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Role of Glu318 at the Putative Distal Site in the Catalytic Function of Cytochrome $P450_d$†

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ABSTRACT: Most microsomal P450s have a conserved "threonine cluster" composed of three Thrs (Thr319, Thr321, Thr322 for $P450_d$) at a putative distal site. An ionic amino acid at 318 is also well conserved as Glu or Asp for most P450s. To understand the role of these conserved polar amino acids at the putative distal site in the catalytic function of microsomal P450, we studied how mutations at this site of $P450_d$ influence the activation of molecular oxygen in the reconstituted system. Catalytic activity (0.02 min^{-1}) toward 7-ethoxycoumarin of the Glu318Ala mutant of $P450_d$ was just 6% of that (0.33 min^{-1}) of the wild type, while those of Glu318Asp, Thr319Ala, and Thr322Ala were comparable to or even higher than that of the wild type. Consumption rates of O_2 and formation rates of H_2O_2 of those mutants varied in accord with the catalytic activities. Especially, the efficiency (0.5%) of incorporated oxygen atom to the substrate *versus* produced H_2O_2 for the Glu318Ala mutant was much lower than that (3.7%) of the wild type, while that (58.8%) for the mutant Glu318Asp was 16-fold higher than that of the wild type. In addition, the autoxidation [Fe(II) → Fe(III)] rate (0.074 s^{-1}) of the Glu318Ala mutant was much lower than those (0.374–0.803 s^{-1}) of the wild type and other mutants. Thus, we strongly suggest that Glu318 plays an important role in the catalytic function toward 7-ethoxycoumarin of microsomal $P450_d$.

For monooxidation reactions catalyzed by cytochrome P450, molecular oxygen must be activated on the heme plane for transferring activated oxygen atom to the organic substrate (Guengerich, 1991; Ortiz de Montellano, 1986; Porter & Coon, 1991). From the crystal structure of water-soluble $P450_{cam}$,[1] it was suggested that highly conserved Thr252 (numbered for $P450_{cam}$) (Figure 1) in the distal site must be very important for the activation of the molecular oxygen (Poulos et al., 1985, 1987). This suggestion was proven by site-directed mutagenesis of the distal Thr252 of $P450_{cam}$ (Imai et al., 1989; Martinis et al., 1989). Namely, the Thr252Ala mutant of $P450_{cam}$ had a very low catalytic activity as compared with that of the wild type, and consumed O_2 in the enzyme solution was recovered as H_2O_2 (Imai et al., 1989; Martinis et al., 1989) and H_2O (Martinis et al., 1989). In addition, the autoxidation rate of the O_2 complex of the Thr252Ala mutant was much faster than that of the wild type (Imai et al., 1989; Martinis et al., 1989).

Although microsomal membrane-bound P450s monooxygenate organic substrates as does $P450_{cam}$, the detailed mechanism of the monooxidation reaction involved with microsomal P450 may be different from that of $P450_{cam}$ in some respects. For example, electrons necessary for the reaction are supplied from a flavoprotein, cytochrome P450 reductase, for microsomal P450s, while those are supplied from a nonheme iron protein, putidaredoxin, for $P450_{cam}$ (Guengerich, 1991; Ortiz de Montellano, 1986; Porter & Coon, 1991). O_2 in the enzyme solution is not efficiently used, and the production of H_2O_2 is accompanied with the hydroxylation reaction for microsomal P450s (Ingelman-Sundberg & Johansson, 1984; Gorsky et al., 1984), whereas O_2 in the enzyme solution is efficiently used for the reaction of $P450_{cam}$ (Atkins & Sligar, 1988; Imai et al., 1989; Martinis et al., 1989). The autoxidation rate of the O_2 complex of microsomal P450s is fast ($1–10^{-1}$ s^{-1}) (Bonfils et al., 1979; Estabrook et al., 1971; Guengerich et al., 1976; Oprian et al., 1983), while that of $P450_{cam}$ is slow ($10^{-2}–10^{-3}$ s^{-1}), and thus the absorption spectrum of the O_2-bound complex is obtained with a conventional spectrometer (Gettings et al., 1990; Ishimura et al., 1971; Lipscomb et al., 1976; Peterson et al., 1972).

