no direct measurements have been carried out concerning oxygen concentration effects on the electronic state of the P-450 heme. Flash photolysis of the P-450·CO complex is particularly sensitive to probe the environment of the heme moiety including the electronic state of the heme. For example, the photodissociability and recombination kinetics of the heme·CO complex for rabbit liver P-450$_{MC}$[1] (Imai et al., 1982) and bovine adrenocortical P-450s (Mitani et al., 1985) are greatly affected by the presence of various substrates electrically interacting with the heme. Here, we have extended these studies to investigate changes in the electronic state of the heme of P-450 in liver microsomes due to different oxygen concentrations.

The present study demonstrates that both the relative photodissociability of the cytochrome P-450·CO complex and the recombination kinetics of CO with cytochrome P-450 are significantly decreased due to the increase of the oxygen concentration both in the presence and in the absence of aminopyrine.

Experimental Procedures

Materials

Liver microsomes were prepared from male Wistar rats (6 weeks, 150 g) that had been pretreated with sodium phenobarbital (60 mg/kg in saline, ip, 3 days). Protein content was determined according to the method of Lowry et al. (1951). Standard gases (1.0 and 20.0% O_2 in N_2) were purchased from Takachiho Chemical, and we obtained gas mixtures of other desired oxygen concentrations (0.4% and 8% O_2 in N_2) with a gas divider apparatus (SGD-XC, STEC Inc.). Aminopyrine was purchased from Wako Pure Chemicals. NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma. All other chemicals were of the highest purity commercially available.

Methods

Assay of Substrate Hydroxylation Activity. By monitoring of oxygen concentration with a Clark oxygen electrode, the oxygen concentrations in the assay solutions equilibrated with the gases (0.4% and 20.0% O_2 in N_2) were determined to be 5 and 205 μM, respectively. The Clark oxygen electrode was calibrated by determining the response of the electrode using diluted oxygen-saturated solutions with oxygen-free solutions based on the standard oxygen solubility table (Nishiki & Ogata, 1976). The assay system is illustrated in Figure 1. The reaction was initiated by the addition of NADP and glucose 6-phosphate to the reaction mixture with a gas-tight syringe, following preincubation for 5 min. The reaction was carried out at 37 °C with continuous gassing (100–200 mL/min), stirring, and monitoring of oxygen concentration. Assays of metabolites were performed according to the method of Nash (1953) by measuring formaldehyde formation.

Flash Photolysis of the Cytochrome P-450·CO Complex. The principle of the flash photolysis apparatus was described in detail elsewhere (Cherry, 1978; Kawato et al., 1988). Briefly, the sample was photolyzed at 532 nm by a depolarized flash of 8-ns duration from a Nd/YAG laser (Quanta-Ray, DCR-2). Absorbance changes due to photolysis of the heme·CO complex were measured at 450 nm. The signals were analyzed by calculating the total absorbance change, $A(t)$, for depolarized flash excitation given by

$$A(t) = 2A_V(t) + A_H(t) \quad (1)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorption changes for vertical and horizontal polarization at time t after the flash. In each experiment, 8192 signals were averaged with a Toyo 2805 transient memory. No degradation of cytochrome P-450·CO complex during the experiment was observed spectrophotometrically. The present measurements of the total absorbance change are theoretically accurate, avoiding the contribution of P-450 rotation in microsomes whose rotational correlation time is comparable to the decay of $A(t)$. The decay parameters of photolysis and recombination of CO with reduced P-450 were obtained by fitting the data to the double-exponential equation:

$$A(t) = A(0)[\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)] \quad (2)$$

The average lifetime, $\langle\tau\rangle$, is defined as

$$\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2 \quad (3)$$

where α_1 and α_2 are exponential fractions and $\alpha_1 + \alpha_2 = 1$. Curve fitting of the data based on eq 2 was accomplished by a PDP-11/73 minicomputer.

