Articles

CO Binding Studies of Engineered Cytochrome P-450$_d$s: Effects of Mutations at Putative Distal Sites in the Presence of Polycyclic Hydrocarbons†

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Received December 12, 1990; Revised Manuscript Received February 22, 1991

ABSTRACT: The kinetic parameters of CO binding to genetically engineered cytochrome P-450$_d$ (P-450$_d$) and two putative distal mutants, Glu318Asp and Thr322Ala, have been evaluated in the presence and absence of polycyclic hydrocarbons. The dissociation constant (K_d) of CO from wild-type P-450$_d$ was decreased by half (from 1.8 μM to approximately 0.9 μM) in the presence of phenanthrene or anthracene but was increased to 11 μM in the presence of 1,2:3,4-dibenzanthracene or 7,8-benzoflavone. These changed K_d values were not altered markedly by mutations at the putative distal site. In contrast, the recombination rate constants (k_{on}) of CO to the Glu318Asp mutant in the presence of phenanthrene (15.5×10^5 M^{-1} s^{-1}) and 7,8-benzoflavone (0.75×10^5 M^{-1} s^{-1}) were much larger than those for the wild type. Similar but smaller increases of the k_{on} values were observed for the Thr322Ala mutant. It was suggested that phenanthrene and anthracene distort the Fe–C–O bond and/or affect the access of CO to the wild-type P-450$_d$ in an opposite way from 1,2:3,4-dibenzanthracene and 7,8-benzoflavone. Glu318 and Thr322 may be located so close to a CO binding channel in ferrous P-450$_d$ that mutations of these residues can open the sterically hindered CO channel caused by the hydrocarbons.

Protoheme-containing enzymes, collectively called cytochrome P-450 (P-450),[1] catalyze a variety of oxidation reactions (Sato & Omura, 1978; Ortiz de Montellano, 1986). Substrate specificities and regiospecificities of P-450s seem to be closely correlated with local amino acid sequences and/or three-dimensional structures near the heme where substrates such as molecular oxygen and organic substances bind to cause the hydroxylation reaction.

Binding kinetics of O_2, CO, or other axial ligands of the heme iron in hemoproteins give valuable information about the heme environment (for example, Olson et al. (1987), Jongeward et al. (1988), and Egenberg et al. (1990), and references cited therein). It was reported that both equilibrium constants and rate constants of CO binding to reduced P-450s in the absence of substrates are remarkably different from those in the presence of substrates (Peterson & Griffin, 1972; Imai, 1982; Imai et al., 1982; Tuckey & Kamin, 1983; Mims et al., 1983; Mitani et al., 1985).

Cytochrome P-450$_d$ is purified from isosafrole-induced rat liver microsomes (Ryan et al., 1980; Guengerich et al., 1982; Waxman, 1986). The three-dimensional structure of this membrane-bound enzyme is not known yet. However, through site-directed mutagenesis, we obtained valuable information about the distal and proximal structures of this enzyme in terms of optical absorption spectra (Shimizu et al., 1988, 1989, 1991), electron-spin resonance (Sotokawa et al., 1990), and substrate specificities (Furuya et al., 1989a,b). Especially, the role of the putative distal region from Glu318–Thr322 in the ligand binding to the oxidized P-450$_d$ has been elucidated (Shimizu et al., 1991).

In the present paper, we studied CO binding kinetics for reduced wild-type and two putative distal mutants, Glu318Asp and Thr322Ala, of P-450$_d$. It is shown here that the CO binding rate to the heme iron of P-450$_d$ is remarkably changed by displacements of amino acids at the putative distal region in the presence of polycyclic hydrocarbons. We discussed the structure–function relationship of P-450$_d$ on the basis of these findings.

Experimental Procedures

Wild-type and mutant P-450s of the high-spin state were purified as previously described (Shimizu et al., 1986, 1988, 1991). Purified wild-type and mutant P-450$_d$s were suspended in 100 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% (w/v) Emulgen 913. For the titration experiments, 0.7–2.0 μM P-450$_d$ solutions in 1-cm cells were used in the same buffer. For titration and flash photolysis studies, we always kept each solution at least 10 min at 25 °C to reach equilibrium after saturated amounts of polycyclic hydrocarbons were added to the solution. We also kept each solution of ferrous P-450$_d$s for 3 min to reach equilibrium after adding the CO stock solution. The concentration of CO of the stock solution was

† This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (02217102) from the Ministry of Education, Science, and Culture of Japan to T.S.