Microsomal P450s have a conserved "threonine cluster" at the putative distal site (Nelson & Strobel, 1988) (Figure 1). Thr319, Thr321, and Thr322 apparently compose the Thr cluster for $P450_d$. Prior to the conserved Thr (Thr252 for $P450_{cam}$, Thr319 for $P450_d$; note that the cluster is absent from $P450_{cam}$), the polar amino acid such as Glu318 for $P450_d$ or Asp251 for $P450_{cam}$ is fairly conserved for P450s (Figure 1). To understand the role of these polar amino acids at the putative distal site of membrane-bound $P450_d$, we changed the

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[1] Abbreviations: P450, cytochrome P450; $P450_d$, rat liver microsomal cytochrome $P450_d$ (which corresponds to P4501A2 or CYP1A2; Nebert et al., 1991); $P450_{cam}$, cytochrome P450 purified from *Pseudomonas putida* grown in the presence of camphor (which corresponds to P450101 or CYP101; Nebert et al., 1991); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; Emulgen 913, poly(oxyethylene)-*p*-nonylphenyl ether containing 13.1 oxyethylene units on average; DLPC, dilauroyl-L-α-phosphatidylcholine; methyl viologen, 1,1-dimethyl-4,4′-bipyridinium dichloride hydrate; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone.

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Table I: Turnover Numbers toward 7-Ethoxycoumarin, Consumption Rates of O_2, Formation Rates of H_2O_2, and Autoxidation Rates of O_2-Bound Complexes

$P450_d$	turnover number[a] (A) (min^{-1})	consumption rate of O_2[b] (B) (min^{-1})	formation rate of H_2O_2[c] (C) (min^{-1})	autoxidation rate of O_2-bound complex[d] (s^{-1})	C/B (%)	A/B (%)	A/C (%)
wild type	0.33	20	6.3	0.41	31.5	1.7	5.2
Glu318Ala	0.02	6	3.1	0.07	51.7	0.3	0.6
Glu318Asp	1.44	27	2.2	0.80	8.1	5.3	65.5
Thr319Ala	0.90	24	2.5	0.37	10.4	3.8	36.0
Val320Ser	0.02	11	1.6	0.37	14.5	0.2	1.3
Thr321Ala	0.11	7	4.3	0.44	61.4	1.6	2.5
Thr322Ala	0.71	17	5.9	0.38	34.7	4.2	12.0

[a] Turnover numbers toward 7-ethoxycoumarin in the reconstituted system (0.3 μM $P450_d$, 500 μM 7-ethoxycoumarin, 0.9 μM P450 reductase, 48 μM DLPC, and 1 mM NADPH) were determined at 25 °C as previously reported (Shimizu et al., 1991c). It should be noted that catalytic activities in yeast microsomes and reconstituted systems are different from each other in some cases (Furuya et al., 1989; Brian et al., 1990). The buffer (pH 7.2) consisted of 0.1 M potassium phosphate, 4% glycerol, and 0.2 mM DTT. [b] Consumption rates of O_2 in the reconstituted system were determined with a Clark-type oxygen electrode (Central Kagaku Corp., Oxygraph-9, UD-1+UD-901) at 25 °C. [c] Formation rates of H_2O_2 in the reconstituted system were determined at 25 °C as described previously (Getting et al., 1990). [d] Autoxidation rates of the O_2-bound complex were determined by monitoring the absorption peak of 418 nm at 4 °C with a stopped-flow spectrometer (Union Rapid Analyzer RA-601) equipped with micromixing cells as described previously (Shimizu et al., 1991c). $P450_d$ (1 μM) in 400–500 μL of buffer (pH 7.2) consisting of 0.1 M potassium phosphate, 20% glycerol, 1 mM EDTA, 1 μM methyl viologen, and 0.4% Emulgen 913 was purged by pure Ar gas for 5 min in an air-tight capped cell. The $P450_d$ solution was then carefully reduced by 10–15 μL of 50 mM sodium dithionite. After it was confirmed that $P450_d$ was fully reduced, saturated O_2 in the same buffer solution made separately was rapidly mixed with the $P450_d$ solution (Shimizu et al., 1991c). Rate constants were evaluated from the fast phase.

polarity of the amino acids of this site of $P450_d$ and studied how these mutations influence catalytic activities of this enzyme in relation to the activation of the oxygen molecule. We suggest here an important role of the polar amino acid, Glu318, in the catalytic function of $P450_d$, associated with the activation of the oxygen molecule.

