Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 96-102

ROLE OF THR-252 IN CYTOCHROME P450$_{CAM}$: A STUDY WITH UNNATURAL AMINO ACID MUTAGENESIS

Yoko Kimata, Hideo Shimada, Tadaaki Hirose*
and Yuzuru Ishimura[1]

Department of Biochemistry and *Pharmaceutical Institute, School of Medicine, Keio University, Shinanomachi 35, Shinjuku-ku, Tokyo 160, Japan

Received January 27, 1995

Replacement of Thr-252 in the active center of cytochrome P450cam with a non-hydroxy amino acid residue such as Ala and Val by conventional site-directed mutagenesis converted this monooxygenase to an NADH oxidase (Imai, M. *et al. Proc. Natl. Sci. U. S. A.* **86**, 7823-7827, 1989). In this study, a mutant enzyme with a methoxy group in place of the hydroxy group of Thr-252 (OMe-mutant) was synthesized by the method of unnatural amino acid mutagenesis (Noren, C. J. *et al., Science* **244,** 182-188, 1989). Unlike other site-directed mutants without a hydroxy group at the position, the OMe-mutant retained a considerably high monooxygenase activity, yielding a stoichiometric amount of 5-*exo*-hydroxycamphor to that of the oxygen consumed. Thus a free hydroxy group at this position is not an indispensable requisite for the monooxygenase to cleave the O-O bond of molecular O_2 as previously proposed.

Cytochrome P450cam (P450cam; CYP101) of *Pseudomonas putida* is a heme-containing monooxygenase that catalyzes the reaction: *d*-camphor + NADH + H^+ + O_2 → 5-*exo*-hydroxycamphor + NAD^+ + H_2O. In this reaction, the O-O bond of the dioxygen (O_2) is cleaved at the expenses of two protons and electrons. Subsequently, one of the oxygen atom from O_2 is incorporated into *d*-camphor, while the other atom is reduced to H_2O. Thus the mechanism by which the cleavage of the O-O bond is coupled to the transfers of the reducing equivalents (electrons and protons) is the central question to understand the monooxygenase reaction catalyzed by this enzyme.

On the other hand, site-directed mutagenesis studies [1-7], which were based on the results of X-ray structural analyses [8,9], indicated that a conserved residue Thr-252 at the active site of cytochromes P450 (Fig. 1A) plays a crucial role in the catalysis. For example, several mutants such as Thr252Ala and Thr252Gly showed an uncoupling of O_2 consumption from *d*-camphor hydroxylation; most of O_2 consumed was converted to H_2O_2 without cleaving the O-O bond (Table 1) [1,3]. Among the 252-mutants studied so far, only Thr252Ser mutant retained a high ratio in the coupling of dioxygen consumption to *d*-camphor hydroxylation (85 %, Table 1),

[1]To whom correspondence should be addressed. Fax: 81-3-3358-8138.

Abbreviations used:P450cam: cytochrome P450cam; OMe-mutant: P450cam mutant with OMe-Thr at position 252; PCR: polymerase chain reaction.

