High-Pressure Flash Photolysis Study of Hemoprotein: Effects of Substrate Analogues on the Recombination of Carbon Monoxide to Cytochrome $P450_{CAM}$†

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ABSTRACT: The effects of camphor and camphor analogues on the CO recombination kinetics of ferrous cytochrome $P450_{CAM}$ ($P450_{CAM}$) at 293 K have been studied as a function of hydrostatic pressure (0.1–200 MPa) by means of flash photolysis. At 0.1 MPa, the association rate constant (k_{on}) for substrate-free $P450_{CAM}$ is 8.5×10^6 M^{-1} s^{-1}. Measurements as a function of pressure lead to a determination of the activation volume ($\Delta V^{\ddagger}$) of +4 cm^3 mol^{-1} for substrate-free protein. This positive $\Delta V^{\ddagger}$ is interesting because the CO association reaction of various hemoproteins, such as myoglobin and hemoglobin, exhibit negative $\Delta V^{\ddagger}$ values [Adachi, S., & Morishima, I. (1989) *J. Biol. Chem. 264*, 18896–18901; Unno, M., Ishimori, K., & Morishima, I. (1990) *Biochemistry 29*, 10199–10205]. The binding of *d*-camphor and some camphor analogues (*d*-fenchone, 3-*endo*-bromocamphor, and 3,3,5,5-tetramethylcyclohexanone) into the heme pocket strongly influences the kinetics, i.e., k_{on} is reduced ($(1–10) \times 10^5$ M^{-1} s^{-1}) and $\Delta V^{\ddagger}$ is altered to a negative value (−14 to −32 cm^3 mol^{-1}). The negative $\Delta V^{\ddagger}$ suggests that the effects of camphor and these camphor analogues are due to an increase in the iron–ligand bond formation barrier. On the other hand, the binding of adamantane and norcamphor does not affect the kinetics. This result is particularly surprising because both substrate analogues are located in the immediate vicinity of the CO binding site. Since both adamantane and norcamphor show high mobility in the heme active site, we conclude that a substrate fluctuation at the heme active site is an important determinant of the rate of the bond formation process.

Cytochrome $P450_{CAM}$, a hemoprotein from *Pseudomonas putida*, catalyzes the regio- and stereospecific hydroxylation of its substrate, *d*-camphor, at the 5-*exo* position (Ortiz de Montellano, 1986). Because of its ready availability, soluble nature, and extensive characterization, it has become a prototype for the entire P450 family of enzymes. The molecular structure of $P450_{CAM}$[1] has been well established by numerous spectroscopic studies [Dawson and Sono (1987) and references therein] and more recently by X-ray crystal structure determinations [Poulos and Raag (1992) and references therein]. These studies show that the camphor molecule is buried in an internal pocket just above the heme distal surface adjacent to the ligand binding site. The substrate molecule is held in place by a hydrogen bond between the hydroxyl group of Tyr-96 and the camphor carbonyl oxygen atom, in addition to complementary hydrophobic contacts between the camphor molecule and the neighboring hydrophobic residues (Figure 1).

One of the interesting aspects of $P450_{CAM}$ is the substrate dependence of its reactivity and physical properties. The binding of the substrate camphor lowers the ligand affinity of ferrous $P450_{CAM}$ for carbon monoxide by 10-fold, and this lower affinity is mainly attributed to a decrease in the association rate by 100-fold (Peterson & Griffin, 1972). Since the substrate molecule is present in the immediate vicinity of the ligand binding site (Figure 1), the substrate-dependent

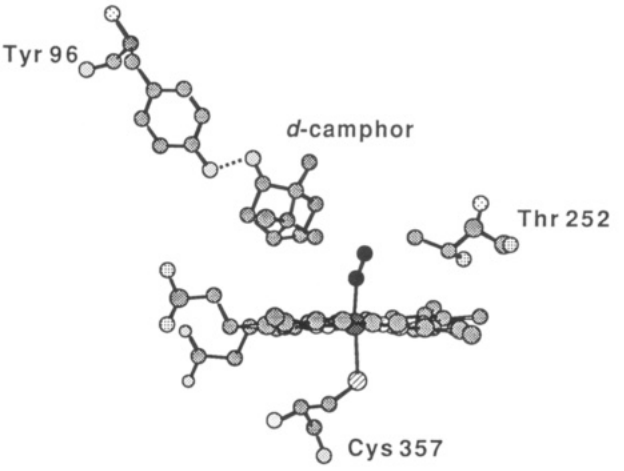

FIGURE 1: Heme environmental structure of the CO complex of $P450_{CAM}$ (Raag & Poulos, 1989). The heme and some selected residues, including camphor and bound carbon monoxide (black atoms), are shown. The dotted line connecting the Tyr-96 hydroxyl group and the camphor carbonyl oxygen atom indicates a hydrogen bond.