Experimental Procedures

Site-directed mutagenesis, DNA sequencing, expression of $P450_d$ mutants in yeast, and purifications of $P450_d$ mutants were carried out as previously described (Furuya et al., 1989; Shimizu et al., 1991a,b). Mutant Glu318Ala was purified as the low-spin form with the Soret peak at 416 nm, but it contained nearly 10–15% high-spin form. The wild type and other mutants were purified as the high-spin form with the Soret peak at 393 nm with the low-spin content less than 5%. Concentrations of the $P450_d$ mutants were obtained from molar absorptivity, 1.09×10^5 M^{-1} cm^{-1} at 393 nm of the high-spin oxidized form or 1.20×10^5 M^{-1} cm^{-1} at 447 nm of the CO-reduced form (Shimizu et al., 1991a–c). A denatured form, P420, did not exist for the present solutions in terms of the optical absorption spectra of the CO-reduced form. To avoid the denaturation during spectral measurements, every experiment was carried out at 25 °C except for measurements of autoxidation rates of the O_2 complexes at 4 °C. More details are described in the table.

Results

Turnover numbers of the mutants Glu318Ala and Val320Ser toward 7-ethoxycoumarin were 6% of that of the wild type, while that of mutant Thr321Ala was 30% of that of the wild type (Table I). Turnover numbers of the mutants Glu318Asp, Thr319Ala, and Thr322Ala toward the substrate were higher than that of the wild type.

Consumption rates of O_2 in the enzyme solution for the mutants Glu318Ala and Thr321Ala were nearly 30% of that of the wild type, while those of the mutants Glu318Asp, Thr319Ala, and Thr322Ala were a little higher or comparable to that of the wild type (Table I). These variations apparently correspond with the catalytic activities of the mutant enzymes.

Formation rates of H_2O_2 of the mutants Glu318Ala, Glu318Asp, Thr319Ala, and Val320Ser were less than half of that of the wild type, while those of the mutants Thr321Ala and Thr322Ala were a little less than that of the wild type.

Autoxidation rates of the mutants Thr319Ala, Val320Ser, Thr321Ala, and Thr322Ala were comparable to that of the wild type, while that of the mutant Glu318Ala was very slow and was just 18% of the wild type. It was also noted that the autoxidation rate of the mutant Glu318Asp was twice that of the wild type.

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NDIFGAGFET319 VTTAIFW   P450d(rat)
FDLFGAGFDT    ITTAISW   P450c(rat)
LDLFGAGFDT    VTTAISW   P450pl(mouse)
LDLFGAGFDT    VTTAISW   P450pl(human)
LDLFGAGFDT    VTTAISW   P4506(rabbit)
NDIFGAGFDT    VTTAISW   P4504(human)
NDIFGAGFDT    ITTALSW   P4504(rabbit)
NDIFGAGFDT    ITTALSW   P450LM4(rabbit)
NDIFGAGFDT    VTTAITW   P450p3(mouse)
TDVFGAGTET    TSTTLRY   P450PBcl(rabbit)
SDVFMAGTET    TSTTLRY   P450PBc2(rabbit)
SDLFGAGTET    TSTTLRY   P4501(rabbit)
MDLIGAGTET    MSTTLRY   P450f(rat)
TDLFGAGTET    TSTTLRY   P450PBl(rat)
WDVFSAGTDT    TSNTLKF   P4503b(rabbit)
ADMFFAGTET    TSTTLRY   P4503a(rabbit)
ADLFFAGTET    TSTTLRY   P450j(rat)
ADLFFAGTET    TSTTLRY   P450j(human)
LDLFLAGTGT    TSTTLRY   P450(chicken)
LSLFFAGTET    GSTTLRY   P450e(rat)
LSLFFAGTET    SSTTLRY   P450b(rat)
LSLFFAGTET    TSTTLRY   P450LM2(rabbit)
LNLFIGGTET    VSTTLRY   P450(human)
GDIFGAGVET    TTSVIKW   P45017α(bovine)
GDIFGAGVET    TTSVVKW   P45017α(human)
VDLLIGGTET    TANTLSW   P450C21(human)
VDLFIGGTET    TASTLSW   P450C21(bovine)
VDLFIGGTET    TATTLSW   P450C21(mouse)
IIFIFAGYET    TSSVLSF   P450HLp(human)
IIFIFAGYEP    TSSTLSF   P450p(rat)
DIFMFEGHDT    TASGVSW   P450LAW(rat)
TENLAGGVNT    TSMTLQW   P450scc(bovine)
TEMLAGGVDT    TSMTLQW   P450scc(human)
GLLLVGGLDT252 VVNFLSF   P450cam(P.putida)
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FIGURE 1: Amino acid sequences of the (putative) distal site of P450s (Nelson & Strobel, 1988). Boldfaced letters of $P450_d$ are amino acids mutated in the present study.