0006-291X/95 $5.00

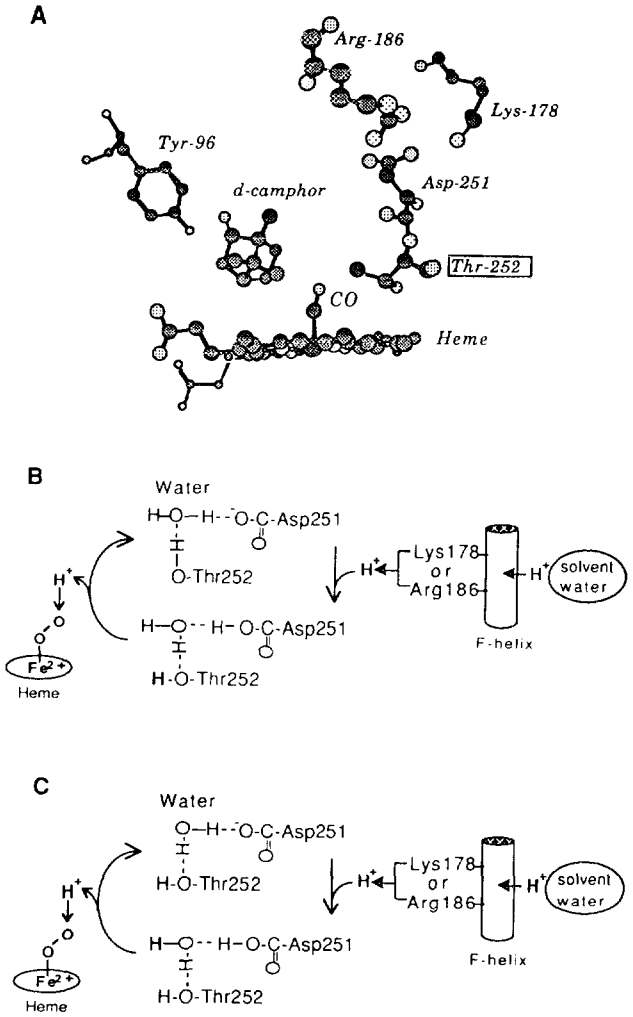

Fig. 1. **The active site of P450cam complexed with *d*-camphor and carbon-monoxide (CO) (A) and proposed proton relay systems in previous papers (B) and in this communication (C).** Physiological substrate of dioxygen binds to the heme iron where carbon monoxide is bound in diagram of panel A. Thr-252 is located close to the oxygen binding site. In the proposed proton relay systems of 450cam in previous papers (panel B)[5,7] and in this communication (panel C), the direct proton donor to the heme-bound oxygen is the hydroxy group of Thr-252 and the water molecule, respectively. The system in panel C is to explain the result that the OMe-mutant also works well with a methoxy group (R-O-CH_3) in place of the hydroxy group (R-OH) of Thr-252.

suggesting the requirement of a hydroxy group at this position for an efficient and proper O-O bond scission [1,3]. A model has been proposed, therefore, that Thr-252 participates in a proton transfer pathway through a hydrogen-bonding network from the outside of the protein to the interior heme pocket (Fig. 1B)[5,7]. Then the hydroxy group of Thr-252, which was assumed to form a hydrogen bond to the heme-bound oxygen, has been proposed to function as an acid catalyst that directly donates protons for the cleavage of the O-O bond (Fig. 1B)[3-5,7]. As shown in the figure, the proton transfer pathway is proposed to consist of Asp-251 of the I-helix, and Lys-178 or Arg-186 of the F-helix.

Table 1. **Enzyme activity of the wild type and mutant 450cam proteins synthesized *in vivo* and *in vitro*.** Oxygen consumption rate is expressed as the turnover number per holo 450cam. The product per O_2 consumed was calculated based on the oxygen consumed during the reaction. The OMe-mutant, as well as the wild type enzyme, formed stoichiometric amount of hydroxylated camphor to that of oxygen consumed during the reaction.

$P450_{cam}$ species	O_2 consumption rate	Product formation per O_2 consumed		References
		5-OH-camphor	H_2O_2	
	μM/min/μM $P450_{cam}$	%		
wild type from *P. putida*	1370	100	2	[1]
wild type*	1330	96	5	[1]
wild type#	1340	100	ND	this study
252OMe-Thr#	410	100	ND	this study
252Ala*	1150	5	89	[3]
252Gly*	1090	3	88	[3]
252Ser*	830	85	15	[3]

*: P450cam expressed in *E. coli* [1,3].
#: P450cam synthesized *in vitro*.
ND: not determined.

In an attempt to test the proposed role of Thr-252, we have substituted the amino acid residue with a threonine analogue O-methyl-threonine, in which the free hydroxy group is replaced by a methoxy group, and hence is no more able to donate proton to the reaction system. The method used to synthesize such a mutant was that of "biosynthetic incorporation of unnatural amino acids into proteins" developed by Schultz et al. [10]. This method, in which an arbitrarily-designed amino acid is co-translationally incorporated into a protein, provides a more promising tool to study the detailed chemistry of an enzyme-catalyzed reaction as compared to the conventional mutagenesis. From the analyses of the resulting mutant (OMe-mutant), a question against the previously proposed role of Thr-252 [3-5,7] has been emerged, leading us to propose a new hypothesis on the role of Thr-252 of $P450_{cam}$.

EXPERIMENTAL PROCEDURES

Construction of expression plasmids and site-directed mutagenesis——The P450cam expression vector (ppk450cam) derived from pUC19 has been described [1]. The plasmid pT252am containing mutant P450cam gene with amber codon at position 252 was generated from ppk450cam with the method using sequential PCR steps [11]. The synthetic oligonucleotides used for the PCR were: M13 forward- and reverse primers (Takara Shuzo), and 5'-TGGATTAGGTGGTCAATTTC-3', 5'-CCACCTAATCCAGGCCGCCG-3', where underlined bases denote mismatches to the wild type sequences.