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[1] Abbreviations: P-450, cytochrome P-450; P-450$_d$, rat liver cytochrome P-450$_d$ induced by isosafrole (which corresponds to P-450IA2) (Nebert et al., 1989); P-450$_{cam}$, cytochrome P-450 purified from *Pseudomonas putida* grown in the presence of camphor (which corresponds to P-450CI) (Nebert et al., 1989); K_d, dissociation constant; k_{on}, recombination rate constant; EDTA, ethylenediaminetetraacetic acid; Emulgen 913, poly(oxyethylene)-*p*-noxylphenyl ether containing 13.1 oxyethylene units on average; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone.

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Table I: Dissociation Constants (K_d) (μM) of CO for Wild-Type and Mutant P-450$_d$s[a]

		+phena	+anthr	+dibenzan	+7,8-benzofla
wild type	1.81	0.77	0.99	10.82	11.41
Glu318Asp	0.30	0.47	0.61	7.90	6.33
Thr322Ala	0.20	0.93	0.43	7.14	9.09

[a] The titration study was repeated at least three times for each mutant, and their averaged values are shown. Experimental errors for K_d values were less than 10%. Concentrations of polycyclic hydrocarbons were phenanthrene, 0.5 mM; anthracene, 20 μM; 1,2:3,4-dibenzanthracene, 20 μM; and 7,8-benzoflavone, 0.1 mM. These concentrations were at nearly saturated concentration in the buffer solution. The abbreviations used are phena, phenanthrene; anthr, anthracene; dibenzan, 1,2:3,4-dibenzanthracene; and 7,8-benzofla, 7,8-benzoflavone.

determined spectrophotometrically each time by titrating a solution of reduced horse heart myoglobin with the CO stock solution. Bindings of polycyclic hydrocarbons to P-450$_d$s were confirmed by the spin change from the partial low-spin complex to the high-spin complex with Soret absorption spectra (Shimizu et al., 1991). Cytochrome P-450 concentration was spectrally determined from the ferrous–CO complex versus the ferrous difference spectra with a Soret $\Delta\epsilon_{447-490nm}$ value of 93 mM^{-1} cm^{-1} or from a molar absorptivity of 109 mM^{-1} cm^{-1} of the Soret peak of the ferric high-spin form (Shimizu et al., 1991).

Absorption spectra were recorded with a Shimadzu UV spectrophotometer (UV-365) equipped with an end-on photomultiplier (Hamamatsu Photonics, R-375) or a Hitachi U-2000 spectrophotometer.

Chemicals of the highest guaranteed grade were purchased from Wako Pure Chemicals (Osaka) and were used without further purification.

The flash photolysis reactions were performed at 25 ± 1 °C throughout this work. The photoexcitation of reduced P-420 for monitoring the absorption around 447 nm was eliminated by use of a 420-nm cutoff filter. The CO binding reactions were conducted under pseudo-first-order conditions in excess CO. The CO-bound P-450$_d$ complexes were prepared in air-tight capped cells by the addition of 20–200 μL of approximately 1.1 mM CO stock solution to solutions of P-450$_d$ (0.7–2.0 μM) reduced by sodium dithionite.

Flash photolysis experiments were carried out with a homemade flash spectrophotometer. Details of this machine were described elsewhere (Ito, 1983). Briefly, this spectrophotometer consists of two flash photolysis lamps (Xenon Corp. N-851C) with a half-duration of 8 μs and with a flash energy of 100 J. Recovery of the P-450–CO complex was monitored with continuous light (447 nm) by a photomultiplier (Hamamatsu Photonics, R-955). Kinetic data were directly transferred from a digital storage scope (Kawasaki, KDS-102) to a personal computer (NEC, PC-9801), by which first-order plots were calculated. A total of 2000 data points in 200–500 ms were collected; these were fit by a least-squares procedure.