Discussion

It appeared that the turnover number of each mutant is closely correlated with the rates of O_2 consumption and H_2O_2 formation for each mutant in the reconstituted system.

In the monooxidation reaction of bacterial $P450_{cam}$, production of H_2O_2 is not observed. However, when Thr252 of

the distal site of $P450_{cam}$ was changed to Ala, remarkable production of H_2O_2 was observed (Imai et al., 1989; Martinis et al., 1989). It was indicated that consumed O_2 in the enzyme solution was mostly used for the production of H_2O_2 rather than incorporated into the substrate for the Thr252Ala mutant of $P450_{cam}$ (Imai et al., 1989; Martinis et al., 1989). For microsomal P450s, in contrast, H_2O_2 is generally produced in the reaction cycle (Ingelman-Sundberg & Johansson, 1984; Gorsky et al., 1984). The production of H_2O_2 was observed for the engineered wild-type and mutant $P450_d$s as well. However, this production was decreased for most of the mutants studied here except for the mutant Thr322Ala. Thus, the conserved Thr319 of $P450_d$, which corresponds to Thr252 of $P450_{cam}$, may not be so significant for the activation of the oxygen molecule to cleave the O–O bond as is the Thr252 in $P450_{cam}$. In accordance with this idea, the Thr mutants of other microsomal P450s have catalytic activities toward specific substrates in the reconstituted system (Imai & Nakamura, 1989).

To know the efficiency of O_2 consumed, we evaluated (1) percentages of H_2O_2 production and catalyzed substrate per O_2 consumed and (2) percentages of catalyzed substrate per H_2O_2 production for the wild type and the mutants (Table I). For the Glu318Ala and Val320Ser mutants, the consumed O_2 was used for the production of H_2O_2 relatively more efficiently than for the hydroxylation reaction toward the substrate. In contrast, for the Glu318Asp, Thr319Ala, and Thr322Ala mutants, consumed O_2 was relatively more efficiently used for the hydroxylation reaction rather than for the production of H_2O_2. When the length of the side chain at position 318 is shortened by the mutation Glu → Asp, the enzyme may take a more appropriate conformation at the distal site for the activation of the oxygen molecule. This conformational change at the distal site may increase apparent catalytic activity toward 7-ethoxycoumarin.

The autoxidation rate for the Thr252Ala mutant of $P450_{cam}$ was 1 order higher than that of the wild type (Imai et al., 1989; Martinis et al., 1989). The autoxidation rate of the corresponding Thr319Ala mutant of $P450_d$ was comparable to that of the wild type. Other Thr mutants such as Thr321Ala and Thr322Ala of $P450_d$ also had an oxidation rate comparable to that of the wild type. Thus, the conserved Thr319, Thr321, and Thr322 may not be directly involved in the autoxidation of microsomal $P450_d$, in contrast with the conserved Thr252 of $P450_{cam}$. On the other hand, for the Glu318Ala mutant the autoxidation rate was much lower than that of the wild type, while the rate was 2-fold higher than that of the wild type for the Glu318Asp mutant. Thus, it seems very likely that the carboxyl group of the conserved Glu318 will significantly contribute to the autoxidation rate of microsomal $P450_d$.