kinetics may be due to a strong interaction of the substrate with CO. Further evidence that the CO ligand interacts with the substrate is given by resonance Raman studies of the ferrous $P450_{CAM}$–CO complex, which show that the ν_{Fe-CO} mode is highly sensitive to the substrate structure (Uno et al., 1985; Wells et al., 1992). The C–O stretching IR mode of the carbon monoxide ligand in ferrous $P450_{CAM}$ in the absence or presence of camphor and in the presence of various camphor analogues was also examined (Jung et al., 1992). These Raman and IR studies demonstrate that steric hindrance and substrate mobility markedly affect the iron-bound CO molecule. Therefore, studies on the effects of substrate and substrate analogues allow us to examine how the steric hindrance of the

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; Hb, hemoglobin; Mb, myoglobin; TMCH, 3,3,5,5-tetramethylcyclohexanone; $P450_{CAM}$, cytochrome $P450_{CAM}$.

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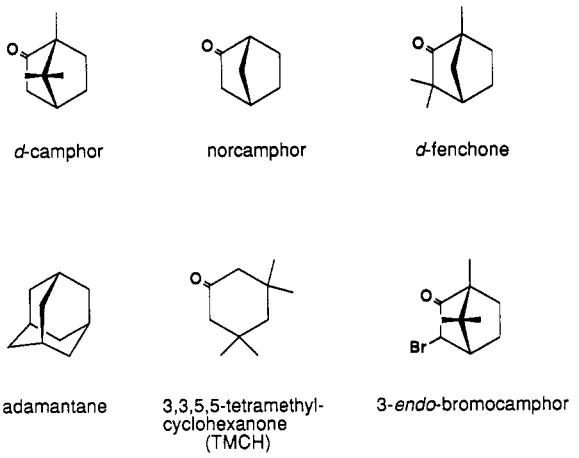

FIGURE 2: Substrate and substrate analogue structures.

heme environment and the mobility of the substrate influence the kinetics of CO binding to P450$_{CAM}$.

Recently, the recombination of ligands with a variety of model hemes and hemoproteins under high pressure has been investigated by our group (Adachi & Morishima, 1989; Unno et al., 1990, 1991; Adachi et al., 1992) and others (Projahn et al., 1990; Taube et al., 1990; Frauenfelder et al., 1990; Projahn & van Eldik, 1991). In analogy to the way temperature-dependence studies help determine the energetics of the ligand binding process, pressure-dependence studies reveal information on the volume profile of the reaction. The activation volume, the difference in the partial molar volume between the activated complex and the reactants, is sensitive to the dynamical aspects of the biological reaction; that is, the observed pressure effects are attributed to the contributions of bond formation and cleavage, solvation effects, changes in the spin state of the heme iron, and changes in the protein conformation. In this study, we examine the recombination reaction of carbon monoxide with ferrous P450$_{CAM}$ in the presence or absence of camphor and in the presence of five camphor analogues (Figure 2) under various pressures (0.1–200 MPa). The influence of reduced putidaredoxin, a physiological electron donor of P450$_{CAM}$, on the kinetics is also investigated. On the basis of these kinetic investigations, we discuss the details of the dynamics of CO binding to P450$_{CAM}$.

MATERIALS AND METHODS

Preparation of Proteins. P450$_{CAM}$ was prepared and purified as described elsewhere (Gunsalus & Wagner, 1978). The purified enzyme preparations with >1.4 *RZ* value[2] were employed for the experiments. Finally, the protein was dialyzed against 0.05 M Tris·HCl–0.05 M KCl buffer (pH 7.4) and concentrated by ultrafiltration to a concentration of ~1 mM.

Camphor-bound P450$_{CAM}$ solution for the laser photolysis measurements was obtained from dilution of the concentrated protein into 0.05 M Tris·HCl buffer, 0.2 M KCl, 5% glycerol, and 1 mM *d*-camphor (pH 7.4). The substrate-free form of P450$_{CAM}$ was prepared by passage over a Sephadex G-25 column previously equilibrated with 0.05 M Tris·HCl buffer containing 5% glycerol at pH 7.4, 277 K. Then, 2 M KCl was added to substrate-free enzyme solution to yield a final K^+ concentration of about 0.2 M. Norcamphor, *d*-fenchone, 3-*endo*-bromocamphor, and 3,3,5,5-tetramethylcyclohexanone were obtained from Aldrich Chemie. *d*-Camphor and adamantane were from Wako Pure Chemical Industries, Ltd. These substrates and substrate analogues were used without further purification. The substrate analogue-bound P450$_{CAM}$ was formed by proper dilution of a 0.1–1 M stock solution of substrate analogue/*N,N*-dimethylformamide (Nacali Tesque, Inc.) into the substrate-free protein solution. The final concentration of norcamphor, fenchone, and TMCH was 2 mM. For adamantane- and bromocamphor-bound complexes, the sample solutions were saturated with these reagents and the excess solid was removed by centrifugation. UV/vis absorption spectra for the ferric species were recorded on a Hitachi U-3210 spectrophotometer. The fraction of the high-spin ferric P450$_{CAM}$ was obtained from optical spectra by using established extinction coefficients for pure high-spin and low-spin species (Sliger, 1976). The estimated fractions of the high-spin species are listed in the last column of Table 1, and the values are almost identical to those of Fisher and Sligar (1985). Since the ferric high-/low-spin equilibrium is sensitive to the presence of substrate, we exclude the possibility that the protein is not saturated with the substrate analogue in our experimental conditions.