Amino acyl tRNA synthesis——Unacylated suppressor tRNA with amber anticodon was synthesized *in vitro* from the synthetic DNA containing mutant yeast $tRNA^{Phe}$ sequence under T7 promoter [13]. Suppressor tRNA acylated with O-methyl-threonine (Sigma) was prepared using the methods of the chemical aminoacylation of the dinucleotide dpCpA and enzymatic RNA ligation of the acylated dpCpA and the unacylated suppressor tRNA, as described elsewhere [12].

In vitro protein synthesis and partial purification——*In vitro* protein synthesis was performed in *E. coli* S30 coupled transcription translation system as described [10] with the exception that the reaction mixture contained following chemicals to increase the efficiency of the synthesis of holo P450cam; 800 μM isopropyl-1-thio-β-D-galactopyranoside, 640 μM cyclic AMP, 10 μM hemin

and 100 μM *d*-camphor. The S30 cell extracts for the protein synthesis were prepared from the cell culture of *E. coli* strain D-10 (*rna-10, relA1, spoT1, metB1*)[12]. P450cam synthesized *in vitro* was detected with Western blot analysis after 11% SDS-polyacrylamide gel electrophoresis. The polyclonal anti-P450cam antibody used for the blot analysis was raised in domestic rabbits against purified P450cam from *P. putida*. Synthesized P450cam proteins were partially purified by polyethylenimine treatment of the *in vitro* reaction mixtures [14] and then chromatography of the supernatant on DEAE-HPLC column, monitoring the absorbance at 391 nm, the soret absorption maximum of camphor-bound ferric P450cam.

Optical measurements and enzyme assay—The specific content of P450cam proteins in the partially-purified preparations was estimated from the absorption difference at 446 and 500 nm in the CO-difference spectra (ε_{mM} = 93)[15]. Catalytic activity of P450cam was assayed by measuring the oxygen consumption and formation of hydroxylated camphor in the presence of 360 μM NADH, 14 μM putidaredoxin and 0.12 μM putidaredoxin reductase in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM *d*-camphor and 50 mM KCl at 20 °C. Oxygen consumption was measured using a Clark-type oxygen electrode. The hydroxylated camphor in the reaction mixture was extracted with chloroform and measured on a Shimazu GC-14A gas chromatograph with a capillary column, Shimazu CBP-1 W25 [1].

RESULTS

In vitro synthesis of OMe-mutant—Introduction of OMe-Thr into the position 252 of P450cam was accomplished by *in vitro* suppression of an amber nonsense mutant at position 252 with a suppressor tRNA which was chemically acylated with OMe-Thr. Figure 2A shows Western blot analyses on SDS-PAGE of the reactions that were programmed with plasmid containing P450cam genes of the wild type (lane 1) or the 252 amber-mutant (lanes 2-8) under the conditions specified in "Experimental procedures". The most eminent band observed in the lane 1 was at 45 kDa with the same mobility as that of the wild type P450cam synthesized either *in vivo* in the *E. coli* system or in *P. putida* (data not shown). Lane 2 shows the result of the reaction performed with the mutant gene in the absence of suppressor tRNA; no synthesis of full-length (45 kDa) product was observed. The band at 30 kDa detected in the lane 2, as well as those in the lanes 3-8, is probably a truncated N-terminal half of P450cam protein due to the amber-stop codon at position 252. The origin of the 30 kDa band which was also seen in the lane 1 was unknown. An addition of 5-15 μg of unacylated suppressor tRNA to the same reaction system (lanes 3-5) again resulted in no detectable amount of 45 kDa product synthesized.

On the other hand, additions of 5, 10 and 15 μg of suppressor tRNA that was acylated with OMe-Thr to the reaction system (lanes 6-8) yielded increasing amounts of the material corresponding to P450cam, as the amounts of the suppressor tRNA added were increased. Maximum synthesis of the full-length product was achieved with the addition of 15 μg of the acylated suppressor tRNA to the reaction, and more supplementation did not cause a further increase in the synthesis of the 45 kDa material under the present experimental conditions. The P450cam mutant that was obtained could not be heterogeneous because the control experiments described above (lanes 2-5) showed that the levels of protein synthesized by amber suppression either with noncognate tRNAs or with unacylated suppressor tRNA were negligible. From these results, therefore, we believe that OMe-Thr was incorporated into 252 amber mutant site of the P450cam gene.

Partial purification and spectroscopic characterization of the in vitro synthesized P450cam—Samples of P450cam synthesized *in vitro* were partially purified from the reaction mixtures with DEAE-HPLC column chromatography after a polyethylenimine treatment [14].