For flash photolysis measurements, the sample (0.5 mg/mL microsomal protein) was preincubated for 5 min at 20 °C in 100 mM sodium phosphate buffer (pH 7.4) containing 0.5 unit of glucose-6-phosphate dehydrogenase in an optical cuvette without sealing; 40 μM NADP and 4 mM glucose 6-phosphate were then added to reduce cytochrome P-450 followed by incubation under a gas stream of different oxygen content for 10 min in an optical cuvette sealed with a rubber cap, preventing penetration of air oxygen. P-450·CO complex was prepared in the same optical cuvette by a gentle bubbling of microsomal suspensions with CO for 30 s, and the sample was incubated for 5 min to complete P-450·CO complex. Immediately, flash photolysis was then performed and was completed within 5 min during which the spectral amount of cytochrome P-450·CO complex remained unchanged for another sample prepared in the same way as that for flash photolysis. CO concentration was kept saturated by sealing a cuvette with a rubber cap. To measure the substrate effect on flash photolysis, 1 mM aminopyrine was added to the microsomal suspension. A pure N_2 gas stream, an 8% O_2 in N_2 gas stream,

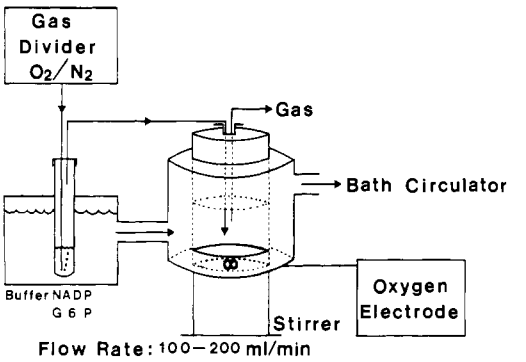

FIGURE 1: Schematic illustration of the apparatus to measure drug oxidation activity under various oxygen concentrations. A Clark oxygen electrode was placed below the reaction vessel. The flow rate of the gas was 100–200 mL/min, and the temperature of the bath circulator was 37 °C. Two gassing towers were sealed with a rubber cap and connected with stainless-steel tubes. The oxygen concentration in the assay solution equilibrated by the gas was kept constant during the reaction.

[1] Abbreviations: P-450$_{MC}$, methylcholanthrene-inducible cytochrome P-450; P-450$_{cam}$, cytochrome P-450 from *Pseudomonas putida*; Ab, absorption difference between 450 and 490 nm in CO difference spectra of cytochrome P-450 in microsomes.

Table I: Decay Parameters of the Flash-Induced Absorption Change, $A(t)$, of Cytochrome P-450·CO in Liver Microsomes Analyzed According to Equations 2 and 3[a]

oxygen content (μM) of sample[b]	$A(0) \times 10^3$	$\langle\tau\rangle$ (μs)	k_{on}[c] $\times 10^6$ (s^{-1} M^{-1})	α_1 (%)	τ_1 (μs)	α_2 (%)	τ_2 (μs)
			Without Substrate				
0	27.1 (0.1)[d]	752 (46)	1.33 (0.09)	49 (2.7)	269 (16)	51 (2.7)	1372 (52)
90	20.7 (0.3)	849 (45)	1.18 (0.05)	48 (3.4)	250 (3.2)	52 (3.4)	1406 (7.5)
115	15.3 (0.4)	889 (17)	1.12 (0.03)	46 (0.3)	256 (7.2)	54 (0.3)	1429 (20)
155	6.2 (2.4)	904 (40)	1.11 (0.05)	50 (1.6)	248 (8.5)	50 (1.6)	1547 (46)
			Aminopyrine				
0	27.3 (1.0)	786 (39)	1.27 (0.06)	29 (2.1)	217 (2.2)	71 (2.1)	1009 (31)
90	13.9 (0.8)	719 (18)	1.39 (0.04)	39 (1.0)	213 (6.6)	61 (1.0)	1044 (33)
115	7.2 (0.03)	1114 (16)	0.90 (0.01)	47 (0.5)	234 (6.0)	53 (0.5)	1899 (41)
155	3.8 (0.3)	1901 (57)	0.53 (0.01)	44 (1.5)	225 (4.5)	56 (1.5)	3199 (43)

[a] The measurements were carried out as described under Experimental Procedures. [b] The experimental error of the oxygen concentration was observed to be 5 μM. [c] k_{on} was calculated according to the equation $k_{on} = 1/\langle\tau\rangle$[CO] where [CO] is 1 mM in the present experimental conditions. [d] Numbers in parentheses are the standard error over several experiments.