The CO complexes of ferrous P450$_{CAM}$ used for the laser photolysis measurements were prepared as follows. The air above the protein solution was replaced with CO to remove oxygen. Sodium dithionite was added to the O_2-free, CO-equilibrated solution to reduce the ferric iron to the ferrous CO adduct at room temperature (~293 K). Putidaredoxin was isolated from bacterial cultures, purified, and prepared as described by Gunsalus and Wagner (1978).

Flash Photolysis Measurements. The experimental procedure for determining the CO association rate constant was described previously (Unno et al., 1990, 1991). Rhodamine 6G (Kodak) in methanol was used to produce an excitation flash at 590 nm, with a half-peak duration of 1 μs. The monitoring beam was generated by a xenon lamp. The high-pressure photolysis experiments took place by using a four-window high-pressure cell and its inner capsule (Hara & Morishima, 1988). All experiments were performed in 0.05 M Tris·HCl–0.2 M KCl buffer and 5% glycerol at pH 7.4. This buffer solution has been shown to exhibit no pressure-dependent pH change in the pressure region examined here (Neuman et al., 1973). The protein concentration was 5–10 μM.

Evaluation of Kinetic Parameters. CO rebinding to the substrate- and substrate analogue-bound P450$_{CAM}$'s at various pressures was monitored at 446 nm and analyzed by fitting to

$$\Delta A_t = \Delta A_0 \exp(-k_{app}t) \quad (1)$$

where ΔA_t is the absorbance change at any time t, ΔA_0 is the total change (absorbance at $t = 0$ minus absorbance at $t = \infty$), and k_{app} is the observed first-order rate constant. Because the camphor-free form is unstable, the pressure experiment produced inactivated protein. Therefore, the time courses for the recombination of CO with camphor-free P450$_{CAM}$ were fit to

$$\Delta A_t = \Delta A_f \exp(-k_f t) + \Delta A_s \exp(-k_s t) \quad (2)$$

where k_f and k_s are the observed fast and slow first-order rate constants, respectively, and ΔA_f and ΔA_s are the amplitudes of the exponential expressions for the fast and slow phases, respectively.

[2] The *RZ* value is the ratio A_{392}/A_{280}, where A is the absorbance.

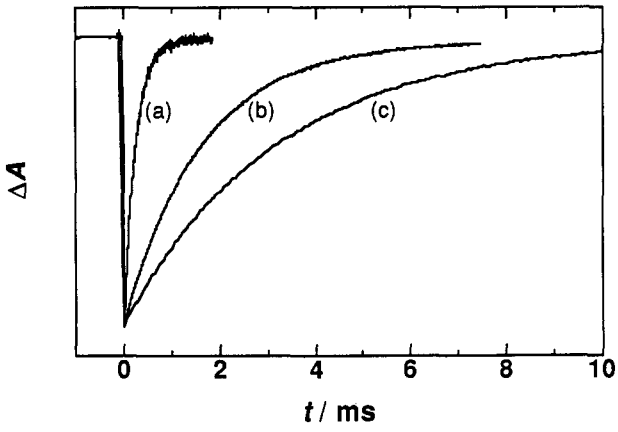

FIGURE 3: Rebinding of CO to norcamphor (a), fenchone (b), and TMCH (c) complexes of $P450_{CAM}$ at 293 K, 0.1 MPa. The reactions were carried out in 0.05 M Tris·HCl–0.2 M KCl buffer at pH 7.4 containing 5% glycerol and were monitored at 446-nm absorption. The concentration of the substrate analogues was 2 mM. Photolysis was accomplished with a 1-μs laser flash (590 nm).

The activation volume is given by

$$\Delta V^{*} = -RT\left(\frac{\partial(\ln k_P/k_1)}{\partial P}\right)_T \qquad (3)$$

where R is the gas constant (= 8.314 J K^{-1} mol^{-1}), and k_1 and k_P are observed first-order rate constants at 0.1 and P MPa, respectively. The slope, $[\partial(\ln k_P/k_1)/\partial P]_T$, for the camphor-, fenchone-, TMCH-, and bromocamphor-bound complexes at atmospheric pressure was calculated from the optimized third-order polynomial function (van Eldik et al., 1989). Linear plots were used for the camphor-free and adamantane-, and norcamphor-bound $P450_{CAM}$'s.