In our previous papers, we suggested an important role of Glu318 in the binding of external axial ligands such as metyrapone, 2-phenylimidazole, and CO to the heme iron of $P450_d$ (Shimizu et al., 1991a,b). The Glu318 also seems to be very important to keep an appropriate active distal structure of $P450_d$. Namely, the Glu318Ala mutant was purified as the low-spin form in contrast with the wild type and other mutants of $P450_d$ (Krainev et al., 1991; Shimizu et al., 1991b,c; Sotokawa et al., 1990), suggesting that the structure of the putative distal environment may be largely changed by the mutation. Thermodynamic parameters for the binding of phenyl isocyanide to the heme of the Glu318Ala and Glu318Asp mutants also suggest that an important conformational change(s) at the putative distal site was induced by the Glu318 mutations (Krainev et al., 1991). Even so, it should be noted that a denatured form, P420, was not observed in terms of the absorption spectrum of the CO-reduced form of this protein. Asp251 of $P450_{cam}$ forms an ion pair with Lys178 and Arg186 in the distal site, keeping the important distal pocket for the catalytic activity of $P450_{cam}$ (Poulos et al., 1985, 1987). A very similar ionic interaction of Glu318 with Lys and/or Arg may be formed in the distal site of $P450_d$. The Glu318 → Ala mutation would eliminate these interactions, creating a new distal conformation for the oxygen atom of water or an amino acid to coordinate the heme to make the low-spin complex, since the newly created low-spin complex is the oxygen-bound form (Krainev et al., 1991; Shimizu et al., 1991b).

The latest X-ray crystallographic studies of $P450_{cam}$ suggest an important role of a solvent network of the distal site in the activation of the oxygen molecule (Raag & Poulos, 1991; Raag et al., 1991). It was suggested that conserved Thr252 and Glu366 of $P450_{cam}$ form the important distal structure for the solvent channel, which serves as the source of protons required to cleave the O–O bond in the catalytic function. Asp251 interacts with Arg186, adding stability to the distorted region of the distal helix of $P450_{cam}$ (Poulos et al., 1985; Raag & Poulos, 1991). If we assume that the putative distal structure of $P450_d$ is similar to, if not identical to, that of $P450_{cam}$, it seems very likely that the Glu318 of $P450_d$ will form a solvent network in the distal site in $P450_d$, which must be very important in the activation of the oxygen molecule. The spin change from the high-spin state to the low-spin state caused by the Glu318 → Ala mutation again supports the suggestion that Glu318 is important for keeping an active distal structure of $P450_d$.

The conserved Thr319 and Thr322 may not significantly contribute to the catalytic function of microsomal P450s from the various parameters obtained in the present study. Although catalytic activity and other parameters were remarkably changed by the Val320 → Ser mutation, it is not clear at this point how Val320 contributes to the catalytic function of $P450_d$.

Conclusions

Well-conserved polar amino acids at position 318 (Glu or Asp of $P450_d$ and other P450s; Asp251 or $P450_{cam}$) prior to the conserved Thr (Thr319 for $P450_d$; Thr252 for $P450_{cam}$) will significantly contribute to the activation of the oxygen molecule in the catalytic function toward 7-ethoxycoumarin of P450s. It may be implied that this polar amino acid may work for the water molecule to serve as the proton donor to cleave the oxygen molecule (Raag & Poulos, 1991; Raag et al., 1991).

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Supplementary Material Available

Figures and legends of Soret absorption spectra of the ferric and ferrous CO-bound Glu318Ala mutant, the Soret absorption spectrum of the ferrous O_2-bound wild type, and traces of the Soret absorption peak of the ferrous O_2-bound wild type and Glu318Ala mutant (3 pages). Ordering information is given on any current masthead page.

Registry No. P450, 9035-51-2; Glu, 56-86-0; Fe, 7439-89-6; Asp, 56-84-8; 7-ethoxycoumarin, 31005-02-4; monooxygenase, 9038-14-6.

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