and a 20% O_2 in N_2 gas stream were used to modulate the oxygen concentration. To exclude oxygen completely in some samples, sodium dithionite was used to reduce cytochrome P-450. Time dependences of the CO difference spectra and the oxygen concentration of the sample during the time course corresponding to flash photolysis measurements were examined in separate experiments using the samples prepared in the same way as that used for flash photolysis. The oxygen concentrations in samples at 5 min after CO bubbling preincubated under pure N_2, 8% O_2, and 20% O_2 gas streams were determined to be 90, 115, and 155 μM O_2, respectively, using a Clark oxygen electrode. Saturated CO concentration in a sample was measured by flash photolysis of whale myoglobin·CO complex reduced with dithionite and bubbled with CO for 30 s. CO concentration was calculated to be 0.93 ± 0.10 mM from the monoexponential recombination of CO to myoglobin, having the lifetime of τ = 2150 ± 250 μs and CO recombination rate $k_{on} = 5 \times 10^5$ M^{-1} s^{-1} (Antonini & Brunori, 1971) using the relationship:

$$[CO] = 1/\tau k_{on} \quad (4)$$

The treatment of a 20% oxygen stream for 10 min for dithionite-reduced myoglobin prior to CO bubbling did not alter the above lifetime within experimental error, indicating that CO concentration should be the same over all present microsome samples independent of the oxygen concentration. For these experiments, a trace amount of dithionite was used to prevent oxygen consumption in solutions besides reduction of myoglobin.

Results and Discussion

Drug Oxidation Activity. The time course of the formation of formaldehyde from aminopyrine was measured under different oxygen concentrations. The rate of formaldehyde formation was significantly increased according to the increase in the oxygen concentration. Figure 2 shows typical results for the time course of the formation of formaldehyde from aminopyrine by phenobarbital-induced rat liver microsomes at 205 μM O_2 (20% O_2 in N_2) and 5 μM O_2 (0.4% O_2 in N_2).

The results imply that oxygen concentration affects N-demethylation of aminopyrine as well as metabolism of anisole and *N*-methylaniline (Takahara et al., 1986; Soma et al., 1989) and that cytochrome P-450 in rat liver microsomes is considerably active even at a low oxygen concentration of 5 μM.

Oxygen Dependence on the Relative Photodissociability of the Cytochrome P-450·CO Complex. Decay curves of the flash-induced absorption change for the cytochrome P-450·CO complex in rat liver microsomes were measured at different oxygen concentrations (see Figure 3). $A(0)$ was obtained

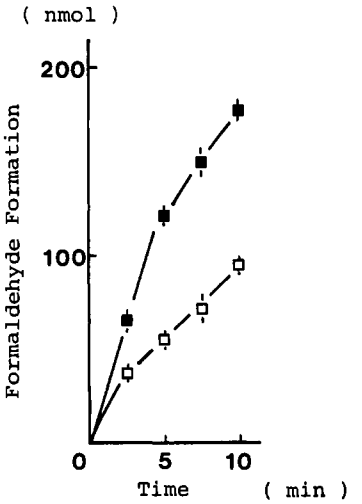

FIGURE 2: Time courses of formaldehyde formation from aminopyrine by phenobarbital-induced rat liver microsomes at 5 and 205 μM O_2. The incubations were carried out at 37 °C for 5 μM O_2 (□) and 205 μM O_2 (■). The bars express the standard deviation over three experiments. The concentration of microsome was 1.6 mg of protein/mL and 1.5 nmol of P-450/mg of protein.

directly as the initial absorbance change at time zero without the curve-fitting procedures of eq 2. It should be noted that $A(t)$ includes two independent physical events: one is $A(0)$, which results from the photodissociation of P-450·CO, and the other is CO recombination to photodissociated P-450, which is characterized by α_i and τ_i (i = 1, 2). Since $A(0)$ happens first and then is followed by CO recombination, the value $A(0)$ is not affected by the CO recombination process which happens after CO dissociation and was completed within 30 ms as judged by $\tau_i < 3$ ms because CO concentration was saturated (see Table I). The initial absorbance change $A(0)$ was decreased significantly as oxygen concentration was increased in both the presence and absence of aminopyrine. For example, $A(0)$ at 155 μM O_2 in the absence of aminopyrine was reduced to 23% of $A(0)$ at 0 μM O_2 (Table I). $A(0)$ is closely correlated with photodissociability of the P-450·CO complex. It must, however, be taken into account that $A(0)$ is also positively proportional to the amount of cytochrome P-450·CO.