RESULTS

Time courses for CO rebinding to ferrous $P450_{CAM}$ in the presence of excess substrate and substrate analogues are shown in Figure 3. Simple exponential time courses were observed for all substrate- and substrate analogue-bound complexes, and no evidence for kinetic heterogeneity was observed. On the basis of the CO concentration dependence of the rebinding rate, we have determined the bimolecular association rate constant (k_{on}), and the values are listed in the first column of Table 1. Figure 3 and Table 1 indicate that k_{on} is highly sensitive to the substrate structure. For example, the alteration of a substrate from camphor to adamantane causes a 130-fold increase in k_{on}, corresponding to a ~12 kJ mol^{-1} reduction in the kinetic barrier. In contrast to the substrate- and substrate analogue-bound forms, the recombination of CO with substrate-free $P450_{CAM}$ exhibits biphasic kinetics (Figure 4A). Because the substrate-free form is unstable, inactivated protein is readily produced. It is therefore likely that the fast and slow processes correspond to the rebinding of CO to native and to inactivated proteins, respectively. In accord with this view, k_{on} for the fast process is 8.5×10^6 M^{-1} s^{-1}, and this value is almost identical to the literature value of 5.1×10^6 M^{-1} s^{-1} (Peterson & Griffin, 1972).

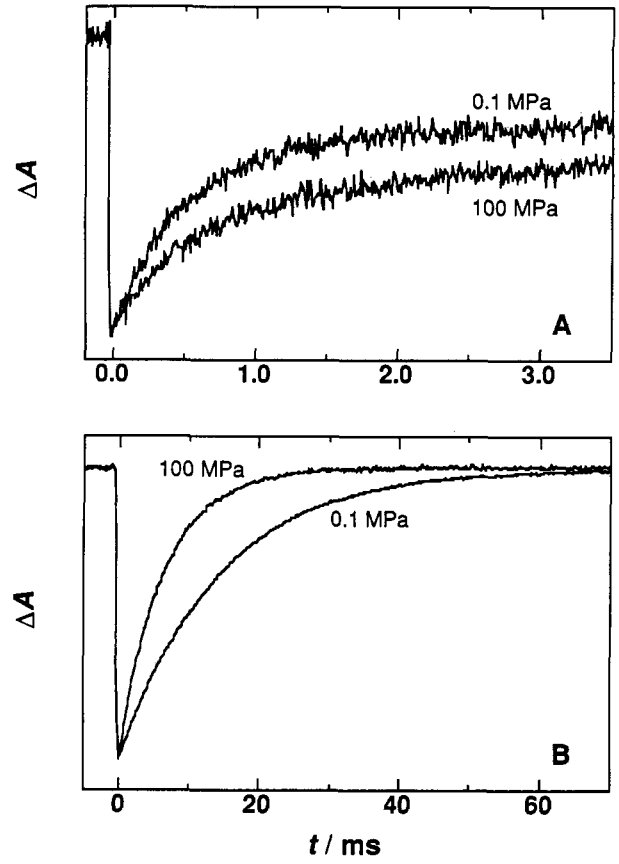

FIGURE 4: Effect of hydrostatic pressure on the recombination kinetics of CO to substrate-free (A) and camphor-bound (B) $P450_{CAM}$'s at different pressures. Experimental conditions are as listed for Figure 3. For substrate-free $P450_{CAM}$, the fractions of the slow phase at 0.1 and 100 MPa are 0.36 and 0.58, respectively.

We have examined the CO binding kinetics of $P450_{CAM}$ as a function of hydrostatic pressure. As an example, we illustrate the effect of pressure on the normalized time courses for

Table 1: Various Substrate-Dependent Parameters for Cytochrome $P450_{CAM}$'s

substrate	$k_{on} \times 10^{-5}$ [a] (M^{-1} s^{-1})	ΔV^{*} [a] (cm^3 mol^{-1})	ν_{C-O} [b] (cm^{-1})	high spin[c] (%)
adamantane	130 ± 20	+7 ± 3	1929 (16%) 1940 (24%) 1955 (60%)	69
norcamphor	100 ± 10	+3 ± 2	1947 (100%)	53
substrate-free	85 ± 1	+4 ± 1	1918 (9%) 1942 (63%) 1955 (16%) 1964 (12%)	4.0
fenchone	15 ± 1	−20 ± 1	1945 (100%)	60
TMCH	7.5 ± 0.1	−14 ± 1	1934 (100%)	26
bromocamphor	5.5 ± 0.1	−32 ± 2	1914 (6%) 1934 (94%)	74
camphor	1.0 ± 0.1	−31 ± 2	1941 (100%)	99

[a] Experimental conditions were 0.05 M Tris·HCl–0.2 M KCl buffer and 5% glycerol at pH 7.4, 293 K. The values for CO binding kinetics at 0.1 MPa are listed in this table. The errors are listed as the standard deviations from the means. [b] Jung et al. (1992). [c] Experimental conditions were 0.05 M Tris·HCl–0.2 M KCl buffer and 5% glycerol at pH 7.4, 293 K.