Results

Typical spectral change at the Soret region of P-450$_d$ caused by the addition of CO and double-reciprocal plots of the spectral change at 447 nm versus the concentration of the free CO ligand are shown in Figure 1. The K_d value (1.81 μM) of the wild type was decreased nearly 50% by the presence of phenanthrene and anthracene (0.77–0.99 μM), while that was increased 6 times by the presence of 1,2:3,4-dibenzanthracene and 7,8-benzoflavone (10.82–11.42 μM) (Table I). The K_d value (1.81 μM) of the wild type was remarkably decreased by the mutations at Glu318 (0.30 μM) and Thr322 (0.20 μM)

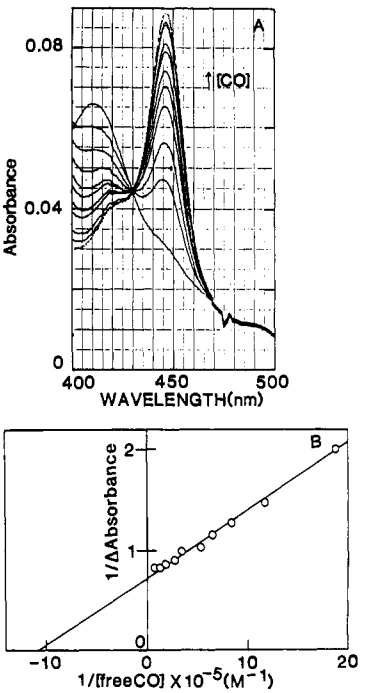

FIGURE 1: Absorption spectral change of the mutant Thr322Ala. (A) Absorption spectral changes at the Soret region of the reduced mutant Thr322Ala caused by the addition of CO in the presence of 0.5 mM phenanthrene. The mutant Thr322Ala was reduced by sodium dithionite in 100 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% (w/v) Emulgen 913. (B) Double-reciprocal plots of the absorption intensity around 447 nm versus the concentration of free CO in the presence of 0.5 mM phenanthrene.

Table II: Recombination Rate Constants (k_{on}) (10^5 M^{-1} s^{-1}) of CO to Wild-Type and Mutant P-450$_d$s[a]

		+phena	+anthr	+dibenzan	+7,8-benzofla
wild type	1.86	0.52	1.86	0.17	0.07
Glu318Asp	1.59	15.50	2.95	0.23	0.75
Thr322Ala	0.67	2.65	2.72	0.18	0.12

[a] The flash photolysis study was repeated at least three times for each mutant, and their averaged values are shown. Experimental errors were less than 20%. Other experimental conditions were the same as in Table I.

(Table I). However, in the presence of polycyclic hydrocarbons, the mutations at Glu318 and Thr322 did not markedly influence the K_d value of the wild type (Table I).

Recombination rate constant (k_{on}) of CO to the wild-type and mutant P-450$_d$s were obtained by the flash photolysis method (Figure 2 and Table II). The observed time courses for CO binding to the wild-type and mutant P-450$_d$s were monophasic (at more than 90% absorption) and fit well to single-exponential expressions. The resultant pseudo-first-order rate constants linearly depended on the CO concentration. The k_{on} value of the wild type (1.86×10^5 M^{-1} s^{-1}) was decreased remarkably by the addition of 1,2:3,4-dibenzanthracene and 7,8-benzoflavone ($(0.07–0.17) \times 10^5$ M^{-1} s^{-1}), while that was not markedly changed by the addition of phenanthrene and anthracene ($(0.52–1.86) \times 10^5$ M^{-1} s^{-1}) (Table II). Mutations at Glu318 and Thr322 in the absence of those polycyclic hydrocarbons decreased the k_{on} value ($(0.67–1.59) \times 10^5$ M^{-1} s^{-1}) to a certain extent. Similar results were also observed for the Glu318 and Thr322 mutants in the presence of anthracene and 1,2:3,4-dibenzanthracene. In contrast, the k_{on} value (0.52×10^5 M^{-1} s^{-1}) in the presence of phenanthrene was increased 30 times by the Glu318Asp mutation (15.50×10^5 M^{-1} s^{-1})