It was demonstrated that the amount of cytochrome P-450·CO complex was decreased to some extent by increasing oxygen concentration (see Figure 4). Relative photodissociability may be obtained by normalization of $A(0)$ with the amount of P-450·CO complex measured as $\Delta OD_{450-490nm}$(Ab) from CO difference spectra. $A(0)$/Ab, thus calculated, should be independent of the amount of P-450·CO complex. Oxygen dependency of the normalized relative photodissociability

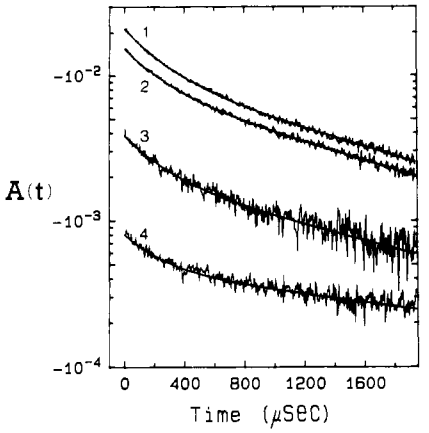

FIGURE 3: Decay curves of the flash-induced absorption change $A(t)$ of cytochrome P-450·CO complex in phenobarbital-induced rat liver microsomes at different oxygen concentrations. Measurements were performed as described under Experimental Procedures. The concentration of microsomes is 0.5 mg/mL. Curve 1, 90 μM O_2; curve 2, 115 μM O_2; curve 3, 155 μM O_2; curve 4, 155 μM O_2 in the presence of aminopyrine. Curves 1–3 are without aminopyrine. The zigzag lines are experimental data, and solid curves were obtained by fitting the data to eq 2. The vertical axis has a negative scale. Although the observed initial absorbance change was $A(0) = -0.0039$ for the sample at 155 μM O_2 with aminopyrine, curve 4 is artificially displaced vertically for illustrative purposes.

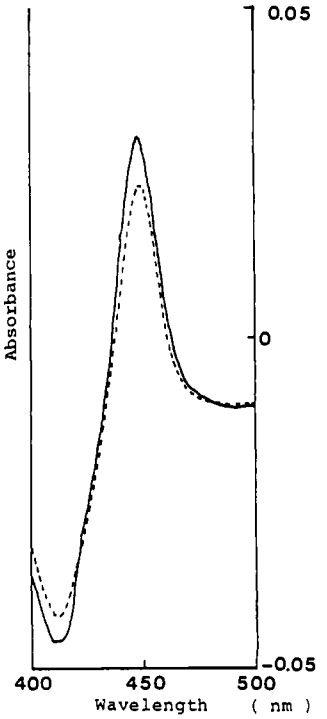

FIGURE 4: CO difference spectra of cytochrome P-450 in rat liver microsomes at different oxygen concentrations. Difference spectra were measured under the same conditions as flash photolysis experiments: 90 μM O_2 (solid line) and 155 μM O_2 (dashed line). Samples were reduced by an NADPH-regenerating system consisting of 0.4 mM NADP, 4 mM glucose 6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase. The concentration of microsome was 2 mg/mL and 1.5 nmol of P-450/mg of protein.

$A(0)/Ab$ of the cytochrome P-450·CO complex is illustrated in Figure 5. As the oxygen concentration was increased from 0 to 155 μM, the relative photodissociability was decreased both in the presence and in the absence of aminopyrine. For example, $A(0)/Ab$ at 155 μM O_2 in the absence of aminopyrine was 51% of that at 0 μM O_2. The addition of aminopyrine enhanced the effect of the oxygen-induced decrease in the relative photodissociability. In the presence of aminopyrine, $A(0)/Ab$ at 155 μM O_2 did decrease to 29% of $A(0)/Ab$ at 0 μM O_2.