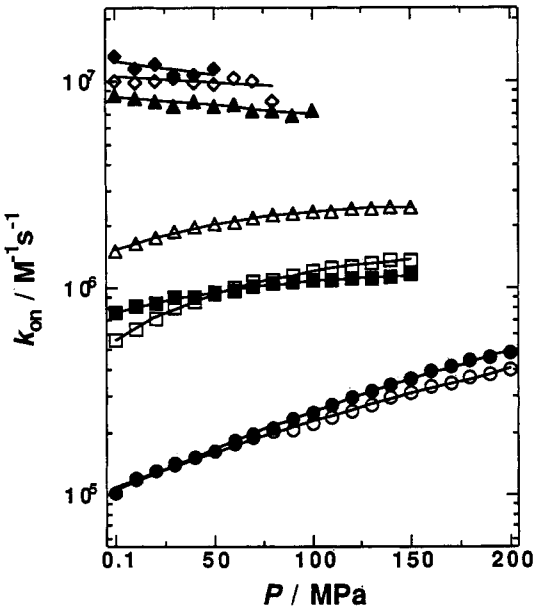

FIGURE 5: Logarithmic plots of the rate constants for the bimolecular CO association reaction vs hydrostatic pressure for substrate-free (▲) and adamantane- (◆), norcamphor- (◇), fenchone- (△), TMCH- (■), bromocamphor- (□), and camphor-(○) bound P450$_{CAM}$'s. The rate constants for camphor-bound complex in the presence of reduced putidaredoxin (●) are also shown in this figure.

substrate-free and camphor-bound P450$_{CAM}$'s in Figure 4. One characteristic feature of the pressure-dependence studies is a decrease in the photoinduced absorbance change (which we call the apparent quantum yield) at elevated pressure (not shown). Such an apparent quantum yield change caused by pressure was observed for all P450 samples, and this change was almost fully reversible. Because pressure-induced conversion between P450 and P420 [an inactive form of P450 (Gunsalus et al., 1974; Wells et al., 1992)] is an irreversible process (Hui Bon Hoa et al., 1989), the low apparent quantum yield under high pressure is not due to the formation of P420 species. Furthermore, we observed little effect of pressure (up to 200 MPa) on the UV/vis absorption spectrum of the camphor-bound P450$_{CAM}$–CO complex. This is direct evidence that the low apparent quantum yield at high pressure does not arise from generation of P420.

Pressure causes changes in the bimolecular association rate for CO binding to P450$_{CAM}$. The association rate constants under various pressures are estimated and the values are given in Figure 5, where we present the log plots of k_{on} against hydrostatic pressure, P. The activation volumes were determined from the slope (eq 3), and the resultant values at 0.1 MPa are listed in the second column of Table 1. According to the effect of pressure on k_{on}, the CO binding kinetics may be classified into two groups: (i) the first group includes the kinetics of substrate-free and adamantane- and norcamphor-bound complexes. In this group, k_{on} is slightly reduced by pressure, implying a positive activation volume (+3–7 cm^3 mol^{-1}). For instance, k_{on} for the substrate-free P450$_{CAM}$ was decreased from 8.5×10^6 (0.1 MPa) to 7.2×10^6 M^{-1} s^{-1} (100 MPa), and ΔV^* is estimated to be +4 cm^3 mol^{-1}. (ii) On the other hand, the second group consists of the fenchone-, TMCH-, bromocamphor-, and camphor-bound proteins. These complexes exhibit a negative activation volume ranging between −14 and −32 cm^3 mol^{-1}. Upon elevation of the pressure from 0.1 to 200 MPa, k_{on} for the camphor-bound complex was increased from 1.0×10^5 to 4.0×10^5 M^{-1} s^{-1}, and ΔV^* at 0.1 MPa is calculated as −31 cm^3 mol^{-1}. In addition to the sign of ΔV^*, the two groups exhibit different association rate constants; k_{on} for group i, greater than $\sim 1 \times 10^7$ M^{-1} s^{-1}, is distinctly larger than that for group ii, less than $\sim 1 \times 10^6$ M^{-1} s^{-1}.