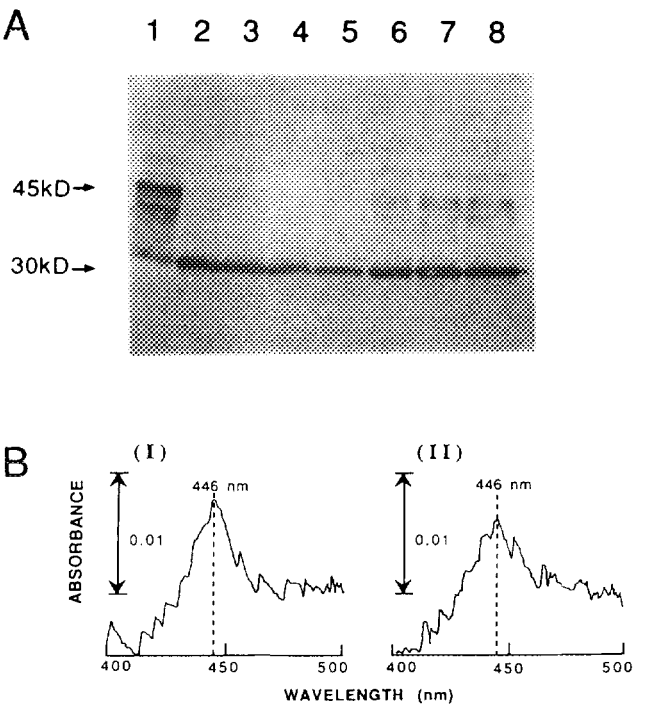

Fig. 2. **Western blot analyses of reactions of protein synthesis *in vitro* (A) and reduced-CO difference spectra of P450cam proteins synthesized *in vitro* (B).** *Panel A*, protein synthesis reactions (10 μl) in *E. coli* S30 system contained the following plasmids and tRNAs : lane 1, ppk450cam (wild type P450cam); lanes 2 through 8, pT252am (amber mutant at position 252) with plasmid only (lane 2), 5 μg, 10 μg and 15 μg of unacylated suppressor tRNA (lanes 3, 4 and 5, respectively) and 5 μg, 10 μg and 15 μg of suppressor tRNA acylated with OMe-Thr (lanes 6, 7 and 8, respectively). Reactions were incubated for one hour at 37 ° C and analyzed by Western blot after 11% SDS-polyacrylamide gel electrophoresis, using rabbit polyclonal anti-450cam antibody. *Panel B*, P450cam proteins partially purified from *in vitro* transcription-translation system were used for spectroscopic analysis. Reduced-CO difference spectra of the wild type (**I**) and OMe-mutant (**II**) are shown. An absorption maximum at 446 nm indicates the presence of P450cam in both cases. The spectra are essentially indistinguishable between those of the wild type and the mutant enzymes.

Authentic and *in vitro* synthesized wild type P450cam and OMe-mutant protein showed the same chromatographic property. Figure 2B depicts reduced-CO difference spectra of the wild type and OMe-mutant proteins synthesized *in vitro*. The absorption spectra of the ferric, ferrous and ferrous CO forms of the OMe-mutant were indistinguishable from those of authentic and wild type P450cam synthesized both *in vivo* and *in vitro* (data not shown).

Catalytic activity of the in vitro synthesized enzyme——Catalytic activities of the partially purified P450cam proteins were assessed by measuring the rate of oxygen consumption and the amount of 5-*exo*-hydroxycamphor formed during the reaction (Table 1). As seen in the table, the oxygen consuming activity of the OMe-mutant was about one-third of that of the wild type. Nevertheless, it showed 100% coupling of the oxygen consumption to the formation of 5-*exo*-hydroxycamphor; no other 252-mutants can preserve such a complete coupling activity. 252Ala- and 252Gly-mutants in the table showed only 5 and 3 % of the coupling, respectively, and even

252Ser-mutant with a hydroxy group has considerably less coupling of 85 %. We thus conclude that the free hydroxy group at position 252 is not prerequisite for the O-O bond cleavage in the P450cam reaction.

DISCUSSION

The results described here indicated that the previously proposed role of the hydroxy group of Thr-252 in the I helix of 450cam as an acid catalyst for the O-O bond scission [3-5,7] must be reconsidered; the OMe-mutant synthesized in this study was shown to retain considerable monooxygenase activity, giving one third of wild type activity without causing the uncoupling phenomenon. Thus, question arises as to the role of Thr-252 of which mutation uncouples the oxygen consumption from *d*-camphor hydroxylation.