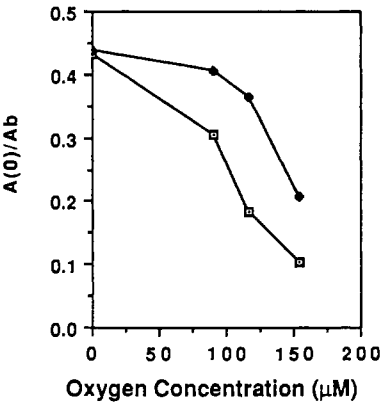

FIGURE 5: Effect of oxygen concentration on the normalized relative photodissociability $A(0)/Ab$. To exclude the contribution of the amount of P-450·CO complex, which depends upon the O_2 concentration, the observed initial absorbance change $A(0)$ was normalized with $Ab = \Delta OD_{450nm-490nm}$ which reflects the amount of P-450·CO complex. (□) In the presence of aminopyrine; (■) in the absence of aminopyrine. The vertical axis is drawn in arbitrary units.

The decrease in the P-450·CO complex by the presence of oxygen as shown in Figure 4 may suggest the presence of cytochrome P-450·O_2. A possible contribution of cytochrome P-450·O_2 complex to the laser flash-induced absorbance change was therefore carefully examined. In the absence of CO, no absorbance change at 450 nm [$A(t) \leq 0.0007$] was observed within experimental error for reduced microsomes at 0, 90, and 155 μM O_2, excluding the possibility that flash photolysis of the P-450·O_2 complex may affect the observed $A(t)$. Although direct spectral examination of the cytochrome P-450·O_2 complex was difficult because of instability of P-450·O_2, an absorbance difference possibly due to P-450·O_2 was observed to be smaller than about $^1/_{30}$th of that for P-450·CO from difference spectra of reduced P-450 at 155 μM O_2 minus reduced P-450 for the wavelength range 400–500 nm. The present results are not conflicting with the reported effect of P-450·O_2 by Roesen and Stier (1973) on the flash-induced absorbance change $A(t)$ at 445 nm, suggesting a possible rapid O_2 recombination and a slow CO recombination with reduced P-450. This is because their experimental conditions are very much different from our ones as follows: The presence of hexobarbital elevated the spectral amount of cytochrome P-450·O_2 complex to be about 20–40% of that of P-450·CO, and they measured $A(t)$ at 445 nm where P-450·O_2 showed significant absorption.

The remarkable decrease in $A(0)$ by oxygen cannot be completely explained by the decrease in P-450·CO to some extent. For example, although $A(0)$ for microsomes was reduced to 23% (−aminopyrine) and 14% (+aminopyrine) of that in the absence of O_2 by going from 0 to 155 μM O_2, the amount of P-450·CO (Ab) was decreased to 45% (−aminopyrine) and 48% (+aminopyrine). Therefore, we introduced $A(0)/Ab$ to quantitatively explain the O_2-induced decrease in the relative photodissociability free from contribution of the decreased Ab. We have concluded that molecular oxygen decreases the relative photodissociability of cytochrome P-450·CO, because $A(0)/Ab$ did decrease significantly with increasing oxygen concentration. Even in the presence of 155 μM O_2, the amount of cytochrome P-450·CO complex (Ab) remained unchanged before and after the 5-min flash photolysis measurements, excluding the possibility that the decreased $A(0)/Ab$ may be due to a decrease in the amount of CO-accessible ferrous P-450 during flash photolysis when molec-

ular oxygen is present. Metabolized endogenous substrates by cytochrome P-450 during incubation with oxygen could decrease $A(0)/Ab$ by preventing the photodissociation of P-450·CO. This possibility is also excluded, because the initial absorbance change $A(0)$ of microsomal P-450·CO in the absence of oxygen (i.e., reduced with dithionite and bubbled with CO) after 10-min incubation under a 20% O_2 stream was the same as that of microsomes reduced by dithionite without incubation under the oxygen stream.