Table 2: Kinetic Parameters for Myoglobin and Hemoglobin

sample	ligand	$k_{on} \times 10^{-5}$ (M^{-1} s^{-1})	ΔV^* (cm^3 mol^{-1})
sperm whale Mb[a]	O_2	240	+5
	CO	6.7	−9
horse Mb[a]	O_2	290	+4
	CO	6.8	−13
dog Mb[a]	O_2	300	0
	CO	8.5	−19
human Mb[b]	O_2	130	+4
	CO	7.0	−21
R-state Hb[c]	CO	91	−9
T-state Hb[c]	CO	4.2	−32
Hb α-chain[d]	CO	51	−18
Hb β-chain[d]	CO	130	−22
R-state Hb (α-subunit)[e]	O_2	26	+8
R-state Hb (β-subunit)[e]	O_2	68	+6

[a] Adachi & Morishima, 1989. [b] Adachi et al., 1992. [c] Unno et al., 1990. [d] Unno et al., 1991. [e] Unno et al., unpublished results.

Finally, we compare the effect of pressure on the recombination of CO with camphor-bound complex in the presence and absence of reduced putidaredoxin. The concentration of putidaredoxin was ~5-fold larger than that of P450 protein. The reaction cycle of P450$_{CAM}$ involves the electron donation process from putidaredoxin to P450$_{CAM}$ (Ortiz de Montellano, 1986). It has been suggested that complex formation of P450$_{CAM}$ with putidaredoxin causes an active site structural change (Makino et al., 1984; Shiro et al., 1989). From infrared and resonance Raman studies of the CO adduct of ferrous P450$_{CAM}$, Makino et al. (1984) suggested that putidaredoxin binding alters the iron-bound CO configuration. Thus, we may anticipate an influence of putidaredoxin on the CO binding kinetics. However, Gunsalus and Sligar (1978) reported that k_{on} at ordinary pressure is almost unaltered by the presence of putidaredoxin. Here we show that putidaredoxin binding does not affect the activation volume (Figure 5). The kinetics and activation volumes at 0.1 MPa in the presence and absence of putidaredoxin are nearly indistinguishable, although k_{on} at elevated pressure is slightly increased by putidaredoxin binding.

DISCUSSION

Rate-Limiting Step for CO Rebinding to P450$_{CAM}$. Studies on the pressure dependence of ligand binding to Mb and Hb (Table 2) can be used to determine the rate-limiting process. In Figure 6, we display the values of ΔV^* for various samples at 293 K as a function of k_{on}. As illustrated in the figure, the ligand binding reaction of hemoproteins could be divided into two cases and their intermediate. The reaction, a, showing positive ΔV^* and large k_{on}, greater than $\sim 10^7$ M^{-1} s^{-1}, represents the case where ligand entry into the heme pocket contributes to the rate of ligand association [e.g., O_2 binding to Mb and R-state Hb] (Adachi & Morishima, 1989; Taube et al., 1990; Unno et al., unpublished results). It was reported that a diffusion-controlled reaction, such as CO binding to protoheme in highly viscous solvents, gives a positive ΔV^* (~2–14 cm^3 mol^{-3}) (Caldin & Hasinoff, 1975). Further, the positive ΔV^* for reaction a could be partly explained by a gatelike conformational change in the protein matrix. On the other hand, the rate-limiting step for reaction b, showing negative ΔV^* and smaller k_{on}, less than $\sim 10^6$ M^{-1}s^{-1}, is the iron–ligand bond formation process [e.g., CO rebinding to

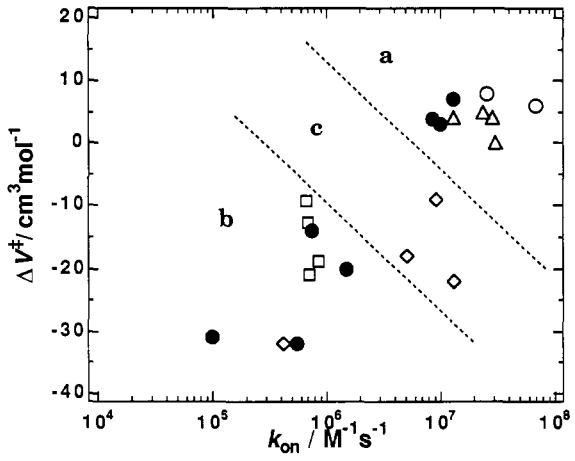

FIGURE 6: $\Delta V^{\ddagger}$ vs k_{on} plots for ligand binding to various hemoproteins: ●, CO binding to P450$_{CAM}$; □, CO binding to Mb; △, O_2 binding to Mb; ◇, CO binding to Hb; ○, O_2 binding to Hb. The points were taken from Tables 1 and 2.