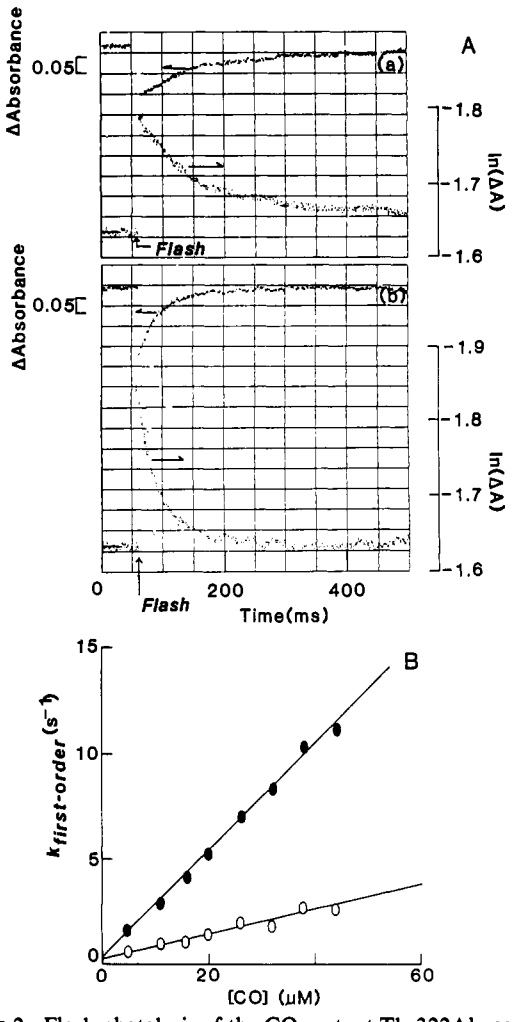

FIGURE 2: Flash photolysis of the CO mutant Thr322Ala complex. (A) Flash photolysis time course curves at 447 nm for the CO binding to the reduced mutant Thr322Ala (0.77 μM)–0.5 mM phenanthrene complex in the presence of 5 μM CO (a) and 32 μM CO (b) (see the left-hand scale) and their semilogarithmic plots directly calculated from digitalized data (see the right-hand scale). First-order constants ($k_{first\text{-}order}$) for the CO binding to the wild-type and mutant P-450$_d$s were obtained by a least-squares fitting to the flash photolysis data. (B) Dependences of the first-order rate constants of the CO binding to the mutant Thr322Ala on the CO concentration in the absence (○) and presence (●) of 0.5 mM phenanthrene.

Table III: Calculated Dissociation Rate Constants (k_{off}) (s^{-1}) of CO from Wild Type- and Mutant P-450$_d$s[a]

		+phena	+anthr	+dibenzan	+7,8-benzofla
wild type	0.34	0.04	0.18	0.19	0.08
Glu318Asp	0.06	0.72	0.18	0.18	0.48
Thr322Ala	0.01	0.25	0.12	0.13	0.11

[a] The k_{off} values were calculated from the equation $K_d = k_{off}/k_{on}$.

(Table II). A very similar, but less remarkable, increase of the k_{on} value in the presence of 7,8-benzoflavone was observed for the Glu318Asp mutation. Less marked increases of the k_{on} value in the presence of phenanthrene and 7,8-benzoflavone were observed for the Thr322Ala mutation as well (Table II).

The k_{off} values (Tables III) in the equilibrium state were calculated from the equation $K_d = k_{off}/k_{on}$. The calculated k_{off} value (0.34 s^{-1}) of the wild type was decreased by the mutations (0.06–0.01 s^{-1}) in the absence of polycyclic hydrocarbons. In contrast, in the presence of phenanthrene and 7,8-benzoflavone, the calculated k_{off} values (0.04–0.08 s^{-1}) were largely increased by the mutation at Glu318 (0.72–0.48 s^{-1}) (Table III) while those (0.18–0.19 s^{-1}) were not essentially changed by the same mutation in the presence of anthracene and 1,2:3,4-dibenzoanthracene (0.18 s^{-1}). Here again, it is noted that the mutation Glu318Asp largely influenced the k_{off} value in the presence of phenanthrene and 7,8-benzoflavone.

Discussion

Addition of smaller hydrocarbons such as phenanthrene and anthracene decreased the K_d value of the wild-type P-450$_d$, while addition of larger hydrocarbons such as 1,2:3,4-dibenzoanthracene and 7,8-benzoflavone increased the K_d value. The K_d value of the ligand for the hemoprotein will reflect the structure of the final bound state (Egenberg et al., 1990; Mims et al., 1983). Thus, it seems that these bindings of larger hydrocarbons may distort the approximate linearity of the Fe–C–O bond in P-450$_d$ (Raag & Poulos, 1989). Addition of 1,2:3,4-dibenzanthracene and 7,8-benzoflavone decreased the k_{on} value corresponding with the increase of the K_d value by addition of these hydrocarbons. The change of the k_{on} value will reflect the change of the access channel of CO in P-450$_d$ (Egenberg et al., 1990; Mims et al., 1983). Thus, these larger hydrocarbons may impose steric constraint at the CO access channel of P-450$_d$ by limiting the distal space for the ligand approach to the heme iron. On the other hand, the binding of smaller hydrocarbons may rather enlarge the CO access channel of P-450$_d$ perhaps in an indirect way. The decrease of the K_{on} value was more remarkable than the corresponding increase of the K_d value by the addition of those hydrocarbons. Thus, it seems that addition of hydrocarbons to P-450$_d$ influenced the k_{on} value or the CO access channel more sensitively than the K_d value or the final bound state.