In the presence of oxygen, three different species of P-450 (i.e., P-450^{2+}·CO, P-450^{3+}, and P-450^{2+}·O_2) are possibly present. Since the absorbance at 532 nm was observed to be almost the same between the presence and absence of oxygen, the photoabsorption by P-450·CO cannot be disturbed by the presence of P-450^{2+} and P-450·O_2 converted from P-450·CO, excluding the possibility that a decrease in photoabsorption of P-450·CO at 532 nm causes a decrease in $A(0)$.

The initial absorbance change $A(0)$ at 155 μM O_2 in the presence of 1 mM *N*-methylaniline, known as a type II substrate, was extremely decreased to about 5% of that in the absence of *N*-methylaniline (data not shown). However, the amount of cytochrome P-450·CO complex in the presence of *N*-methylaniline remained about 97% of that in the absence of *N*-methylaniline observed by CO difference spectra. These two independent phenomena may not be conflicting by interpreting as follows: Although CO binds with the heme iron to replace *N*-methylaniline, *N*-methylaniline still keeps interaction with the heme iron or the heme porphyrin ring, resulting in the significant decrease of P-450·CO photolysis by a laser flash at 532 nm, which might be due to the strengthened Fe–CO bond by interacting with *N*-methylaniline. The transient relative photodissociability obtained from flash photolysis is therefore strongly regulated by interactions between the heme and substrates independently of the steady-state amount of P-450·CO complex.

Such an effect of substrate-induced regulation in photodissociability has also been shown for cytochrome P-450_{MC} purified from rabbit liver microsomes by Imai et al. (1982). For example, photodissociability of P-450_{MC}·CO was significantly increased by 7–10-fold in the presence of 3-methylcholanthrene and 7,8-benzoflavone, whereas pyrene and triphenylene strongly inhibited photolysis of P-450_{MC}·CO. It should be noted that spectral amounts of P-450_{MC}·CO were not changed significantly by the presence of the above substrates, supporting further that the binding efficiency of CO to P-450 is independent of the photodissociability of P-450·CO. So far, however, there were no reports for the effect of oxygen concentration on flash photolysis of cytochrome P-450. This study implies that oxygen concentration as well as various substrates significantly affects the relative photodissociability of the heme·CO complex and suggests that increasing amounts of oxygen tighten the Fe–CO bond by affecting the electronic state of heme of the cytochrome.

Recombination of CO with Cytochrome P-450 in the Presence of Various Concentrations of O_2. The recombination of CO with reduced cytochrome P-450 after photolysis was apparently biphasic in phenobarbital-induced rat liver microsomes at different O_2 concentrations (see Figure 3) (Kawato et al., 1982). The total absorbance change $A(t)$ was analyzed by eq 2, and an average lifetime $\langle\tau\rangle$ of the photodissociated P-450 was calculated according to eq 3. Decay parameters are summarized in Table I. The average relaxation time $\langle\tau\rangle$ in the course of recombination of CO increased with increasing oxygen concentration from 0 to 155 μM. It was indicated that recombination of CO was interfered with increasing oxygen concentration. $\langle\tau\rangle$ was increased by 1.2 times from 752 μs at 0 μM O_2 to 904 μs at 155 μM O_2. In the presence of aminopyrine, $\langle\tau\rangle$ was increased by 2.4 times from 786 μs at 0 μM O_2 to 1901 μs at 155 μM O_2. The association of oxygen with ferrous P-450 is known to be much faster than that for CO association with ferrous P-450 by the stopped-flow kinetic studies of Stier and co-workers (Rosen & Stier, 1973). The observed oxygen-induced slowdown in CO recombination rate, which is inversely proportional to $\langle\tau\rangle$ as $k_{on} = 1/(\langle\tau\rangle \times 10^{-3})$, could be explained by interference due to rapid O_2 association of the cytochrome to form a possible transient cytochrome P-450·O_2 complex. Thereafter, O_2 of the P-450·O_2 complex would be slowly replaced by CO having much higher affinity for P-450, resulting in complete recovery of the P-450·CO complex within about 10 ms after photolysis of P-450·CO as judged by the relaxation time of $\langle\tau\rangle \lesssim 2$ ms needed for CO recombination to cytochrome P-450. At high oxygen concentrations of 155 μM O_2, there might be free O_2 molecules associable to the ferrous heme in the heme pocket of cytochrome P-450 when CO is dissociated.