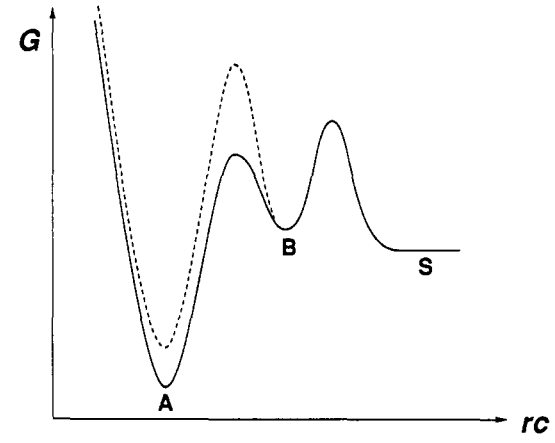

FIGURE 7: Relative free energy (G) plotted schematically plotted vs the reaction coordinate, rc; A represents the CO-bound state, B, the geminate state where CO is still in the heme pocket, and S is in the solvent phase. The solid and dashed curves represent the free energies for groups i and ii, respectively. Since there is not enough information, we cannot illustrate the free energy at state B and the S→B kinetic barrier for group ii.

Mb and T-state Hb] (Adachi & Morishima, 1989; Taube et al., 1990; Unno et al., 1990). It is thought that the negative $\Delta V^{\ddagger}$ is mainly contributed by the iron–ligand bond formation and heme structural changes from out-of-plane to in-plane structures. The intermediate situation appears in reaction c [e.g., CO binding to R-state Hb and Hb isolated chains], which is characterized by a negative $\Delta V^{\ddagger}$ and a k_{on} lager than that of b. In this case, we showed that both ligand entry and the bond formation process affect the association rate constant comparatively (Unno et al., 1990, 1991).

When we discuss the ligand binding process in terms of $\Delta V^{\ddagger}$, the contribution of a water molecule should not be overlooked. From a series of kinetic studies of mutant Mb, Olson and co-workers have shown that one of the dominant factors governing the CO association rate appears to be water displacement from the distal pocket (Smerdom et al., 1991; Li et al., 1994). For P450$_{CAM}$, if a substrate is not structurally complementary to the active site, the water molecule retains partial access to the heme iron (Fisher & Sligar, 1987). Thus, the water replacement in the heme pocket could contribute to the observed $\Delta V^{\ddagger}$. However, Table 1 shows that $\Delta V^{\ddagger}$ and the high-spin character, which is a good measure of the water accessibility of the active site (Fisher & Sligar, 1987), are independent quantities. For instance, the adamantane complex is characterized by a positive $\Delta V^{\ddagger}$ (+7 cm^3 mol^{-1}) and exhibits 69% high spin. On the other hand, the bromocamphor complex shows essentially the same high-spin character (74%); however, $\Delta V^{\ddagger}$ is negative value (−32 cm^3 mol^{-1}). It is therefore likely that the effect of water displacement on $\Delta V^{\ddagger}$ is negligible in the first approximation. According to the above considerations, the kinetics of hemoproteins could be classified in the $\Delta V^{\ddagger}$ vs k_{on} plot on the basis of the rate-limiting process. In short, $\Delta V^{\ddagger}$ for the iron–ligand bond formation process is a negative value, while the ligand entry process exhibits a positive $\Delta V^{\ddagger}$.

In the present study, the CO binding kinetics for group ii, fenchone-, TMCH-, bromocamphor-, and camphor-bound complexes, is characterized by a negative $\Delta V^{\ddagger}$. Although the active site structure of P450$_{CAM}$ is different from those of Mb and Hb, the negative value is consistent with the iron–ligand bond formation being rate-limiting. In contrast, a positive $\Delta V^{\ddagger}$ is observed for group i: substrate-free and adamantane-, and norcamphor-bound P450$_{CAM}$'s. The positive value implies a contribution of the entry process to k_{on}. The observation of positive $\Delta V^{\ddagger}$ is unexpected since the bimolecular association reaction of CO with various other hemoproteins is characterized by a negative $\Delta V^{\ddagger}$ and the bond formation process is rate-determining (Adachi & Morishima, 1989; Taube et al., 1990; Unno et al., 1990, 1991).

To discuss such different behavior between groups i and ii, we utilize the free energy diagram of the ligand binding process (Figure 7). For group i, ligand entry into the heme pocket is the rate-limiting step and it is represented as a solid curve. Taking into account that k_{on} for group ii is smaller than that for group i, the free energy profile for group ii is depicted by the dashed curve, in accordance with substrate-dependent switching of the rate-limiting step from a ligand entry to a bond formation process. From X-ray crystallographic investigations (Poulos & Raag, 1992), it has been demonstrated that the bound camphor and its various analogues are located adjacent to the CO binding site. Thus, it is reasonable that the binding of substrate and substrate analogues for group ii increases the iron–ligand bond formation barrier. However, the lack of an effect of adamantane and norcamphor binding on the kinetics is surprising. In what follows, we will discuss why these two substrate analogues do not affect the kinetics of P450$_{CAM}$.