First of all, one can consider the possibility that the side-chain of Thr-252 merely functions as a structural factor to ensure the efficient coupling instead of participating in the bond scission. In this regard, it has been argued that the activity for the O-O bond cleavage can be explained by the effect of an axial ligand cystein, a characteristic heme ligand of P450 family, which has been supposed to provide enough electron density to push and cleave the O-O bond [16]. Secondly, it is also possible that the electron attracting ability of amino acid residues at distal side, along with the effect of the proximal cystein ligand, allows for an efficient and rapid oxygen bond cleavage, as proposed for the peroxidase system [17]. In an agreement with the latter notion, site-directed mutation of Asp-251, in proximity of Thr-252, to non-dissociable residues dramatically decreased the steady-state rate of the catalysis [3,4,7], suggesting the crucial role of Asp-251. Thus a hypothetical hydrogen-bonding network for the transfer of protons, shown in figure 1B, was proposed [5,7].

On the basis of above discussions, and to explain the result of current study, we propose here another hypothetical proton relay system shown in figure 1C; the side-chain oxygens of Thr-252 and Asp-251 function to keep a water molecule through hydrogen bonds, and the water molecule, instead of the hydroxy group of Thr-252 as proposed previously (Fig. 1B), acts as the terminal proton donor for the bond cleavage. To verify these possibilities, further study is in progress in our laboratory.

ACKNOWLEDGMENTS

We thank Dr. Barbara J. Bachmann (*E. coli* Genetic Stock Center) for providing *E. coli* strain D-10, Dr. Peter G. Schultz and Dr. Virginia W. Cornish (University of California, Berkeley) for the technical advice for preparing the aminoacyl dpCpA, Peg Kopps (Promega Corporation) for the technical advice for developing *in vitro* translation system, and Dr. Hideho Miki for preparing anti-P450cam antibody. This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan, by the Special Coordination Funds of the Science and Technology Agency of Japanese Government, and by grants from Keio University.

REFERENCES

1. Imai, M., Shimada, H., Watanabe, Y., Matsushima-Hibiya, Y., Makino, R., Koga, H., Horiuchi, T., and Ishimura, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7823-7827.

2. Martinis, S. A., Atkins, W. M., Stayton, P. S., and Sligar, S. G. (1989) *J. Am. Chem. Soc.* **111,** 9252-9253.
3. Shimada, H., Makino, R., Imai, M., Horiuchi, T., and Ishimura, Y. (1990) In *International Symposium on Oxygenases and Oxygen Activation* (Nozaki, M., Yamamoto, S., and Ishimura, Y. *eds*) 133-136, Yamada Science Foundation.
4. Gerber, N. C., and Sligar, S. G. (1992) *J. Am. Chem. Soc.* **114,** 8742-8743.
5. Shimada, H., Makino, R., Unno, M., Horiuchi, T., and Ishimura, Y. (1993) In *Cytochrome P450 Biochemistry, Biophysics and Molecular Biology* (Lechner, M. C. *eds*) 299-306, John Libbey.
6. Makino, R. and Shimada, H. (1993) In *Cytochrome P450, second edition* (Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y. *eds*) 17-30, Kodansha.
7. Gerber, N. C., and Sligar, S. G. (1994) *J. Biol. Chem.* **269,** 4260-4266.
8. Loew, G. H., and Rohmer, M. M. (1980) *J. Am. Chem. Soc.* **102,** 3654-3657.
9. Poulos, T. L., Finzel, B. C., and Howard, A. J. (1987) *J. Mol. Biol.* **195,** 687-700.
10. Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C. and Schultz, P. G. (1989) *Science* **244,** 182-188.
11. Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1991) in *Current Protocols in Molecular Biology*, unit 8.5, pp. 1-9. John Wiley & Sons, Inc.
12. Ellman, J. A., Mendel, D., Anthony-Cahill, S. J., Noren, C. J., and Schultz, P. G. (1991) *Methods Enzymol.* **202,** 301-336.
13. Noren, C. J., Anthony-Cahill, S. J., Suich, D. J., Noren, K. A., Griffith, M. C. and Schultz, P. G. (1990) *Nucleic Acids Res.* **18,** 83-88.
14. Mendel, D., Ellman, J. A., and Schultz, P. G. (1991) *J. Am. Chem. Soc.* **113,** 2758-2760.
15. Omura, T. and Sato, R. (1962) *J. Biol. Chem.* **237,** 1375-1376.
16. Dawson, J. H. (1988) *Science* **240,** 433-439.
17. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem.* **255,** 8199-8205.