Why is CO recombination to P-450 biphasic? There are several possible explanations. One possibility is that in PB microsomes there are several chemically different species of P-450_{PB}, e.g., P-450b, P-450e, etc., which show different kinetics for recombination with CO (Oertle et al., 1985; Lu & West, 1980). There are also other factors which induce the complex CO recombination kinetics. Partial saturation of cytochrome P-450_{PB} with different substrates such as phenobarbital and endogenous substrates probably might induce multiphasic rebinding of CO. Low-spin–high-spin equilibrium of P-450_{PB} may result in two different recombination kinetics with CO. Different aggregated states observed in PB microsomes (Kawato et al., 1982) might cause different recombination kinetics of CO. Therefore, the expected time course of CO recombination should be essentially multiphasic. However, practically the double-exponential approximation with eq 2 fits sufficiently well to the experimental data, and approximation consisting of more than triple-exponential terms was not necessary. Therefore, we took the double-exponential approximation for curve fitting using eq 2. The observed two fractions of the decay do not correspond to distinct P-450 fractions but might reflect the average of the above different states of P-450_{PB}.

Many investigations demonstrate effects of substrates on the recombination of CO with cytochrome P-450, although there were no reports treating oxygen as a substrate. Imai and co-workers (Imai et al., 1982) have studied the various effects of different hydrocarbon substrates on the recombination of CO with cytochrome P-450_{MC}. Dibenzanthracenes and 3-methylcholanthrene were observed to significantly decrease the CO recombination rate to about $^1/_{10}$th to $^1/_{20}$th of the control, while phenanthrene and anthracene increased the recombination rate by about 20–30 times. It was also shown that the binding of *d*-camphor to P-450_{cam} greatly reduced the CO recombination rate to $^1/_{100}$th of the control (Peterson & Griffin, 1972).

Hypothesis. Here we propose a hypothesis explaining a dramatic decrease in the photodissociability, but a small effect on CO recombination, of cytochrome P-450·CO complex by increasing the oxygen concentration. A few oxygen molecules could be present in the active site of cytochrome P-450·CO when the oxygen concentration is increased, because the size of the oxygen molecule is much smaller than that of substrates of P-450_{PB} such as PB, aminopyrine, and benzphetamine. As judged from the crystalline structure P-450_{cam}, there is enough

space for more than several oxygen molecules present in the heme pocket of the cytochrome (Poulos et al., 1985, 1987; Poulos & Howard, 1987). Also, dynamic fluctuation of the protein structure could allow frequent diffusion-controlled exchange of several oxygen molecules into and from the heme pocket of P-450 (Nakanishi & Tsuboi, 1978). There are reports which suggest significant oxygen diffusion within proteins, as judged from the dynamic quenching of tryptophan fluorescence of many different types of proteins by oxygen molecules (Lackowicz & Weber, 1973). Since the π-electron orbital covers the heme plane and Fe–CO, interactions of O_2 with the heme plane could modulate electronic state of the heme to strengthen the Fe–CO bond, resulting in decreased photodissociation. Another possible interpretation is that the neighboring O_2 molecules quench the alteration of the electronic bond of Fe–CO immediately after absorption of the laser flash energy by the heme, preventing the photodissociation of heme–CO. The other least likely case is that interactions of O_2 with the heme might change the conformation of the heme pocket, resulting in an increase of the steric hindrance, offered by the distal amino acids, preventing photodissociation.

Only a small effect of O_2 on the recombination rates of CO should be due to much higher affinity of CO to the P-450 heme than O_2. Of course, neighboring O_2 in the substrate binding pocket would bind to the heme iron immediately after photodissociation of CO; this O_2 can be easily replaced by CO coming back, resulting in only a small decrease in the CO recombination rates.

Further biophysical investigations are necessary to get a better idea about the effect of nonreactive oxygen on the electronic state of the heme of cytochrome P-450. The present observation of an oxygen-induced decrease in the relative photodissociability of P-450·CO shows an interesting example of possible effects of nonreactive oxygen molecules around the heme of P-450.

Registry No. P-450, 9035-51-2; CO, 630-08-0; O_2, 7782-44-7; heme, 14875-96-8; aminopyrene, 64990-23-4.

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