Influence of Substrate Mobility on the CO Binding Kinetics. IR spectroscopy of hemoproteins can provide unique information about the structure of the heme moiety. The C–O stretching frequency is an indirect measure of the angle of the CO dipole relative to the heme normal (Ormos et al., 1988; Moore et al., 1988), which reflects the steric interaction between the bound CO and neighboring residues (Yu et al., 1983).[3] In the case of MbCO, it has been suggested (Morikis et al., 1989) that the high-frequency band (1965 cm^{-1}) corresponds to the widely opened distal pocket, whereas the low-frequency band (1945 cm^{-1}) is related to the restricted pocket. For P450$_{CAM}$, Jung et al. (1992) measured the CO stretching mode in the absence or presence of camphor and in the presence of camphor analogues (their results are summarized in the third column of Table 1). Comparison of

[3] It is necessary to comment here on the recent studies of Braunstein et al. (1993) and Li et al. (1994). They examined IR spectra of several mutants of MbCO and demonstrated that the Fe–C–O configuration does not arise from the steric constraints imposed by the distal pocket residue. This implies that we may not discuss the steric hindrance at the heme active site with the IR absorption of the CO ligand.

the kinetic measurements reported here with the IR data clearly demonstrates that k_{on} is not entirely correlated to ν_{C-O}, and we may not relate the association rate with the steric constraints at the active site. For example, the substrate-free and adamantane-bound $P450_{CAM}$'s are characterized by a high-frequency stretching band. However, the norcamphor complex does not show such a high-frequency band, although k_{on} is almost identical to those of substrate-free and adamantane-bound complexes. The above conclusion appears to be further supported by the finding that there is no correlation between the molecular volume of the substrate and k_{on}, e.g., the order of molecular volume is camphor (315 Å^3) > adamantane (293 Å^3) > norcamphor (236 Å^3), whereas that of k_{on} is adamantane (1.3×10^7 M^{-1} s^{-1}) > norcamphor (1.0×10^7 M^{-1} s^{-1}) ≫ camphor (1.0×10^5 M^{-1} s^{-1}). We therefore conclude that the steric constraint is not a main determinant of the CO association rate of $P450_{CAM}$.

Unlike the natural substrate camphor, which is hydrogen-bonded to Tyr-96 (Figure 1), adamantane does not hydrogen bond to the protein. Raag and Poulos (1991) demonstrated that the absence of a substrate–protein hydrogen bond allows greater mobility of the substrate in the heme pocket, indicated by the crystallographic temperature factor of 30.1 Å^2 [the temperature factor for the bound camphor is 16.2 Å^2 (Poulos et al., 1985)]. The multiplicity and the larger half-width of the CO stretching band also conform with its high mobility. Similarly, the bound norcamphor also exhibits a high temperature factor of 33.5 Å^2 (Raag & Poulos, 1989). Since norcamphor lacks the 8-, 9-, and 10-methyl groups of camphor, specific interactions between these groups and Phe-87 and Val-247 and -295 are missing, and hence it is reasonable that norcamphor is more "loosely" bound than camphor. This conclusion is also suggested by a recent molecular dynamics simulation of the norcamphor–$P450_{CAM}$ complex (Bass et al., 1992). Case and Karplus (1979) demonstrated that motion of a small ligand inside a protein may be possible only through protein conformational fluctuations. Thus, it is reasonable that the higher fluctuation of the bound substrate leads to a lower kinetic barrier (Figure 7).

At this stage, we should comment that our results may be partly explained by the possibility that the photolysis of CO ligand in $P450_{CAM}$ induces dissociation of the substrate molecule from the heme active site. This view was suggested by resonance Raman (Wells et al., 1992) and photoacoustic calorimetric (Di Primo et al., 1993) studies of CO–$P450_{CAM}$. Although we cannot rule out that the photolysis of CO ligand causes complete escape of the substrate, this may allow us to expect two-exponential kinetics, i.e., recombination with the substrate-free (fast phase) and substrate-bound (slow phase) complexes. However, because no kinetic heterogeneity was observed for any of the substrate- and substrate analogue-bound complexes (Figures 3 and 4), we would exclude the effect of substrate dissociation on the kinetics.

Conclusions. The most characteristic feature of the present kinetic experiments of $P450_{CAM}$ is the lack of an effect of adamantane and norcamphor binding on CO recombination kinetics. The pressure dependence of the CO binding rate for substrate-free and substrate analogue-bound complexes is also interesting; a positive ΔV^* is obtained unexpectedly, while the other substrate analogue-bound $P450_{CAM}$'s exhibit negative ΔV^* values. Since both adamantane and norcamphor have substantial freedom for fluctuation in the heme pocket, we conclude that the higher mobility of the substrate causes a lower bond formation barrier.

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