Although a three-dimensional structure of membrane-bound P-450$_d$ has not been known, it is possible to speculate the structure of this protein by referring to alignments of amino acid sequences of P-450s (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989) and the crystal structure of water-soluble P-450$_{cam}$ (Poulos et al., 1985). Thr322 (numbered for P-450$_d$) is well conserved for most of the microsomal P-450s (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989). Glu318 (numbered for P-450$_d$) is also well conserved as either Glu or Asp for all P-450s (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989). From the crystal structure of water-soluble P-450$_{cam}$, it was indicated that these residues are located at the distal site of the heme (Poulos et al., 1985). The distal site of P-450 will be very important for the activation of molecular oxygen and the substrate specificity of P-450. The present study indicates that the K_d value of CO was remarkably decreased by the mutations at Glu318 and Thr322 in the absence of polycyclic hydrocarbons (Table I). It is suggested that these mutations may change the Fe–C–O structure in P-450$_d$. It is also shown here that the ligation rate (k_{on}) of CO to the ferrous P-450$_d$ is also largely influenced by the mutations specifically at Glu318 as well as at Thr322 in the presence of phenanthrene and 7,8-benzoflavone (Table II). The sterically hindered CO access channel in the 7,8-benzoflavone-bound P-450$_d$ may be opened by the Glu318Asp mutation. The CO channel similarly perturbed by phenanthrene may be largely changed in favor of CO access by the Glu318Asp mutation. Thus, it is suggested here that Glu318 must be located at the nearest neighbor of the CO access channel of ferrous P-450$_d$. The effect of the Thr322Ala mutation on the CO recombination rate constant (k_{on}) in the presence of these hydrocarbons may be explained in a similar way. Mutations at the Glu318–Thr322 region of P-450$_d$ also directly influence the binding behavior of nonlinear ligands such as metyrapone, 2-phenylimidazole, and acetanilide to

ferric P-450$_d$ (Shimizu et al., 1991). It was reported that bindings of linear ligands such as the CO molecule to the heme in the hemoprotein are dominated more by electrostatic effects than by steric effects (Smith & McLendon, 1980). However, it seems that steric effects influenced the K_d and k_{on} values of CO for the wild-type and mutant P-450$_d$s more than electrostatic effects since, for example, the mutation Glu318Asp may not remarkably change the electrostatic environment of the CO access channel.

The calculated k_{off} value of the wild type was decreased by the addition of hydrocarbons (Table III). The k_{off} value was decreased by mutations at Glu318 and Thr322, too. However, the k_{off} value in the presence of anthracene and 1,2:3,4-dibenzanthracene were not markedly changed by the same mutations, as observed for the K_d and k_{on} values of the corresponding complexes. In contrast, the k_{off} values in the presence of phenanthrene and 7,8-benzoflavone were remarkably increased by the mutation at Glu318 (Table III). This result is very similar to that observed for the k_{on} values, again suggesting the significance of Glu318 in the CO access channel.

In conclusion, (1) phenanthrene and anthracene change the K_d and k_{on} values of CO for the wild-type P-450$_d$ so as to distort the Fe–C–O bond and/or affect the access of CO in an opposite way from 1,2:3,4-dibenzanthracene and 7,8-benzoflavone; (2) k_{on} and calculated k_{off} values of CO for the wild type were remarkably changed by the putative distal mutations at Glu318 and Thr322 in the presence of phenanthrene and 7,8-benzoflavone so as to open the sterically hindered CO channel; and (3) these findings reinforce the suggestion that the Glu318–Thr322 region is located at the distal site and/or at the CO access channel of the membrane-bound P-450$_d$, as has been suggested from alignments of amino acid sequences of P-450s (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989) and the X-ray crystal structure of water-soluble P-450$_{cam}$ (Poulos et al., 1985; Raag & Poulos, 1989).

Registry No. CO, 630-08-0; cytochrome P450, 9035-51-2; phenanthrene, 85-01-8; anthracene, 120-12-7; 1,2:3,4-dibenzanthracene, 215-58-7; 7,8-benzoflavone, 604-59-1.

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